Bioavailability and bioefficacy of β-carotene measured using ^{13}C-labeled β-carotene and retinol: studies in Indonesian children

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Proefschrift

Ter verkrijging van de graad van doctor
op gezag van de Rector Magnificus
van Wageningen Universiteit,
Prof. Dr Ir L Speelman,
in het openbaar te verdedigen
op vrijdag 9 november 2001
des namiddags te vier uur in de Aula

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Stellingen

1. Voor het elimineren van vitamine A-tekorten kan niet volstaan worden met het stimuleren van de groenten- en fruitconsumptie.

   Dit proefschrift

2. De techniek gebaseerd op 'plateau isotoopverrijking' is de beste techniek voor het bestuderen van de biodoeltreffendheid van carotenoïden.

   Dit proefschrift

3. De huidige richtlijnen van FAO/WHO en het 'Institute of Medicine' in de VS voor de vitamine A activiteit van β-caroteen in een gemengde voeding overschatten de biodoeltreffendheid van β-caroteen met een factor 2 tot 3.

   Onder andere dit proefschrift

4. De internationale gedragscode voor de marketing van borstvoeding-vervangende middelen schiet zijn doel voorbij.

5. Het Angelsaksische PhD-systeem leidt tot publikatie-bias.

6. Het teruglopende aantal leraren met een universitaire opleiding (WO) in het Voorbereidend Wetenschappelijk Onderwijs (VWO) zal negatieve gevolgen hebben voor de kwaliteit van het onderwijs.

7. Nederland zou een groter deel van het budget voor ontwikkelingshulp moeten besteden aan voor ontwikkelingslanden relevant onderzoek.

8. Als je elkaar, in een lange afstandsrelatie, niet meer vertelt dat de melk is overgekookt, is het tijd om elkaar weer op te zoeken.

Stellingen behorend bij het proefschrift 'Bioavailability and bioefficacy of β-carotene measured using ^{13}C-labeled β-carotene and retinol: studies in Indonesian children'

Machteld van Lieshout
Wageningen, 9 november 2001

Propositions

1. Approaches other than the promotion of the consumption of fruit and vegetables are required in order to eliminate vitamin A deficiency.

   This thesis

2. The plateau isotopic enrichment technique is the best technique for studying the bioefficacy of carotenoids.

   This thesis

3. The current guidelines of FAO/WHO and the US Institute of Medicine for the vitamin A activity of β-carotene in a mixed diet overestimate the bioefficacy of β-carotene about 2 to 3 times.

   This thesis and other publications

4. The International Code of Marketing of Breastmilk Substitutes misses its goal.

5. The Anglo Saxon PhD system results in publication bias.

6. The decline in the number of academically trained teachers in high schools will have a negative effect on the quality of education.

7. The Netherlands should spend a larger proportion of its budget for international development aid on scientific research aimed at problems of developing countries.

8. When, in a long distance relationship, one does no longer tell the other that the milk boiled over, it is time to meet up again.

Propositions pertaining to the thesis 'Bioavailability and bioefficacy of β-carotene measured using ^{13}C-labeled β-carotene and retinol: studies in Indonesian children'

Machteld van Lieshout
Wageningen, 9 November 2001

Around 1957, it was thought that the isolation and synthesis of the numerous carotenoids,
although they may provide a happy hunting ground for PhD fellows,
have tended to distract from the central theme, the conversion of β-carotene to retinol.

Around 2001, concerted action by organic chemists, LC-MS analysts and nutritionists
has been directed towards the central theme,
obviously a happy hunting ground for PhD fellows.

Around 1957, it was thought that we might possibly know more about vitamin A,
if all energy could be devoted to the central theme.

After 2001, this energy is still much needed.

Machteld van Lieshout, 2001.
Inspired by the preface to the monograph '*Vitamin A*' by Thomas Moore, 1957.

For those devoting their energy to a good purpose

Bioavailability and bioefficacy of β-carotene measured using ^{13}C-labeled β-carotene and retinol: studies in Indonesian children

PhD thesis by Machteld van Lieshout, Division of Human Nutrition and Epidemiology, Wageningen University, the Netherlands. November 9, 2001.

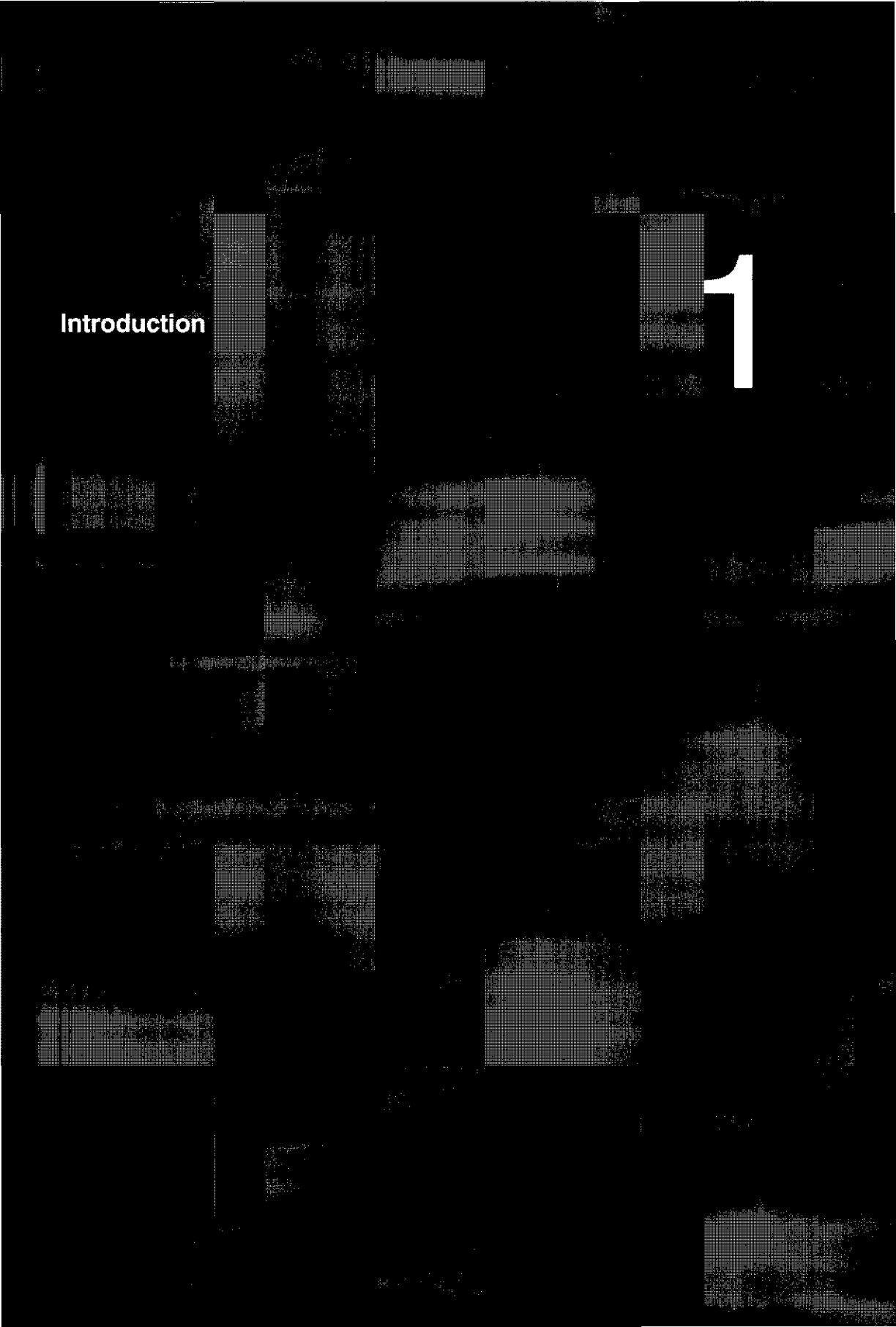
Vitamin A deficiency is a serious health problem in many developing countries. Improved vitamin A status can reduce morbidity and mortality by 23% in developing countries. Vitamin A occurs in food as preformed vitamin A (retinol), present in animal foods and breastmilk, and as provitamin A carotenoids - the major source of vitamin A for a large proportion of the world's population. However, the contribution of plant foods to vitamin A status can only be substantial when not only the consumption and provitamin A content of foods but also the bioefficacy of provitamin A carotenoids in such foods is high. With respect to provitamin A carotenoids, bioefficacy is the product of the fraction of the ingested amount which is absorbed (bioavailability) and the fraction of that which is converted to retinol in the body (bioconversion). Isotopic tracer techniques can supply accurate and precise data on bioavailability, bioconversion and bioefficacy of carotenoids in humans.

A new stable isotope technique - based on reaching plateau isotopic enrichment of β-carotene and retinol in serum during prolonged intake of multiple low doses of β-carotene and retinol, each specifically labeled with 10 ^{13}C atoms - has been developed. In 2 studies, for periods ≤10 wk, >100 Indonesian children (aged 7-13 y), consumed daily 2-3 doses of specifically labeled [$^{13}C_{10}$]β-carotene (≤80 μg/dose) and [$^{13}C_{10}$]retinyl palmitate (≤80 μg/dose). In the second study, half of the subjects (n = 41) daily also received spinach while the others (n = 36) received orange pumpkin. Either in serum or in feces, the degree of isotopic enrichment 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 high performance liquid chromatography with atmospheric pressure chemical ionization liquid chromatography-mass spectrometry (APCI LC-MS). These techniques have emerged as the most effective and convenient for studying the bioavailability, bioconversion and bioefficacy of provitamin A carotenoids. For interpretation of data, a mathematical model has been developed based on assumptions that can be readily justified in the light of present knowledge on carotenoid metabolism.

From the studies presented, it can be concluded that 2.6 μg β-carotene in oil has the same vitamin A activity as 1 μg retinol (bioefficacy of β-carotene in oil, 36%). The bioavailability of β-carotene in oil was found to be 86%. The bioavailability and bioefficacy of β-carotene in pumpkin were 1.7 times those of β-carotene in spinach. Results obtained using data from feces confirmed results obtained using data from serum.

This plateau isotopic enrichment technique can be applied for studying the effect of individual SLAMENGHI factors which affect the bioavailability and bioefficacy of carotenoids in humans (SLAMENGHI is a mnemonic for such factors). The studies presented have provided the most reliable estimates of the bioefficacy of β-carotene in oil available to date. The estimates are most probably applicable to other children in developing countries. Results from recent studies and from those presented in this thesis indicate that the current guidelines that the vitamin A activity of 1 μg retinol can be supplied by 6 μg (FAO/WHO) or 12 μg (US Institute of Medicine) of β-carotene in a mixed diet overestimate β-carotene bioefficacy about 2 to 3 times. This means that approaches other than the promotion of the consumption of fruit and vegetables are required for eliminating vitamin A deficiency.

1 Introduction



To live a life without malnutrition is a fundamental human right. The persistence of malnutrition, especially among children and mothers, in this world of plenty is immoral. Nutrition improvement anywhere in the world is not a charity but a societal, household and individual right. It is the world community's responsibility to find effective ways and means to invest for better livelihood and to avoid future unnecessary social and economic burdens. With collective efforts at international, national and community levels, ending malnutrition is both a credible and achievable goal.

Commission on the Nutrition Challenges of the 21st century. *Ending malnutrition by 2020: an agenda for change in the Millenium.* Geneva: ACC/SCN, 2000

MALNUTRITION: CAUSES, CONSEQUENCES AND CONTROL

Poverty is one of the major causes of malnutrition (WHO, 2001). Malnutrition, in turn, contributes to poverty. Reducing poverty is not the only, nor the most effective, way to reverse this downward spiral. In fact, coordinated action and modest investments by 2020 (Brown, 2000). Malnutrition arises from deficiencies of both macronutrients and micronutrients. Protein-energy malnutrition and micronutrient malnutrition together contribute to half of the ca. 10.4 million children under five years of age who die annually in developing countries (WHO, 2001). Although micronutrients are needed in only small amounts - hence their name - their health effects are far from 'micro'. They enable the body to produce enzymes, hormones and other substances essential for growth, development and the proper functioning of the immune system. Prevention and research programs on micronutrient deficiencies have focused on iodine, iron, zinc, and vitamin A. Dramatic progress has been made in some areas in the past decade, especially in reducing iodine deficiency disorders. The size of the malnutrition problem nevertheless remains vast, and progress in eliminating it is all too slow.

The global goal of virtually eliminating iodine deficiency disorders is within reach, thanks to the introduction of iodized salt to 68% of the 5 billion people living in countries with iodine deficiency disorders (ACC/SCN, 2000a). As a result, prevalence rates of goitre, mental retardation and cretinism are dropping fast.

Iron deficiency is believed to affect 20-50% of the world's population, making it the most common nutritional deficiency in the world (Beard and Stoltzfus, 2001). In children, iron deficiency and its anemia have been associated with premature birth, low birth weight, infections and increased risk of death. Physical and cognitive development in these children is impaired, resulting in lower school performance. Iron deficiency anemia contributes to 20% of all maternal deaths (WHO, 2001). Unlike iodine deficiency, anemia is only partly caused by insufficient nutrient intakes. In many countries, malaria, helminth infections and AIDS are contributing factors. Therefore, food-based approaches alone will be insufficient to control anemia (Gillespie, 1998). This explains in part why most national control programs have not been successful, and why the global prevalence of iron deficiency and anemia has not declined.

Although zinc deficiency is thought to be wide-spread in most developing countries, there is no reliable information on the magnitude of zinc deficiency, mainly because a single sensitive and specific biochemical factor for assessing zinc status is lacking (Umeta et al, 2000). Using the amount of zinc in the food supply as an indicator of zinc status, it has been estimated that nearly half of the world's population is at risk of low zinc intake (Brown and Wuehler, 2000). Insufficient intake of dietary zinc is thought to be the major cause of nutritional zinc deficiency. Because zinc is involved in fundamental activities such as gene expression and enzyme function, mild to severe zinc deficiency disturbs several biological functions such as immunity, growth, pregnancy outcome, taste perception and appetite (Mills, 1996). Supplementation with zinc is effective in alleviating or preventing zinc deficiency. However, because the extent of zinc deficiency and benefits of zinc

supplementation are not clear in many populations (Dijkhuizen and Wieringa, 2001), zinc supplementation and fortification have not been implemented widely.

Because effective and low-cost approaches to controlling vitamin A deficiency are available, the incidence of severe vitamin A deficiency (serum retinol concentration ≤ 0.35 μmol/L) is declining in all regions. However, marginal vitamin A deficiency (serum retinol concentration between 0.35 and 0.70 μmol/L) still affects between 140 to 250 million preschool children in developing countries (ACC/SCN, 2000b). In Indonesia, the prevalence of xerophthalmia (one of the consequences of severe vitamin A deficiency) among preschool children declined from 1.3% in 1977-78 to 0.3% in 1992. This was due to a multitude of health and social changes, including an effective national program involving vitamin A capsule distribution, nutrition education, and social marketing (Muhilal et al, 1994). Despite this progress, half of the preschool children in Indonesia suffer from marginal vitamin A deficiency (WHO, 1995). The causes, consequences and control of vitamin A deficiency will be described in more detail in this chapter because they have a direct bearing on the research described in this thesis.

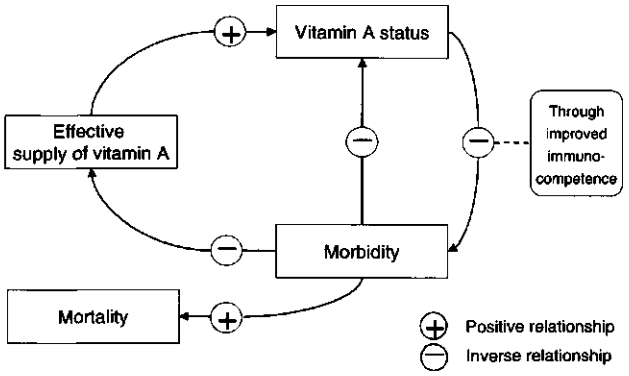


Figure 1-1. The vicious 'circle' of the causes and consequences of vitamin A deficiency. For example, infections might increase urinary excretion of vitamin A , thereby directly decreasing vitamin A status. Some illnesses reduce appetite, thereby decreasing vitamin A intake and status. The effective supply of vitamin A is defined as the amount ultimately available for metabolic functions and storage.

VITAMIN A DEFICIENCY: CAUSES, CONSEQUENCES AND CONTROL

The causes and consequences of vitamin A deficiency are linked in a vicious circle (**Figure 1-1**). Infections, such as measles, increase urinary excretion of vitamin A, thereby contributing to vitamin A deficiency. Illnesses and deficiencies in zinc and other nutrients reduce appetite, thereby decreasing vitamin A intake and status. Improved immunocompetence achieved by vitamin A supplementation in children under five years of age in developing countries has been shown to reduce morbidity and mortality by 23% (Beaton et al, 1993). Vitamin A deficiency is also a problem in other groups of the population (De Pee et al, 1998; West et al, 1999; Dijkhuizen et al, 2001). Improved vitamin A status can be achieved by reducing the body's demand for vitamin A, by increasing the effective supply of vitamin A or by combining both these approaches. Measures to control infectious diseases such as immunization and improved personal hygiene can improve

vitamin A status effectively (ACC/SCN, 2000a), but development of successful public health programs is a long-term process.

The effective supply of vitamin A, which is defined as the amount ultimately available for metabolic functions and storage (**Figure 1-2**), is determined by three factors: the consumption of unmodified and modified foods and of pharmanutrients (dietary supplements); the content of vitamin A or its precursor provitamin A in the food or pharmanutrients consumed; and the bioefficacy of vitamin A or its precursors.

Vitamin A occurs in food as preformed vitamin A (retinol) and as provitamin A carotenoids, such as β-carotene, α-carotene and β-cryptoxanthin (**Figure 1-3**). Apart from vitamin A supplied as supplements or in fortified foods, breastmilk and animal foods particularly cod liver oil, liver, milk, butter and eggs are the only sources of retinol in the diet. Breastmilk and animal foods also contain significant amounts of provitamin A carotenoids. Plant foods do not contain retinol but they are the major source of provitamin A carotenoids. Before provitamin A carotenoids can perform their functions as vitamin A, they have to be converted into retinol in the body. Foods rich in provitamin A carotenoids are red palm oil, dark-green leafy vegetables, yellow and orange fruits, and red and orange roots and tubers, such as carrots and red/yellow sweet potato (FAO/WHO, 1988).

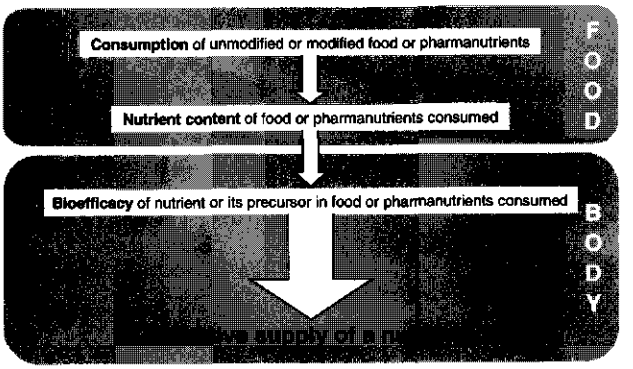


Figure 1-2. Three factors determine the effective supply of a nutrient: consumption, content and bioefficacy.

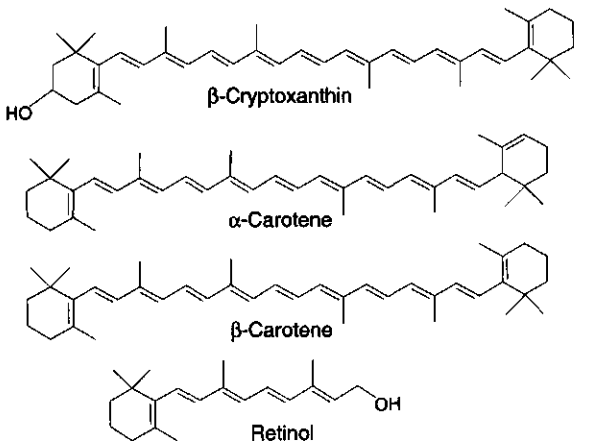


Figure 1-3. Chemical structure of retinol and the most common provitamin A carotenoids in foods, β-carotene, α-carotene and β-cryptoxanthin. Provitamin A carotenoids can be converted into retinol in the body.

The effective supply of vitamin A can be increased by increasing the consumption, content or bioefficacy of vitamin A. Increasing consumption involves introducing policies to increase the production (including promotion of horticulture and where appropriate animal husbandry), availability, palatability and demand of vitamin A-rich foods. Furthermore, social marketing of foods rich in vitamin A and provitamin A may also improve consumption. Increasing the vitamin A content of foods can be achieved by selection or breeding - traditional or genetically engineered – of varieties of plants with a higher content of vitamin A or provitamin A or by fortification of foods with vitamin A or provitamin A. However, unless the bioefficacy is sufficiently high, the effect of the approaches mentioned above on the effective supply of vitamin A will be limited. Bioefficacy is a particular problem with provitamin A carotenoids in plant foods, it is possible for it to be increased (Castenmiller et al, 1999). This is important because plant foods are the major source of vitamin A in the diet of a large proportion of the world's population (IVACG, 1999). Thus, the bioefficacy of provitamin A carotenoids determines the effectiveness (impact under field conditions) of many interventions to increase vitamin A status. Therefore, accurate and reliable data on the bioefficacy of carotenoids in plant foods are required but obtaining such data is proving a challenge.

BIOAVAILABILITY AND BIOEFFICACY OF PROVITAMIN A CAROTENOIDS

Definitions

Bioavailability is the fraction of an ingested nutrient that is available for utilization in normal physiologic functions and for storage (Jackson, 1997). Bioconversion is the fraction of a bioavailable nutrient (here, absorbed provitamin A carotenoids) that is converted to the active form of a nutrient (retinol) in the body. Bioefficacy is the fraction of an ingested nutrient (here, dietary provitamin A carotenoids) that is absorbed and converted to the active form of the nutrient (retinol) in the body (**Figure 1-4; Chapter 2**). Functional bioefficacy (not shown in Figure 1-4) is the fraction of an ingested nutrient that performs a certain metabolic function (Brouwer et al, 2001), such as the ability of ingested provitamin A carotenoids to reverse or prevent abnormal dark adaptation. Dark adaptation is the ability of rod cells of the retina of the eye to take over the function of vision under conditions of low illumination. This function is heavily dependent on an adequate vitamin A status. If the most efficient stoichiometry of cleavage of β-carotene into retinol is used, i.e., 1 mol β-carotene yields 2 mol retinol, 100% bioefficacy would mean that 1 μmol (0.537 μg) β-carotene is absorbed and converted totally to retinol, yielding 2 μmol (0.572 μg) retinol. Thus, if the bioefficacy of β-carotene was 100%, the vitamin A activity of 0.537/0.572 = 0.94 μg β-carotene would have the same vitamin A activity as 1 μg retinol. Consequently, if *y* μg of β-carotene has the same vitamin A activity as 1 μg retinol, the bioefficacy (*z*) can be calculated as (100% × 0.94 μg)/*y* μg = *z*%. According to FAO/WHO (1967; 1988) and official bodies in many countries who have followed their guidelines, the amount (μg) of provitamin A that has the same vitamin A activity as 1 μg retinol is referred to as 1 retinol equivalent (abbreviated as RE).

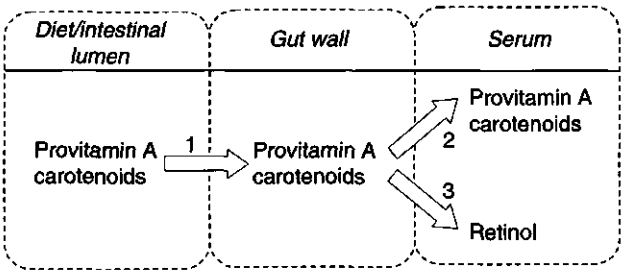


Figure 1-4. The bioavailability and bioefficacy of provitamin A carotenoids. Bioavailability = 1; bioconversion = 3; and bioefficacy = 1 × 3 (for further explanation, see text).

Relationship between bioefficacy and human requirement for vitamin A

The human requirement for vitamin A is defined as the daily intake that will prevent deficiency and provide a safe intake for 95% of the population (FAO/WHO, 1998; Institute of Medicine, 2001). In 1988, the FAO/WHO recommendations for human requirements for vitamin A were revised. Requirements were set at 600 RE for men, 500 RE for women, 400 RE for children under five years of age and 600 and 850 RE for pregnant and lactating women, respectively. The requirements relate to body size and to expected losses of retinol through breast milk. These recommendations can be met easily if animal foods are available. An average egg (50 g) contains ca. 100 and a chicken liver (25 g) contains ca. 3,000 μg retinol, thus ca. 100 and 3,000 RE. Plant food also contribute to vitamin A intake. An average carrot (raw, 20 g), portion of spinach (cooked, 70 g) or a mango (225 g) contain approximately 400, 600 and 450 μg β-carotene, respectively (Voorlichtingsbureau voor de Voeding and Nevo, 1997). With a bioefficacy of 100%, they would supply approximately 400, 600 and 450 RE, respectively. This means that a child could achieve its daily vitamin A requirement by eating either 4 eggs, one-seventh of a chicken liver, one carrot, two-thirds of a portion of spinach or one mango. It is now known however that, β-carotene and other provitamin A carotenoids are absorbed much less efficiently than retinol (Moore, 1957). In other words, provitamin A carotenoids have a bioefficacy much less than 100%. Consequently, the effective supply of vitamin A from provitamin A carotenoids in plant foods is much lower than that from retinol in animal foods.

Factors affecting the bioavailability and bioefficacy of provitamin A carotenoids

The acronym SLAMENGHI lists the various factors known to affect the bioavailability and bioefficacy of carotenoids. S) denotes the **s**pecies of carotenoid, L) the molecular **l**inkage, A) the **a**mount of carotenoids consumed in a meal, M) the **m**atrix in which the carotenoid is incorporated, E) the **e**ffectors of absorption and bioconversion, N) the **n**utrient status of the host, G) the **g**enetic factors, H) the **h**ost-related factors, and I) the mathematical **i**nteractions (De Pee and West, 1996; Castenmiller and West, 1998). Because bioefficacy of provitamin A carotenoids is one of the three determinants of the effective supply of vitamin A, it is important to quantify the role of individual SLAMENGHI factors on bioefficacy. Much attention has been paid to the matrix of plants which seems to trap carotenoids, thus making them unavailable for absorption from the intestine - thereby reducing their bioavailability. The size of this reduction remains to be quantified, as well as the effects of other SLAMENGHI factors individually and interactions between factors.

Taken together this would enable prediction of the bioavailability and bioefficacy of dietary provitamin A carotenoids under specific circumstances (West and Castemiller, 1998). In order to obtain such quantitative data on the SLAMENGHI factors, there is a need for well-designed controlled studies.

Data on the bioavailability and bioefficacy of provitamin A carotenoids

Much data on the bioefficacy of provitamin A carotenoids come from a three-step process. Firstly, examining the bioefficacy of β-carotene in oil compared with that of retinol in oil. Secondly, examining the relative bioavailability of β-carotene in food compared with that of β-carotene in oil. Thirdly, examining the relative bioefficacy of dietary provitamin A carotenoids other than β-carotene, such as α-carotene, cis-isomers of α- and β-carotene, and β-cryptoxanthin, compared with that of β-carotene in food.

Bioefficacy of β-carotene in oil compared with that of retinol in oil

Data for the first step have been derived from a small number of studies (**Table 1-1**). The most influential of these studies was the 'Sheffield Experiment' on vitamin A deficiency conducted in Britain during World War II (Hume and Krebs, 1949). According to Thomas Moore (1957), the principal reason to conduct this study was to establish vitamin A requirements in humans, particularly the relative efficiency of β-carotene and retinol, since β-carotene could be produced in Britain while retinol had to be imported from the USA. Twenty-three adults (20 men who were conscientious objectors to military service and 3 women) participated in this experiment. Sixteen subjects (two women and fourteen men) were asked to consume a vitamin A deficient diet and seven subjects (one woman and six men) served as controls, consuming the same diet, but with additional supplements of retinol or β-carotene for periods ranging from 8.5 to 25 months. Dark adaptation was used as a functional indicator of the bioefficacy. In the control group, 750 μg retinol/d (n = 2) or 3,000 μg β-carotene/d in oil (n = 4) or in margarine (n = 2) prevented impaired dark adaptation. From the other 16 subjects, only three men, who also had the lowest plasma retinol concentrations, developed impaired dark adaptation (depletion phase of the study). Either 390 μg retinol/d (n = 1) or 1,500 μg β-carotene/d in oil (n = 2) were required to reverse this impaired dark adaptation (repletion phase). In the 'Sheffield Experiment' (Hume and Krebs, 1949), 4 μg β-carotene in oil (weighted average of prophylactic and therapeutic tests) had the same vitamin A activity as 1 μg retinol. Thus, the functional bioefficacy of β-carotene in oil was (100% x 0.94 μg)/4 μg = 24%.

In a much shorter depletion-repletion study in 5 adults, Booher and colleagues (1939) concluded that (on a weight basis) 3.5 times as much β-carotene than retinol was required to prevent impaired dark adaptation. This corresponds to a bioefficacy of (100% x 0.94 μg)/3.5 μg = 27%. In another depletion-repletion study conducted by Wagner (1940) shortly before World War II (n = 10) a bioefficacy similar to that in the 'Sheffield Experiment' was found. This study is probably quoted less often because the potency of the vitamin A preparation used in this study was ambiguous. However, according to Hume and Krebs (1949), the potency reported by Wagner (1940) was correct (see footnote Table 1-1). In the 1970s, Sauberlich and colleagues (1974) concluded that (on a weight basis) twice as much

β-carotene than retinol was required to reverse impaired dark adaptation in 8 adult men. This corresponds to a bioefficacy of (100% x 0.94 μg)/2 μg = 47%. A major disadvantage of these studies on the functional bioefficacy is that they yield only crude estimates because only stepwise increased doses of retinol and β-carotene were tested and compared.

The first study to assess the bioefficacy of β-carotene in oil directly was published in 2000 by Tang and colleagues (2000). They conducted a study in one woman using a stable isotope technique. The area under the curve of the concentration of [$^2H_4$]retinol, derived after feeding a single dose of 126 mg and 6 mg [$^2H_8$]β-carotene in crystalline form 2.5 year apart, was compared with the area under the curve of the concentration of [$^2H_8$]retinol, derived after feeding a single dose of 9 mg [$^2H_8$]retinyl acetate 2 year after consumption of the first dose. From this study, it was concluded that, 55 μg (high dose) and 3.8 μg (low dose) β-carotene in oil have the same vitamin A activity as 1 μg retinol. These data correspond with a bioefficacy of β-carotene in crystalline form of 2 and 25%, respectively.

FAO/WHO (1967; 1988) have proposed that 3.3 μg β-carotene has the same vitamin A activity as 1 μg retinol. Recently, the Institute of Medicine (IOM) of the National Academy of Sciences of the USA (2001) revised the estimates of the relative efficiency of retinol and β-carotene to meet the recommended vitamin A intakes. In fact, for the bioefficacy of β-carotene in oil (Table 1-1), the IOM drew only on the functional bioefficacy study of Sauberlich and colleagues (1974) which is at variance with the other functional bioefficacy studies (Table 1-1). The IOM did not quote the papers by Booher et al (1939) and Wagner (1940). In addition, the first release of the IOM report (2001) contained two errors. Firstly, the bioefficacy, as calculated from data from the 'Sheffield Experiment', was overestimated by a factor of 2 because it was assumed that 1 IU of retinol and of β-carotene had the same weight. Secondly, from the study of Tang and colleagues (2000), the amount of β-carotene in oil that has the same vitamin A activity as 1 μg retinol was reported to be 2.6 μg based on a dose of 6 mg (published value was 3.8, see above). It would be interesting to know whether the IOM (2001) would have proposed a bioefficacy of β-carotene in oil of 47% if they were aware of this information at the time of their deliberations. At the time this thesis went to press, discussions were being held with the Institute of Medicine to resolve this issue.

Bioavailability of β-carotene in food compared with that of β-carotene in oil

For a long time, data on the second step - the relative bioavailability of β-carotene in food compared to that of β-carotene in oil - were as scarce as those on the first step. In a number of studies, differences between the amount of β-carotene in food consumed and that excreted in feces (oral-fecal balance technique) were calculated. These differences were assumed to reflect absorbed β-carotene (apparent absorption). In 1967, FAO/WHO reviewed these studies and proposed that 6 μg β-carotene in a mixed diet has the same vitamin A activity as 1 μg retinol. A conclusion, which was confirmed by FAO/WHO in their guidelines of 1988. Thus according to FAO/WHO (1967; 1988), the bioefficacy of β-carotene in food is (100% x 0.94 μg)/6 μg = 16%.

It was not until the 1990s that evidence was mounting that the bioefficacy of provitamin A carotenoids in fruit and vegetables was only 20-50% of the FAO/WHO estimates (Micozzi et al, 1992; De Pee et al, 1995; Törrönen et al, 1996; De Pee et al, 1998; Khan et al,

Table 1-1. Estimates of bioefficacy of β-carotene in oil[1]

Referece	Subjects (country)	Study design	Diet/dose/d	Results	Bioefficacy
Studies designed to quantify functional bioefficacy[2]					
Booher et al, 1939	2 men and 3 women, 21-40 year (United States)	Depletion for 16-124 d; repletion for 3-10 d	Low (<31 μg/d) retinol plus varying doses of retinol and of β-carotene in crystalline form administered to each subject after repletion[3]	Abnormal dark adaptation prevented with 3.5 times (range: 3.2-3.7) as much β-carotene as retinol in each subject, calculated on a weight basis relative to body weight[4]	1:3.5 for prevention of abnormal dark adaptation
Wagner, 1940	10 men, 23-57 year (Germany)	Depletion for 188 d; repletion for 105 d	Low retinol plus doses of 180, 360, 600 and 750 μg retinol (n = 5) or doses of 720, 1,200, 2,520 and 3,000 μg β-carotene in sesame oil (n = 5) administered during repletion	Abnormal dark adaptation reversed with 750 μg retinol (2,500 IU × 0.3 μg/IU)/d[4,5] and with 3,000 β-μg carotene in oil (5,000 IU × 0.6 μg/IU)/d[4].	1:4.0 for reversal of abnormal dark adaptation
Hume and Krebs, 1949	8 men, 1 woman, 19-34 year (United Kingdom)	Depletion for 8.5 to 25 mo; repletion for 3 wk to 6.5 mo	Low (<21 μg/d) retinol plus 370 (or 390) and 750 μg retinol or 750, 1,500 and 3,000 μg β-carotene in arachis oil administered during repletion/prevention	Abnormal dark adaptation reversed with 390 μg retinol (1,300 IU × 0.3 μg/IU)/d[4] (n = 1) and with 1,500 μg β-carotene in oil (2,500 IU × 0.6 μg/IU)/d[4] (n = 2); abnormal dark adaptation prevented with 750 μg retinol (2,500 IU × 0.3 μg/IU)/d[4] (n = 2) and with 3,000 μg β-carotene in oil (5,000 IU × 0.6 μg/IU)/d[4] (n = 4[6]).	1:3.8 for reversal and 1:4.0 for prevention of abnormal dark adaptation

Sauberlich et al, 1974	8 men, 31-43 year (United States)	Depletion for 359 to 771 d; repletion for 9 to 493 d	Low (<23 μg) retinol plus varying doses of retinol or β-carotene in corn oil administered during repletion[7]	Abnormal dark adaptation reversed with 150 μg retinol/d (n =4) and with 300 μg β-carotene/d (n = 2)	1:2 for reversal of abnormal dark adaptation[8]
Studies designed to quantify bioefficacy[9]					
Tang et al, 2000	1 woman, 47 year (United States)	Measurement of [$^2H_4$]- and [$^2H_8$]retinol in serum	Administration of single doses of 126 mg (high dose) and 6 mg (low dose) [$^2H_8$]β-carotene in crystalline form and 9 mg [$^2H_8$]retinyl acetate over a 2.5 year period	Amount of 55 μg (high dose) and 3.8 μg (low dose) of β-carotene in crystalline form has the same vitamin A activity as 1 μg retinol	1:55 (high dose) and 1:3.8 (low dose)

[1] An extended version of this table appeared in West et al, 2001.

[2] Functional bioefficacy, which is defined as the proportion of an ingested nutrient that carries out a given metabolic function, such as the ability of ingested β-carotene to reverse or prevent abnormal dark adaptation (Brouwer et al, 2001). Vitamin A was provided as retinyl esters but the amounts are expressed in terms of retinol.

[3] As an example, one subject received 270, 405, 450 and 540 μg retinol, and 1,274 and 1,770 μg β-carotene.

[4] Based on 1 IU β-carotene is 0.6 μg and 1 IU vitamin A is 0.3 μg as mentioned in each paper.

[5] Hume and Krebs (1949) suggested that there was some confusion about the potency of the retinol preparation ('Vogan') used. However, in personal communications to Hume and Krebs both from Wagner himself and from Scheunert in 1947, it was confirmed that the potency quoted by Wagner (1940) was correct.

[6] In two of those four subjects the same amount of β-carotene in margarine was found to prevent impaired dark adaptation.

[7] Three subjects received 2,400 μg; or 150, 300, 600, 1,200 and 2,400 μg; or 150, 300, 600 and 1,200 μg β-carotene and five subjects received 37.5, 75, 150, 300 and 600 μg; or 2,400 and 25,000 μg; or 150, 300, 600, 1,200, 2,400 μg; or 150, 300, 600 and 1,200 μg; or 75, 150, 300 and 8,300 μg retinol.

[8] The ratio of 1:2 was also obtained with other measures: electroretinograms, prevention of cutaneous lesions, and maintenance of plasma retinol concentrations.

[9] Bioefficacy is defined as the fraction of ingested provitamin A carotenoids absorbed and converted to retinol in the body (Chapter 2).

1998; Castenmiller et al, 1999; Tang et al, 1999a; Van het Hof et al, 1999). Therefore, the IOM (2001) recognized that data on the effective supply of vitamin A from provitamin A carotenoids in foods needed to be revised. Although more studies were available, the IOM used data from one study, conducted by Van het Hof and colleagues (1999), because that was the only study that not only had been conducted in healthy subjects in a developed country but also used a mixture of vegetables and some fruit. In Van het Hof's study (1999), the increase in serum β-carotene concentration after consumption of β-carotene-rich vegetables was one-seventh, i.e. 14%, of the increase after consumption of β-carotene in oil. The IOM (2001) adjusted this to one-sixth, i.e. 17%, because of the low fruit content in the diet used in this study. The IOM (2001) combined the data of the first and second step to calculate the vitamin A activity of β-carotene in a mixed diet to that of retinol in oil. The IOM (2001) concluded that the bioefficacy of β-carotene in oil was 47% (as described above in fact based on a value found only by Sauberlich and colleagues in 1974). Thus, the bioefficacy of β-carotene in a mixed diet was calculated as the product of the two values, that is 17% × 47% = 8%. In other words, (100% x 0.94 μg)/8% = 12 μg β-carotene in food has the same vitamin A activity as 1 μg retinol (IOM, 2001).

Relative bioefficacy of dietary provitamin A carotenoids other than β-carotene

There is even less data available for estimates on the third step - the relative bioefficacy of dietary provitamin A carotenoids other than β-carotene. Using the oral-fecal balance technique mentioned above, it has been assumed that the absorption of other provitamin A carotenoids in food is half that of β-carotene in food (Rao and Rao, 1970). Thus, the FAO/WHO (1988) and the IOM (2001), have proposed that compared to 1 μg retinol, 12 μg (FAO/WHO) and 24 μg (IOM) dietary provitamin A carotenoids other than β-carotene have the same vitamin A activity.

Overview of estimates

An overview of the reported estimates of the bioefficacy of provitamin A carotenoids is given in **Table 1-2**. The investigators of most of the above-mentioned studies recognized that their sample sizes were too limited to yield accurate and precise estimates of the bioefficacy of β-carotene in oil. Despite this, authoritative bodies have used some of these estimates in a modified or unmodified form to formulate guidelines on the relative efficiency of β-carotene and retinol to meet the human requirement for vitamin A. This has led to estimates being regarded as true values (and has thus led to a false sense of security). The IOM data might even be too optimistic, as they still overestimate the bioefficacy of β-carotene, considering evidence that as much as 21 μg β-carotene in a mixed diet (4:1 vegetables to fruit intake) might have the same vitamin A activity as 1 μg retinol (De Pee et al, 1998; Khan et al, 1998). In this case, the bioefficacy of β-carotene in food would be 4.5% (West et al, 2001).

Implications of bioefficacy for human vitamin A requirements

The impact of this low bioefficacy of β-carotene is illustrated using the above example of carrot (20 g), portion of spinach (70 g) and mango (225 g), containing approximately 400,

600 and 450 μg β-carotene, respectively. If the bioefficacy of β-carotene in food was 8%, then 12 μg β-carotene in food would provide 1 RE. Thus the carrot, portion of spinach and mango would supply only 0.08 x 400 = 32 RE, 48 RE and 36 RE, respectively. An unrealistically high consumption of 12 carrots, 8 portions of spinach or 11 mangos would therefore be needed for a child to meet its daily requirement for vitamin A (400 RE). Even larger portions of fruits and vegetables would be required if the bioefficacy of β-carotene in food was less than 8% - which is a likely prospect. Alternatively, these children could consume 4 eggs or one-seventh of a chicken liver per day. However, most impoverished people cannot afford animal products, thus their consumption of food rich in retinol is low.

Table 1-2. Overview of the estimates of the bioefficacy of provitamin A carotenoids used to produce guidelines for human vitamin A requirements

Nutrient and matrix	Amount of carotenoid that has the same vitamin A activity as 1 μg retinol	Bioefficacy, %[1]	References
β-carotene in oil	3.3	28	FAO/WHO, 1967 and 1988[2]
	2[2]	47	IOM, 2001
β-carotene in food	6	16	FAO/WHO, 1967 and 1988
	12[3]	8	IOM, 2001
Other provitamin A carotenoids in food	12	8	FAO/WHO, 1988
	24[4]	4	IOM, 2001

[1] Bioefficacy is the fraction of ingested provitamin A carotenoids absorbed and converted to retinol in the body (**Chapter 2**). If the bioefficacy is 100% and the most efficient stoichiometry of the cleavage of β-carotene is assumed, 1 mol β-carotene would yield 2 mol retinol, i.e., 0.94 μg β-carotene would have the same vitamin A activity as 1 μg retinol.

[2] Although 2 studies are quoted, in fact, the IOM has used a value found only by Sauberlich and colleagues (1974; see text).

[3] Product of the relative bioavailability of β-carotene in food compared with that in oil (17%) and the bioefficacy of β-carotene in oil compared with that of retinol (47%; see footnote 2).

[4] Product of the relative bioefficacy of provitamin A carotenoids, other than β-carotene, compared with that of β-carotene (50%) and the bioefficacy of β-carotene in food compared with that of retinol (8%; see footnote 3).

Methods to assess bioavailability and bioefficacy of provitamin A carotenoids

Up until now, data on the bioavailability and bioefficacy of provitamin A carotenoids in humans have been estimated from depletion-repletion studies (as discussed above), animal models, oral-fecal balance techniques (see above), measurement of response of serum/plasma or chylomicron carotenoid and/or retinol concentration after a single or multiple dose(s) of dietary carotenoids and/or retinol, and techniques using radio- or stable isotopic tracers. Animal models can be very useful for studying qualitative problems (West and Beynen, 1988), but they have limited use for studying quantitative processes. In addition, no appropriate animal model has been found that mimics human carotenoid metabolism (Van Vliet, 1996; Lee et al, 1999). Data obtained from oral-fecal balance techniques are likely to 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, endogenously secreted carotenoids may be excreted in feces, thus leading to underestimation of the absorption of carotenoids. Serum carotenoid and retinol concentrations have been used as measures of relative bioavailability and bioefficacy. Thus, the change in serum carotenoid and retinol concentration after consumption of provitamin A carotenoid-rich food is compared to that after consumption of a synthetic supplement of β-carotene in oil or of preformed retinol. This technique suffers from the homeostatic control of serum retinol concentrations. A modification of this technique involves measurement of chylomicron response after consumption of carotenoids. As many factors affect the bioavailability and bioefficacy of β-carotene, the intra- and interindividual variabilities of techniques measuring serum or chylomicron response are probably high. Therefore, these techniques require studies in large numbers of subjects to yield accurate and precise data.

Isotopic tracer techniques can provide accurate estimates of the bioavailability and bioefficacy of dietary carotenoids with a high precision, thus enabling studies with fewer subjects. In the 1960s, two tracer studies measured the recovery of single orally administered doses of ^{3}H or ^{14}C radioisotopically labeled β-carotene in lymph (Goodman et al, 1966; Blomstrand and Werner, 1967) . A recent study measured the recovery of a single oral dose of [^{14}C]β-carotene and its metabolite [^{14}C]retinol in serum, feces and urine (Dueker et al, 2000). Radioisotopes have been used reluctantly while the true risks of their use in human nutrition research are unknown. In the past decade, increased availability of stable isotope labeled compounds has stimulated their use. To date, nine studies have been conducted using stable isotope tracer techniques for studying the bioavailability and bioefficacy of carotenoids in humans (Parker et al, 1993; Novotny et al, 1995; You et al, 1996; Burri and Park, 1998; Tang et al, 1999b; Lin et al, 2000; Pawlosky et al, 2000; Tang et al, 2000; Yao et al, 2000). Because of their design, five of these studies can only provide qualitative information (Parker et al, 1993; You et al, 1996; Burri and Park, 1998; Pawlosky et al, 2000; Yao et al, 2000). The other four studies were designed to obtain quantitative estimates on bioavailability (Novotny et al, 1995) and bioefficacy (Tang et al, 1999b; Lin et al, 2000; Tang et al, 2000). Only limited numbers of subjects (n = 1-11) were studied, probably, as techniques were still under development. Lin and colleagues (2000), designated 5 of the 11 women as non-responders and concluded from data of the remaining 6 women that the bioefficacy of β-carotene was 74% while the bioavailability of β-carotene was only 6%. Since these results are inconsistent with each other, the assumptions underlying the model will need to be addressed.

In conclusion, although tracer techniques can provide quantitative data on the bioavailability and bioefficacy of dietary carotenoids their use has so far not yielded reliable data. Adequate quantitative isotope techniques are still required for quantification of the bioavailability and bioefficacy of dietary provitamin A carotenoids.

OBJECTIVE AND OUTLINE OF THIS THESIS

The aim of the research described in this thesis was to quantify the bioavailability and bioefficacy of β-carotene in foods typically consumed by school children in Indonesia.

Because accurate techniques to quantify the bioavailability and bioefficacy of β-carotene in foods were lacking, a stable isotope technique was developed for this purpose. In the first study we assessed the time required for the degree of isotopic enrichment in serum of β-carotene and retinol to reach a plateau, the bioefficacy of β-carotene in oil, and the intraindividual variation in response (**Chapter 2**; see also corresponding editorial by Solomons and Russell, 2001). In the second study (**Chapter 3**) we extended this technique to assess the bioavailability and bioefficacy of β-carotene in spinach and pumpkin. The design of the study also enabled the bioavailability and bioefficacy of β-carotene in oil to be re-assessed.

Collection of stool samples has the advantage that they can be collected non-invasively. Estimates of the bioavailability and bioefficacy of β-carotene in oil, spinach and pumpkin obtained using data from feces were compared to estimates obtained using data from serum. Stool samples were collected from a sub-sample of children participating in the second study. An existing HPLC method was adapted to measure in feces the concentration of retinol, retinyl palmitate and various carotenoids (**Chapter 4**). The degree of isotopic enrichment of retinol and β-carotene in serum and feces has been measured by liquid chromatography-mass spectrometry methods that were developed for this purpose (Van Breemen et al, 1998; Wang et al, 2000).

Chapter 5 reviews isotopic tracer techniques for studying the bioavailability and bioefficacy of dietary carotenoids in humans. Recommendations are made for the development of such techniques to obtain accurate data with a high precision. **Chapter 6** summarizes the main findings from the research described in this thesis, and gives the implications of these findings and provides recommendations for further research.

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Bioefficacy of β-carotene dissolved in oil studied in children in Indonesia

2

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

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

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

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

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

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INTRODUCTION

Vitamin A deficiency is a serious problem in many developing countries (Beaton et al, 1993). Provitamin A carotenoids, such as β-carotene, are the major source of vitamin A in the diet of a large proportion of the world's population. Unfortunately, the bioavailability of carotenoids in fruit and vegetables and the conversion of carotenoids to retinol are lower (De Pee et al, 1995; De Pee et al, 1998) than assumed previously (Hume and Krebs, 1949).

Bioavailability is the fraction of an ingested nutrient that is available for utilization in normal physiologic functions and for storage (Jackson, 1997). We define bioconversion as the fraction of a bioavailable nutrient (here, absorbed provitamin A carotenoids) that is converted to the active form of a nutrient (retinol). We define bioefficacy as the efficiency with which ingested nutrients (here, dietary provitamin A carotenoids) are absorbed and converted to the active form of the nutrient (retinol). Because 1 μmol β-carotene theoretically could form 2 μmol retinol, 100% bioefficacy would mean that 1 μmol dietary β-carotene (0.537 μg) is 100% absorbed and converted 100% to retinol, yielding 2 μmol retinol (0.572 μg). Thus, the amount of β-carotene required to form 1 μg retinol would be 0.537/0.572 = 0.94 μg.

Various factors, ordered in the acronym SLAMENGHI, influence the bioefficacy of carotenoids. The factors are S) the species of carotenoid, L) molecular linkage, A) the amount of carotenoids consumed in a meal, M) the matrix in which the carotenoid is incorporated, E) effectors of absorption and bioconversion, N) the nutrient status of the host, G) genetic factors, H) host-related factors, and I) interactions (De Pee and West, 1996a; Castenmiller and West, 1998).

Until now, the bioefficacy of carotenoids was estimated in humans with use of oral-fecal balance techniques, measurement of plasma or chylomicron responses after single or multiple doses of carotenoids, and tracer methods. In the 1960s, 2 tracer studies measured the recovery of [^{14}C]β-carotene in lymph (Goodman et al, 1966; Blomstrand and Werner, 1967). To date, only single doses of specifically extrinsically labeled [$^{2}H_8$]β-carotene (Novotny et al, 1995; Burri and Park, 1998; Tang et al, 2000), intrinsically labeled β-carotene biosynthesized in green algae grown with ^{13}C as the sole carbon source (Parker et al, 1993), and intrinsically labeled β-carotene from spinach grown on 30% $^{2}H_2O$ (Tang et al, 1999) have been administered for studying β-carotene metabolism in a limited number of human subjects (n = 1–5).

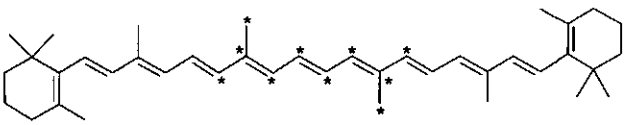
We developed a method that can quantify the bioefficacy of carotenoids with use of multiple low doses of β-carotene and retinol, each specifically labeled with 10 ^{13}C atoms. This enables the isotopic enrichment of both retinol and β-carotene in serum to reach a plateau. From the degree of labeling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

SUBJECTS AND METHODS

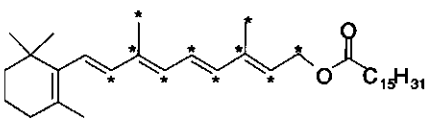
Subjects

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

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



[$^{13}C_{10}$]β-Carotene



[$^{13}C_{10}$]Retinyl palmitate

Figure 2-1. 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.

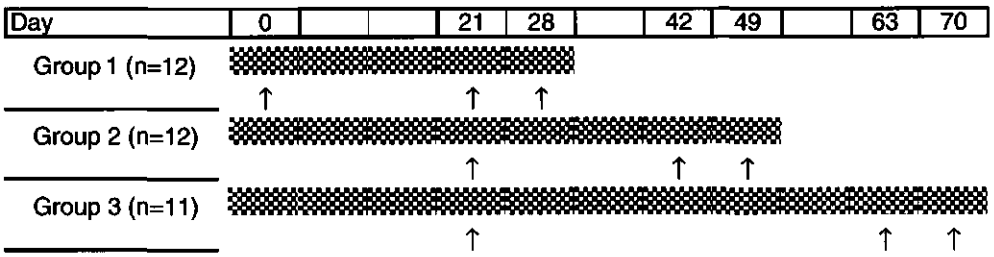


Figure 2-2. Study design of the intervention period. During the period shown by the shaded box, children consumed 2 capsules/d, 7 d/wk, and were provided a low-retinol, low-carotenoid diet 5d/wk. Each capsule contained 80 μg [$^{13}C_{10}$]β-carotene and 80 μg [$^{13}C_{10}$]retinyl palmitate. Three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

Study design

Each child received 2 capsules/d, 7 d/wk. Each capsule contained 80 μg [12,13,14,15,20,12',13',14',15',20'-$^{13}C_{10}$]β-carotene (analyzed value) and 80 μg [8,9,10,11,12,13,14,15,19,20-$^{13}C_{10}$]retinyl palmitate (44 retinol equivalents, or RE; analyzed value) (**Figure 2-1**). The oily mixture for the capsules was prepared by dissolving the labeled β-carotene and retinyl palmitate in highly unsaturated sunflower oil (> 82% oleic acid and > 10% linoleic acid; Hozol RBDW; Contined BV, Bennekom, Netherlands). *all-rac-α*-Tocopheryl acetate (Roche Nederland BV, Mijdrecht, Netherlands) was added to the oil as an antioxidant. These actions were carried out under subdued light. The capsules used in this study were made from bovine gelatin (Capsugel, Bornem, Belgium) and were filled with the oily mixture by multipipette. Each capsule contained 0.36 g oil and 150 μg vitamin E. The $^{13}C_{10}$-labeled retinol and β-carotene were synthesized at the Leiden Institute of Chemistry (Lugtenburg et al, 1999). These compounds were food grade, on the basis of criteria established by the US Pharmacopoeia (1990), the Joint FAO/WHO Expert Committee on Food Additives (1992), and the European Pharmacopoeia (1993).

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

Nonfasting blood samples (8 mL) were collected between 0930 and 1100 from an antecubital vein on days 0, 21, and 28 for group 1; on days 21, 42, and 49 for group 2; and on days 21, 63, and 70 for group 3 (**Figure 2-2**). The children were examined by a physician on the day of blood collection and their weights and heights were measured. While the children wore a school uniform but no shoes, weight was measured to the nearest 0.1 kg with a digital electronic scale (770 alpha; Seca, Hamburg, Germany) and height was measured to the nearest 0.1 cm with a microtoise.

Methods

Estimation of energy and nutrient intakes

Concentrations of retinol and β-carotene in the capsules were analyzed by HPLC (Hulshof et al, 1997). To measure the nutrient contents of the meals, a duplicate sample of a menu was collected at random once per week. The random allocation was done in such a way that each menu was sampled once during the first 5 wk of the intervention and once during the last 5 wk. Duplicate samples of all dishes of both meals of a collection day were weighed before and after removal of the inedible portion (bones, peels, etc). The edible portions were then pooled, blended, and portioned into 3 plastic bags. All bags were stored at –20 °C. Within 3 wk of collection, one bag of each duplicate sample was used to measure fat content by the Soxhlet method, protein content by the micro-Kjeldahl method, water content by evaporating the sample, and ash content by dry ashing the sample (Nio, 1992). Carbohydrate content was calculated by difference. Within 5 wk of collection, another bag of each duplicate sample was used to measure iron content (Nio, 1992). All analyses were carried out in duplicate at the laboratory of the Nutrition Research and Development Centre (NRDC) in Bogor. The last bag of each duplicate sample was stored for 2 mo before being packed on dry ice and transferred to the Division of Human Nutrition and Epidemiology in Wageningen, the Netherlands. There the samples were stored for 2 mo at –80 °C until analyzed for retinol and carotenoids by HPLC (Hulshof et al, 1997).

The energy content of the meals in kJ was calculated by multiplying the weight (in g) of fat, protein, and carbohydrates by 37, 17, and 17, respectively. Results of both duplicate samples per menu were averaged. Time and conditions of storage were adequate to obtain reliable results for the analyses carried out in food and serum (Greenfield and Southgate, 1992; Craft et al, 1988; Comstock et al, 1993). Energy and nutrient intakes during the screening and during the intervention were calculated by using the diaries, 24-h recalls, records of attendance at meals, and a computer program suite (KOMEET, version 2.0c, and VBS-EDIT, version 1.0; B-Ware Nutrition Software, Wageningen, Netherlands) with a nutrient database (Bg95K98) based on that developed by de Pee et al (1996b) to which the energy and nutrient contents of the foods provided (on the basis of the duplicate analyses) were added.

Analysis of blood and stool samples

Immediately after blood was collected, a portion of whole blood was removed to count white blood cells (Pijlman and Sanders, 1976) and measure hemoglobin concentrations (Wintrobe, 1968). These analyses were done on the day of blood collection at the NRDC.

Table 2-1. Description of the β-carotene and retinol at plateau isotopic enrichment (CarRet PIE) mathematical model

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

[1] Sample calculation based on data from a 9-y-old boy on day 21.

[2] Where $M_{279,sR}$ is the signal measured by liquid chromatography-mass spectrometry at m/z 279.

[3] $\overline{F}_{dR/dC}$ represents the mean of averaged values from each subject.

[4] Can be calculated only at the group level.

The remaining blood was placed on ice, protected from light, and centrifuged within a few hours (750 × *g* for 10 min at room temperature) at the NRDC. In serum samples collected during the screening, concentrations of retinol and total carotenoids were measured by HPLC at the NRDC (Thurnham et al, 1988). In serum samples collected during the intervention, concentrations of retinol and various carotenoids were measured by HPLC in Wageningen (Craft et al, 1992). For these latter analyses, serum was stored in a series of containers and frozen at –20 °C for 2 mo before being packed on dry ice and transferred to Wageningen. All containers were then stored at –80 °C. One container was stored for 2 mo until analyzed for both retinol and carotenoids by HPLC. Two other containers were stored for 4 mo before being packed on dry ice and transferred to the Department of Medicinal Chemistry and Pharmacognosy in Chicago. There the samples were stored for 2 mo at –80 °C until analyzed for the degree of isotopic enrichment of retinol (Van Breemen et al, 1998) and β-carotene (Wang et al, 2000) by HPLC coupled with atmospheric pressure chemical ionization liquid chromatography-mass spectrometry (APCI LC-MS).

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

The presence of protozoan cysts and worm eggs was diagnosed in stools by the Ridley method. The load of worm eggs was quantified by the Kato Katz method (Polderman et al, 1993).

Calculation of the bioefficacy of β-carotene converted to retinol

A mathematical model was developed that uses the isotopic enrichment in serum of both β-carotene with [$^{13}C_{10}$]β-carotene and retinol with [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol at plateau isotopic enrichment. This model, which we refer to as CarRet PIE, was used to estimate carotenoid bioavailability and bioconversion and, thus, bioefficacy. This stable-isotope method is based on the isotopic enrichment of retinol and β-carotene in serum reaching a plateau during multiple dosing with [$^{13}C_{10}$]retinol and [$^{13}C_{10}$]β-carotene. The intake of retinol and β-carotene from other sources is kept constant and as low as possible. It is assumed that such retinol and β-carotene mixes completely with labeled retinol and labeled β-carotene. The bioefficacy of dietary provitamin A (in this study, β-carotene in oil) compared with that of dietary retinol was calculated as shown in the first 3 columns of

Table 2-2. Characteristics of population on day 21[1]

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

[1] There were no significant differences among groups.

[2] $\bar{x}$ ± SD.

[3] Median (25th, 75th percentile).

[4] Parasitic infestation, as diagnosed by the Kato Katz and Ridley method (Polderman et al, 1993), and egg load, as quantified by the Kato Katz method (Polderman et al, 1993), were determined at screening only; n = 34. Infestation with *Entamoeba hystolitica* and hookworm was not found in the feces of these children.

[5] Eggs per gram feces as diagnosed by the Kato Katz method (Polderman et al, 1993) for those infested with *Ascaris lumbricoides* and *Trichuris trichuria*, respectively.

Table 2-1. To assess the time required for isotopic enrichment of β-carotene and retinol in serum to reach a plateau, the regression coefficients of vitamin A activity of β-carotene in oil compared with that of retinol in oil were calculated for each group, pooling the data for the second and third time points in groups 2 and 3.

Statistical methods

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

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

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

RESULTS

On the first day of the intervention, we made minor changes in the distribution of the children throughout the 3 groups because some children were absent. As a result, group 1

Table 2-3. Energy and nutrients provided and consumed daily during the screening and the intervention[1]

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

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

comprised 7 children with an initial serum retinol concentration <0.70 μmol/L (low) and 5 children with an initial serum retinol concentration >1.05 μmol/L (high), group 2 comprised 4 children with initial low and 8 children with initial high serum retinol concentrations, and group 3 comprised 6 children with initial low and 6 children with initial high serum retinol concentrations. Serum retinol concentrations of samples obtained during the intervention, which started 2.5 mo after the screening, showed that classification of children according to vitamin A status could not be maintained. Therefore, the effect of vitamin A status on the bioefficacy of β-carotene could not be assessed.

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

The drawing of the third blood sample from 3 children (one from group 1 and 2 from group 2) was postponed for 1 wk because the children had been absent for 4 d during the week before the planned blood drawing day. During the additional week, these children continued on the experimental regimen. Blood was drawn only on days on which a child did not show signs of infection as judged by a physician. White blood cell counts in all blood samples taken were <1 10^{10}/L, indicating lack of apparent infection on the day of blood collection (Pijlman and Sanders, 1976).

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

Energy and nutrient intakes

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

Table 2-4. Serum concentrations of retinol and β-carotene and degree of isotopic enrichment of retinol with [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol and of *all-trans*-β-carotene with [$^{13}C_{10}$]β-carotene[1]

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

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

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

3 $M_{274,sR} / (M_{269,sR} + M_{274,sR} + M_{279,sR})$.

4 n = 10.

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

6 $M_{547,sC} / (M_{537,sC} + M_{547,sC})$.

us was low (15 RE/d). β-Carotene intake was significantly lower during the intervention than during the screening. β-Carotene intake from the food and capsules provided was 223 g β-carotene/d, of which 160 g/d was derived from the capsules and 63 g/d from the foods. During the intervention, β-carotene intake from foods not provided by us was 155 g/d.

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

Bioefficacy of β-carotene converted to retinol

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

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

To calculate the vitamin A activity of β-carotene in oil compared with that of retinol, we used the formulas described in Table 2-1. Data from a 9-y-old boy on day 21 are shown in Table 2-1 as an example. Shown in **Figure 2-3** are values for the mean vitamin A activity of β-carotene in oil compared with that of retinol per group per day. The coefficient of the regression of the vitamin A activity of β-carotene in oil compared with that of retinol was not significantly different among groups. When the 3 groups were pooled, the regression coefficient was not significantly different from 0 (P = 0.137). Thus, in these children, isotopic enrichment of retinol and β-carotene in serum reached a plateau by day 21. On the basis of data from days 21 to 70, the mean vitamin A activity of β-carotene in oil compared with that of retinol was 0.4149 (95% CI: 0.3637, 0.4661). This corresponds to 2.4 μg (95% CI: 2.1, 2.7) β-carotene required to form 1 μg retinol in the body. If the data are recalculated assuming that *all-trans*-β-carotene and *cis*-β-carotene are absorbed to the same extent and

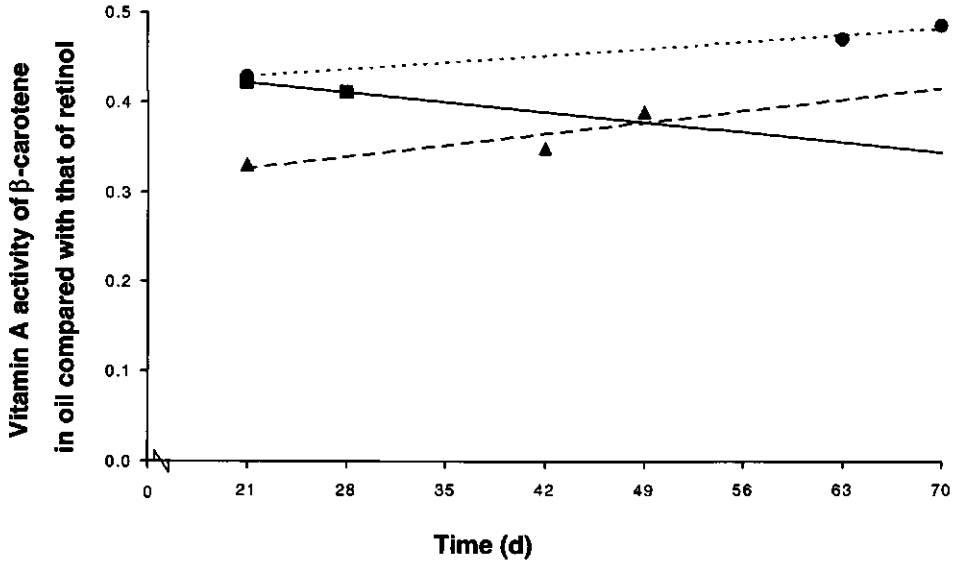


Figure 2-3. Vitamin A activity on a weight basis of β-carotene (with a *cis-trans* ratio of 3/1) dissolved in oil compared with that of retinol. Data are means of values obtained from subjects in each group on various days: for group 1 (■) on days 21 (n = 12) and 28 (n = 10); for group 2 (▲; n = 12)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

that the vitamin A potency of *cis*-β-carotene is 50% of that of *all-trans*-β-carotene, the vitamin A activity of β-carotene in oil compared with that of retinol is higher: 0.6638 (95% CI: 0.5819, 0.7458), corresponding to 1.5 μg (95% CI: 1.3, 1.7) β-carotene required to form 1 μg retinol.

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

Parasitic infestation and anthropometry

The prevalence and intensity of intestinal parasites were low in the children studied. Therefore, we expect that the effect of intestinal parasites on the findings of this study was negligible. On days 0, 21, and 28 there were no significant differences in weight and height among the 3 groups. Within groups 1 and 2, height was constant and body weight increased by 1 kg during the intervention (28 and 49 d, respectively). Within group 3, height increased by 1 cm and body weight by 1 kg during the intervention (70 d). Because these changes were not expected to affect the outcome of the study, no adjustments in the statistical analyses were made for them.

DISCUSSION

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

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

Tang et al (2000) developed a stable-isotope method that uses single doses of [$^{2}H_8$]β-carotene and [$^{2}H_8$]retinyl acetate given on separate occasions up to 2 y apart. It would have been possible to administer [$^{2}H_8$]β-carotene and [$^{2}H_8$]retinyl acetate simultaneously with subsequent measurement of [$^{2}H_8$]retinol and [$^{2}H_4$]retinol, thus eliminating differences related to giving the labeled compounds at different times. However, the low isotopic purity of the ^{2}H-labeled compounds used in Tang et al's method would decrease the signal-to-noise ratio, especially when enrichment approaches baseline. Thus, the use of not only H-labeled compounds but also single doses would possibly contribute to a lower intrinsic accuracy of Tang's method compared with our method.

A fifth advantage of our method is that the APCI LC-MS method developed for this study does not require derivatization or saponification of serum samples before analysis, whereas the use of gas chromatography-mass spectrometry does require these labor-intensive processes (Swanson et al, 1996). Intrinsic labeling yields molecules with a variable number of ^{13}C or ^{2}H atoms. This unnecessarily complicates the use of MS for the detection and subsequent quantification of bioavailability. Finally, for this study, we synthesized extrinsically and specifically labeled retinol and β-carotene. These compounds have several advantages, such as the high incorporation of ^{13}C atoms (99%) and the absence of scrambling (ie, leakage of ^{13}C atoms to positions in the molecule other than those in which they were initially incorporated). Scrambling occurs more often when deuterium is used for labeling, thus requiring measurement at different masses in the MS method. Summation of the signals leads to higher analytic variation. In addition, the labeled

compounds can be synthesized as 95% in the *all-trans* configuration, which is the configuration in which they occur in nature.

The low intraindividual CV in the vitamin A activity of β-carotene in oil compared with that of retinol was derived in part from the experimental design. The high compliance with capsule consumption (96%) also contributed to the low variation observed. Unfortunately, as noted above, only 25% of the labeled β-carotene was present as *all-trans*-β-carotene. Although care was taken to prevent isomerization of synthesized *all-trans*-β-carotene, *cis*-isomerization of labeled β-carotene probably occurred during capsule preparation (Chandler and Schwartz, 1987). Isomerization did not continue after capsule preparation because the ratio of *cis*-β-carotene to *all-trans*-β-carotene in the capsules was measured by HPLC after 9, 22, 29, and 50 d of storage at 4 and 30 °C and remained stable. More knowledge on the metabolism of *cis*-β-carotene is required to decide whether the conversion factor given here may be even lower.

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

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

Estimates of intake of several nutrients derived from the 24-h recalls were lower than the estimates from the dietary records, as reported earlier (Cameron and Van Staveren, 1988; Burk, 1980). The main purpose of the 24-h recall method was to make the children aware of their food intake. Neither of the methods used provides reliable measurements of food intake at the individual level, but both were appropriate for assessing whether differences in food intake existed among the 3 groups during the intervention and between the screening and the intervention.

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

This advanced stable-isotope technique can be extended to address the important question of the vitamin A potency of β-carotene in fruit and vegetables. Although this method has not yet been tested for its ability to measure the bioefficacy of β-carotene in fruit and vegetables, it is thought to be capable of doing so if the following design is used. Subjects would be studied for 2 consecutive periods of a maximum of 3 wk each. During

both periods, the subjects would consume 2 or 3 capsules/d, 7 d/wk, and each capsule would contain small doses (maximum: 50 μg) of [$^{13}C_{10}$]β-carotene and [$^{13}C_{10}$]retinyl palmitate. The subjects would be provided a low-retinol, low-carotenoid diet during both periods; during the second period, this diet would be supplemented with 2 or 3 portions of fruit or vegetables per day. Blood samples, preferably 2 fasting samples taken 2 d apart, would be drawn at baseline and at the end of each period. The degree of isotopic enrichment in serum retinol and β-carotene would be measured as in this study. Then, the change in isotopic enrichment during the second period (compared with the first period) would be calculated. The extent of dilution in isotopic enrichment during the second period indicates the bioefficacy of β-carotene in fruit or vegetables.

Because of the precision of this method, fewer subjects would be required to measure the bioefficacy of dietary β-carotene than were required with the methods used previously. To illustrate this, we performed sample size calculations for a hypothetical study in which the vitamin A activity of β-carotene in oil is assessed in 2 groups, one with high and one with low serum retinol concentrations. To determine which difference in bioefficacy can be expected, we can use the findings of Villard and Bates (1986) that vitamin A–deficient rats had roughly a 27% higher β-carotene 15,15'-dioxygenase activity than did control rats, indicating that vitamin A deficiency increases bioconversion. We can then calculate the minimum sample size required to detect a 27% difference between 2 groups in the vitamin A activity of β-carotene in oil compared with that of retinol, with a power of 0.90 and an α value of 0.05, with the following formula (Kirkwood, 1988):

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

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

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

In conclusion, in this study of 35 children, 2.4 μg (95% CI: 2.1, 2.7) β-carotene (with a *cis-trans* ratio of 3:1) dissolved in oil was equivalent to 1 μg retinol in the body. This is 27% less than the 3.3 μg β-carotene (with an unknown *cis-trans* ratio) dissolved in oil proposed by a committee of the International Union of Pure and Applied Chemistry in 1959, a value that

was based on 2 studies in a limited number of subjects (Hume and Krebs, 1949; Wagner, 1940). This value of 3.3 μg is quoted in the FAO/WHO guidelines of 1967 and 1988. Although our findings are in line with earlier data, this is the first time this method was used. Therefore, our findings need to be confirmed. The magnitude of the effect of several SLAMENGHI factors on carotenoid bioefficacy can be studied quantitatively with this stable-isotope method. This will enable a more accurate evaluation of food-based approaches to eliminating vitamin A deficiency.

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Bioavailability and bioefficacy of β-carotene measured using β-carotene and retinol, labeled with ^{13}C, in Indonesian children

3

Background: Accurate estimates of the bioefficacy of β-carotene in food are needed to formulate appropriate policies for controlling vitamin A deficiency.

Objective: To quantify the bioavailability and bioefficacy of β-carotene in oil, spinach and pumpkin using a stable isotope technique.

Design: During a 3-wk run-in period, 77 Indonesian school children consumed capsules (3/d, 7 d/wk) containing 31 μg [$^{13}C_{10}$]β-carotene and 21 μg [$^{13}C_{10}$]retinyl palmitate in oil. During a subsequent 3-wk treatment period, in addition to the capsules, children daily received either 2 portions of 82 g spinach (n = 41) or 81 g pumpkin (n = 36) containing 1.5 mg and 0.7 mg β-carotene/portion, respectively. At baseline, and at the end of the run-in and treatment periods, blood samples were drawn to measure the degree of isotopic enrichment of retinol with [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol and of β-carotene with [$^{13}C_{10}$]β-carotene using liquid chromatography-mass spectrometry.

Results: An amount of 2.7 μg (95% CI: 2.5, 2.8) β-carotene in oil has the same vitamin A activity as 1 μg retinol. The bioavailability of β-carotene in oil was 81% (95% CI: 73, 89). The bioavailability and bioefficacy of β-carotene in pumpkin were both 1.7 times (interval: 1.3, 2.4) those of β-carotene in spinach.

Conclusions: The bioefficacy of β-carotene in oil is less than that proposed by the US Institute of Medicine. The low bioavailability and bioefficacy of β-carotene from dark-green leafy vegetables compared with that of β-carotene in pumpkin is confirmed. Thus, approaches other than the promotion of the consumption of fruit and vegetables are required to eliminate vitamin A deficiency.

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Submitted

INTRODUCTION

Improved immunocompetence achieved by vitamin A supplementation in children under five years of age in developing countries has been shown to reduce morbidity and mortality by 23% (Beaton et al, 1993). Vitamin A deficiency is also a problem in other groups of the population (De Pee et al, 1998; West et al; 1999; Dijkhuizen et al, 2001). Improved vitamin A status can be achieved by either reducing the body's demand for vitamin A or by increasing the effective supply of vitamin A or by combining both these approaches. For example, the demand can be reduced by preventing and managing infectious diseases by public health measures such as immunization and improved personal hygiene (ACC/SCN, 2000). The effective supply of vitamin A, which is defined as the amount ultimately available for metabolic functions and storage, is determined by three factors: the consumption of foods and pharmanutrients (dietary supplements); the content of vitamin A or its precursor provitamin A in food or pharmanutrients consumed; and the bioefficacy of vitamin A or its precursor in the food or pharmanutrients consumed. Up until now, the bioefficacy of provitamin A carotenoids has received little attention compared to the consumption of vitamin A-containing foods and the vitamin A and provitamin A content of such foods. Animal foods, breastmilk and food fortified with preformed retinol are good sources of vitamin A because of the high content and bioavailability of vitamin A. However, the contribution of plant foods such as red palm oil, dark-green leafy vegetables, and orange fruits can only be substantial when not only the content but also the bioefficacy of provitamin A carotenoids is high (Moore, 1957). There is mounting evidence that as much as 21 μg β-carotene in a mixed diet (4:1 vegetables to fruit intake) has the same vitamin A activity as 1 μg retinol (De Pee et al, 1998; Khan et al, 1998; Tang et al, 1999). Therefore, accurate estimates on the bioefficacy of carotenoids in plant foods are required. This is important because plant foods are the major source of vitamin A in the diet of a large proportion of the world's population (IVACG, 1999).

In a recent study in 35 Indonesian children using a plateau isotopic enrichment technique, we found that 2.4 μg (95% CI: 2.1, 2.7) β-carotene has the same vitamin A activity as 1 μg retinol (**Chapter 2**). The study needed to be repeated because it was the first using this technique. In addition, there is a paucity of reliable data on the bioefficacy of β-carotene in fruit and vegetables. Isotopic tracer techniques can provide such data with high precision. Thus, the aim of this study was to demonstrate the reliability of the plateau isotopic enrichment technique and to quantify the bioavailability and bioefficacy of β-carotene in oil, spinach and pumpkin.

SUBJECTS AND METHODS

Definitions

In this paper, we use the following definitions (**Figure 3-1**): Bioavailability is the fraction of an ingested nutrient that is available for utilization in normal physiologic functions and for storage (Jackson, 1997). Bioconversion is the fraction of a bioavailable nutrient (here,

absorbed provitamin A carotenoids) that is converted to the active form of a nutrient (retinol). It is generally accepted that the gut wall is the primary site of bioconversion of provitamin A to retinol but other tissues such as liver are probably involved (Barua and Olson, 2000). Bioefficacy is the fraction of an ingested nutrient (here, dietary provitamin A carotenoids) that is absorbed and converted to the active form of the nutrient (retinol) (**Chapter 2**). Functional bioefficacy (not shown in Figure 3-1) is defined as the fraction of an ingested nutrient that performs a certain metabolic function (Brouwer et al, 2001) such as the ability of ingested provitamin A carotenoids to reverse or prevent abnormal dark adaptation of the photopigments in the retina.

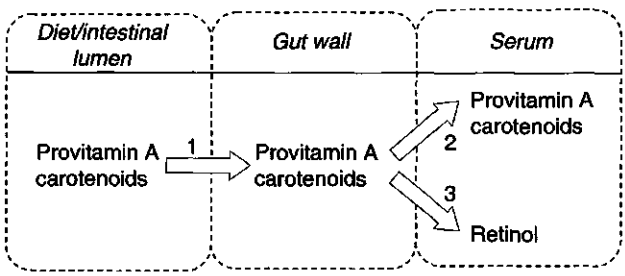

Figure 3-1. The bioavailability and bioefficacy of provitamin A carotenoids. Bioavailability = 1; bioconversion = 3; and bioefficacy = 1 × 3 (for further explanation, see text).

Subjects

The study was conducted from March to May 1999. School children aged 7–13 y (grades 2-5) from a rural village in Bogor District, West Java, Indonesia, were asked to participate in the screening (**Figure 3-2**). The purpose and procedures of the screening and of the study were explained to the parents or guardians of these children who were asked to sign an informed consent form (184 did). Eligibility was assessed in a two-step process. Firstly, based on the prevalence and intensity of infestation with intestinal parasites children were categorized into tertiles. All children in the extreme tertiles were eligible to proceeding further in the study. The purpose and procedures of the baseline and study were explained in more detail to these children and their parents. The parents were asked to sign a second informed consent form. Secondly, as a prerequisite to entering the study the children had to be apparently healthy (as assessed by a physician), after which they had to be prepared to provide a baseline blood and stool sample.

Eighty children started the 3-wk run-in period, at the end of which they were allocated randomly to receive either spinach (n = 41) or pumpkin (n = 39) during the subsequent 3-wk treatment period. To assess the effect of intestinal parasites on bioavailability and bioefficacy of β-carotene in spinach and pumpkin, children with and without parasites were distributed over both groups. We estimated that a sample size of 40 children per treatment group would be sufficient to detect a similar difference in the bioefficacy of β-carotene between spinach and pumpkin as observed by De Pee and colleagues (1998), who found that the bioefficacy of β-carotene in dark green leafy vegetables was 3.6% and that of β-carotene in orange fruit 7.8%. The Medical Ethics Committee of the Ministry of Health, Indonesia; the Indonesian Institute of Science; and the Medical Ethical Committee of the Division of Human Nutrition and Epidemiology of Wageningen University, The Netherlands approved the study.

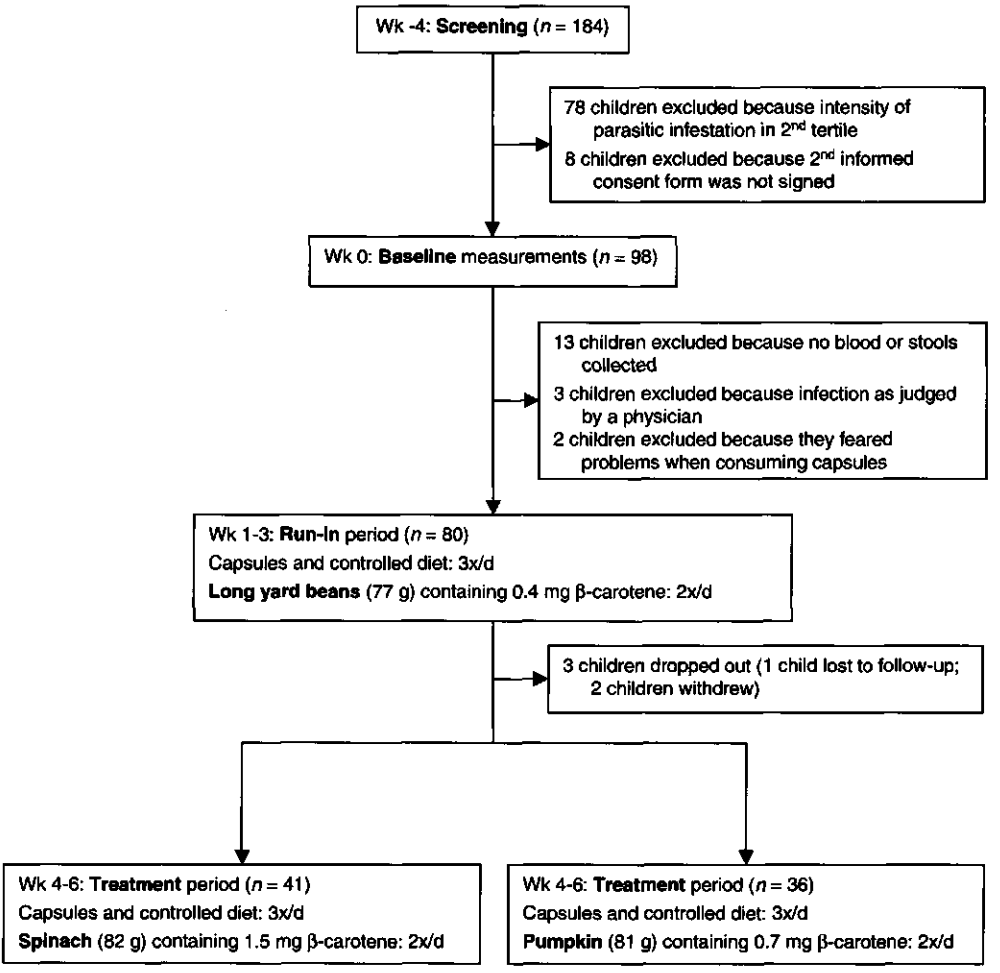

Figure 3-2. Study design. Capsules contained 31 μg [$^{13}C_{10}$]β-carotene and 21 μg [$^{13}C_{10}$]retinyl palmitate. The basal diet was low in retinol and carotenoids. At baseline and at the end of the run-in and treatment periods, blood and stool samples were collected. All children entering the treatment period completed the study.

Study type and intervention

The study was a randomized controlled dietary trial with two treatment arms - spinach and pumpkin - and a parallel design (Figure 3-2). During the 3-wk run-in period and subsequent 3-wk treatment period, each child received capsules (3/d for 7 d/wk) containing 31 μg [12,13,14,15,20,12',13',14',15',20'-$^{13}C_{10}$]β-carotene (analyzed value) and 21 μg [8,9,10,11,12,13,14,15,19,20-$^{13}C_{10}$]retinyl palmitate (calculated based on analysis; equivalent to 12 μg retinol). Children consumed the capsules with a low-retinol, low-carotenoid meal 3 times/d. During the run-in period, the lunch and afternoon meal were each supplemented with a portion of 77 g long-yard beans, containing 0.4 mg β-carotene and no other provitamin A carotenoids/portion (analyzed value). During the treatment period, the long

yard beans at the two meals provided were replaced by either 82 g spinach (spinach group) or 81 g pumpkin (pumpkin group). Spinach and pumpkin contained 1.5 and 0.7 mg β-carotene and 64 and 302 μg other provitamin A carotenoids, respectively (analyzed values). During the study, members of the research team supervised and recorded compliance with consumption of the capsules and meals. Main outcome measures of the study were the dilution of the degree of isotopic enrichment of retinol and β-carotene in serum during the treatment period (compared with the run-in period). In addition, the bioavailability and bioefficacy of β-carotene in oil was assessed using data from the run-in period.

Measurements

During the screening, we recorded age and sex of the children. Children provided a single stool sample that was examined for protozoan cysts and worm eggs. During the baseline measurements, a physician examined the children and we measured each child's weight, height and mid-upper-arm-circumference (MUAC). Children provided two stool samples four days apart that were examined for protozoan cysts and worm eggs. Children also recorded their food intake in a diary for 6 consecutive days. Dieticians interviewed each child four times during this period to improve the quality of the data in the diaries. Three days (half of the group) or two days (the other half of the group) before the start of the run-in period, blood samples were drawn from apparently healthy children (as judged by a physician) and we measured the weight, height and MUAC of the children. In the blood samples we counted leukocytes and measured hemoglobin concentrations and hematocrit. In serum, we measured concentrations of retinol and carotenoids and the degree of isotopic enrichment of retinol and β-carotene.

During the study, children recorded daily in a diary which and how much foods and drinks not provided they had consumed. Dieticians interviewed the children every other day about their food intake. In the last week of the run-in period and of the treatment period, children provided stool samples four days apart. The last two days of the run-in period and of the treatment period, blood samples were drawn from apparently healthy children (one sample/child/period). The same measurements were made in these blood and stool samples as in those collected during the baseline measurements.

To assess the time required for the degree of isotopic enrichment of β-carotene and retinol in serum to reach a plateau, on day 8 and 29, an additional blood sample was drawn from each of a sub-sample of 6 children. In serum from these samples, the concentrations of retinol and various carotenoids and the degree of isotopic enrichment of retinol and β-carotene were measured.

Meal preparation and food consumption

Ten village health workers prepared all meals throughout the study. For each ingredient, the recipes listed the amount to be purchased and the cleaned amount to be cooked. The amount of food to be distributed was listed for each dish. There were four different menus for the basal diet, with fixed combinations of breakfast lunch and an afternoon meal for Monday and Thursday; for Tuesday and Friday; for Wednesday and Saturday; and for

Sunday. Breakfast, lunch and an afternoon meal were served at 0700, 1100 and 1500, respectively. At school, members of the research team recorded for each child when not all of a dish of a meal was eaten. Then the amount consumed was presumed to be half the amount provided. Children had to consume all the spinach and pumpkin completely before they were dismissed.

Methods

Preparation of capsules

The labeled compounds were synthesized at the Leiden Institute of Chemistry (Lugtenburg et al, 1999) and were dissolved in highly unsaturated sunflower oil (>82% oleic acid and >10% linoleic acid; Hozol RBDW; Contined BV, Bennekom, Netherlands). δ-/-α-Tocopheryl acetate (Roche Nederland BV, Mijdrecht, Netherlands) was added to the oil as an antioxidant. Capsules were made from bovine gelatin (Capsugel, Bornem, Belgium) and were filled with the oily mixture by multipipette. Each capsule contained 0.35 g oil and 0.12 mg vitamin E. The oily mixture and the capsules were prepared under subdued light. The capsules and their content were food grade (**Chapter 2**).

Estimation of energy and nutrient intakes

Concentrations of retinol and β-carotene in the capsules were analyzed by HPLC (Hulshof et al, 1997). To measure the nutrient content of meals and vegetable supplements (long yard beans, spinach and pumpkin), duplicate samples were collected after preparation and portioning of the food. Once per week, a duplicate sample of rice was collected. Duplicate samples of the vegetable supplements were collected on 4 days each week. Four duplicate samples of the menus were collected each week in such a way that in each 3-wk period, each menu was sampled 3 times. Duplicate samples of all dishes of all three meals of a collection day were weighed before and after removal of the inedible portion (bones, peels, etc). The edible portions were then pooled (0.5 mL of a 10% (w/v) t-butylhydroquinone solution in ethanol was added per 100 g sample), blended and portioned into 5 labeled plastic bags and stored at -20 °C. Duplicate portions of rice and vegetable supplements were prepared using the same procedure. All bags were stored for 2 mo before being packed on dry ice and transferred to Wageningen. There the samples were stored for 1 mo at -80 °C until analysis for moisture and ash content, protein concentration (Kjeldahl method using a conversion factor of 6.25) (Osborne and Voogt, 1978), and fat by the Folch method (Osborne and Voogt, 1978; Folch et al, 1957). Carbohydrate content was calculated by difference. The energy content (kJ) of the food provided was calculated by multiplying the weight (g) of fat, protein, and carbohydrates by 37, 17, and 17, respectively. The concentrations of retinol and various carotenoids in food were analyzed by HPLC (Hulshof et al, 1997). All 3 duplicate samples of each menu collected within each period were pooled before analysis, thus resulting in one composite sample per menu per period. All 12 duplicate samples of long yard beans were pooled before analysis, resulting in one composite sample of long yard beans. All spinach and pumpkin supplements collected within each week were pooled before analysis, thus resulting in 3 composite samples for each vegetable. Time and conditions of storage were adequate to obtain reliable results for the analyses

carried out in food and serum (Comstock et al, 1993; Craft et al, 1988; Greenfield and Southgate, 1992).

Energy and nutrient intakes at baseline and during the study were calculated by using the diaries, records of attendance and consumption of the menus and supplements, and a computer program suite (KOMEET, version 2.0c, and VBS-EDIT, version 1.0; B-Ware Nutrition Software, Wageningen, Netherlands) with a nutrient database (Bg95k99t; **Chapter 2**) based on that developed by de Pee et al (De Pee et al, 1996) to which the energy and nutrient contents of the foods provided (on the basis of the duplicate analyses) were added.

Collection and analysis of blood and serum

Nonfasting blood samples (8-mL) were collected between 0800 and 1000 from an antecubital vein. Immediately after blood collection, a portion of whole blood was removed to count leukocytes (Pijlman and Sanders, 1976) and to measure hemoglobin concentrations and hematocrit (Wintrobe, 1968). The remaining blood was placed on ice, protected from light, and centrifuged within 3 h (750 × *g* for 10 min at room temperature) at the Nutrition Research and Development Centre. Serum was divided in 1- and 2-mL aliquots in nalgene tubes, stored in containers, and frozen at -20 °C.

Two months after completion of the study, all containers with serum were packed on dry ice and transferred to Wageningen. There the samples were stored for 2 mo at -80 °C until analyzed for concentrations of retinol and various carotenoids by HPLC (Craft et al, 1992). Containers were stored for 1 mo before being packed on dry ice and transferred to Chicago. There the samples were stored for 4 mo at -80 °C until analyzed for the degree of isotopic enrichment of retinol (Van Breemen et al, 1998; Wang et al, 2000) and β-carotene (Wang et al, 2000) by HPLC coupled with atmospheric pressure chemical ionization liquid chromatography-mass spectrometry (APCI LC-MS). For the LC-MS analysis, retinol and β-carotene were extracted from 0.2- and 1.0-mL serum samples, respectively, and analyzed by reversed-phase HPLC with a C_{30} column interfaced to a mass spectrometer equipped with positive ion APCI. To assess the ratio of labeled to unlabeled β-carotene, selected ion monitoring was carried out at mass-to-charge ratios (m/z) of 537 and 547. These ions corresponded to circulating β-carotene and orally administered [$^{13}C_{10}$]β-carotene, respectively. To assess the ratio of labeled to unlabeled retinol, selected ion monitoring was carried out at m/z 269, 274, and 279. These abundant fragment ions corresponded to the loss of water from the protonated molecule of circulating retinol, [$^{13}C_5$]retinol (metabolically formed from orally administered [$^{13}C_{10}$]β-carotene), and [$^{13}C_{10}$]retinol (formed by hydrolysis of orally administered [$^{13}C_{10}$]retinyl palmitate), respectively.

Anthropometry and collection and analysis of stool samples

While the children wore a school uniform but no shoes, weight was measured to the nearest 0.1 kg with a digital electronic scale (770 alpha; Seca, Hamburg, Germany) and height and MUAC were measured to the nearest 0.1 and 0.05 cm, respectively, with an appropriate microtoise. The presence of protozoan cysts and worm eggs was diagnosed in stools by the Ridley concentration method. The load of worm eggs was quantified by the Kato Katz

Table 3-1. Description of the β-carotene and retinol at plateau isotopic enrichment (CarRet PIE) mathematical model (**Chapter 2**)

Symbol	Description	Derivation	Run-in period		Treatment period	
			Spinach group (n = 41)	Pumpkin group (n = 36)	Spinach group (n = 41)	Pumpkin group (n = 36)
a	Retinol intake from diet (μmol/d)[1]		0	0	0	0
b	Retinol intake from capsules (μmol/d)		0.119 (0.116, 0.122)[2]	0.120 (0.118, 0.123)[†]	0.120 (0.118, 0.122)[*]	0.123 (0.122, 0.124)[*†]
d	β-Carotene intake from diet (μmol/d)[1]		1.43 (1.35, 1.52)[†]	1.37 (1.24, 1.50)[†]	5.55 (5.43, 5.68)[*†]	3.35 (3.32, 3.37)[*†]
e	β-Carotene intake from capsules (μmol/d)		0.163 (0.159, 0.168)	0.166 (0.162, 0.169)[†]	0.165 (0.162, 0.168)[*]	0.170 (0.168, 0.171)[*†]
C_{sR}	Total retinol concentration in serum (μmol/L)		0.80 (0.73, 0.87)	0.79 (0.73, 0.86)	0.78 (0.72, 0.85)	0.83 (0.78, 0.87)
$E_{10,sR}$	Enrichment of retinol in serum with [$^{13}C_{10}$]retinol	$\frac{M_{279,sR}}{M_{269,sR} + M_{274,sR} + M_{279,sR}}$ [3]	0.0479 (0.0412, 0.0546)	0.0503 (0.0427, 0.0579)	0.0475 (0.409, 0.541)	0.0499 (0.0446, 0.0552)
$E_{5,sR}$	Enrichment of retinol in serum with [$^{13}C_5$]retinol	$\frac{M_{274,sR}}{M_{269,sR} + M_{274,sR} + M_{279,sR}}$	0.0453 (0.0383, 0.0524)	0.0494 (0.0407, 0.0581)[†]	0.0450 (0.0376, 0.0523)	0.0445 (0.0385, 0.0504)[†]
C_{sC}	Total β-carotene concentration in serum (μmol/L)		0.17 (0.15, 0.20)[†]	0.15 (0.13, 0.17)[†]	0.25 (0.21, 0.29)[*†]	0.44 (0.39, 0.48)[*†]
$E_{10,sC}$	Enrichment of serum β-carotene with [$^{13}C_{10}$]β-carotene	$\frac{M_{547,sC}}{M_{537,sC} + M_{547,sC}}$	0.1411 (0.1184, 0.1628)[†]	0.1630 (0.1388, 0.1872)[†]	0.0850 (0.0676, 0.1023)[*†]	0.0412 (0.0313, 0.0510)[*†]
$F_{dCo/dR}$	Vitamin A activity of β-carotene in oil compared with that of retinol in oil (mol-to-mol basis)	$(E_{5,sR1}/E_{10,sR1}) \times (b_1/e_1)$ [4]	0.713 (0.663, 0.763)[5]	0.700 (0.652, 0.748)	-	-
		Average of total group	0.707 (0.673, 0.741)[5]		-	-
A_{dCo}	Compared to 1 μg retinol, amount (μg) of β-carotene in oil that has the same vitamin A activity	$\left(1/\overline{F}_{dCo/dR}\right) \times (537/286)$ [6]	2.7 (2.5, 2.8)		-	-

B_{dCo}	Bioefficacy (%) of β-carotene in oil	$(X_{dC} \times Y_{dC})/\overline{A}_{dCo}$ [7]	35	-	-
BV_{dCo}	Bioavailability (%) of β-carotene in oil compared with that of retinol in oil	$\frac{[(E_{10,sC1} \times C_{sC1}) + (StC \times E_{5,sR1} \times C_{sR1})] \times b_1}{(E_{10,sR1} \times C_{sR1}) \times a_1} \times 100$ [8]	78 (67, 91)[9]	83 (72, 96)	-	-
		Average of total group	81 (73, 89)[9]		-	-
F_{dCs_p}	Relative vitamin A activity of β-carotene in spinach and pumpkin	$\left[\frac{(E_{5,sR2} \times d_2)}{e_2}\right]_{\overline{sp}} \Big/ \left[\frac{(E_{5,sR2} \times d_2)}{e_2}\right]_{\overline{pu}}$	-	-	1.7 (1.3, 2.3)[*10]
BV_{dCs_p}	Relative bioavailability of β-carotene in spinach and pumpkin	$\frac{\left\{\frac{[(E_{10,sC2} \times C_{sC2}) + (StC \times E_{5,sR2} \times C_{sR2})] \times d_2}{e_2}\right\}_{\overline{sp}}}{\left\{\frac{[(E_{10,sC2} \times C_{sC2}) + (StC \times E_{5,sR2} \times C_{sR2})] \times d_2}{e_2}\right\}_{\overline{pu}}}$	-	-	1.7 (1.3, 2.4)[*10]

[1] Intake from food provided is shown here. Retinol in food provided was below the limit of detection (thus, <3 ng/d). β-Carotene intake is the sum of the intake of β-carotene and half the intake of other provitamin A carotenoids (see **Table 3-3**).

[2] $\overline{x}$, 95% CI.

[3] Where $M_{279,sR}$ is the signal measured by liquid chromatography-mass spectrometry at *m/z* 279. Values at baseline are subtracted from values at the end of the run-in and treatment periods.

[4] Figures 1 and 2 in the subscripts refer to the period from which data were used (1 = run-in and 2 = treatment period).

[5] Data from one child were excluded based on 'extreme studentized deviate' statistics (Rosner, 1995), thus $n = 76$.

[6] Where $\overline{F}_{dCO/dR}$ is the average of values from all children. The molecular weight of β-carotene is 537 and that of retinol is 286.

[7] Where $\overline{A}_{dCo}$ is the average vitamin A activity. X_{dC} is the maximal bioefficacy of β-carotene (100%) if absorption and conversion are both 100%. Y_{dC} is the amount of β-carotene having the same vitamin A activity as 1 μg retinol if the X_{dC} is 100%, 0.94 μg.

[8] Where *StC* refers to the stoichiometry of the cleavage of β-carotene to retinol in the body. We assume the most efficient stoichiometry, i.e. 1 μmol β-carotene yields 2 μmol retinol, thus obtaining minimum estimates for the bioavailability. With this stoichiometry, $StC = 0.5$.

[9] Data from four children were excluded based on 'extreme studentized deviate' statistics (Rosner, 1995), thus $n = 73$. The remaining data were slightly positively skewed, thus data were transformed by taking the square root after which the geometric mean was calculated.

[10] Data are expresses as $\overline{x}$ with an interval of spread. The ratio was calculated using the means of the numerator and denominator. Interval of spread of this ratio was calculated using the upper and lower 95% CI limits of the means of the numerator and denominator (Sokal and Rohlf, 1997). A ratio > 1 indicates that the bioefficacy of β-carotene in spinach is less than that of β-carotene in pumpkin.

* Values within one row within one period differ significantly between the spinach and pumpkin groups ($P < 0.05$).

† Values within one row within one group differ significantly between the run-in and treatment groups ($P < 0.05$).

method (Polderman et al, 1993). Data from two samples collected four days apart were averaged.

Calculation of the bioavailability and bioefficacy of β-carotene

From the degree of isotopic enrichment in serum of β-carotene with [$^{13}C_{10}$]β-carotene and retinol with [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol at plateau isotopic enrichment, during the run-in period, the bioavailability and bioefficacy of β-carotene in oil were calculated using the CarRet PIE mathematical model (**Chapter 2; Table 3-1**). This plateau can be reached during prolonged consumption of multiple doses of [$^{13}C_{10}$]retinol and [$^{13}C_{10}$]β-carotene. In comparison to our previous study, we extended the CarRet PIE mathematical model to quantify the bioavailability of β-carotene in oil, for which we assumed the most efficient stoichiometry possible, i.e., 1 mol β-carotene would yield 2 mol retinol, thus obtaining estimates of the minimum bioavailability.

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

Statistical methods

Data are shown as means and 95% CI or SDs (in the case of descriptive measures). The run-in period serves as a control for the treatment period, therefore, data collected during the run-in period are shown separately for each treatment group. Because they might be related to the outcome parameters of the treatment period, results obtained using data from the run-in period were compared between the spinach and pumpkin groups for the following variables: intake of retinol and carotenoids from food provided, concentrations in serum of retinol and carotenoids, degree of isotopic enrichment in serum of retinol and β-carotene, parasite status, bioavailability of β-carotene in oil and vitamin A activity of β-carotene in oil. Bioavailability and vitamin A activity of β-carotene in oil were compared between those with and without intestinal parasites. Results obtained using data from the treatment period were compared between the spinach and pumpkin groups for the following variables: intake of retinol and carotenoids from food provided, concentrations in serum of retinol and carotenoids, degree of isotopic enrichment in serum of retinol and β-carotene, parasite status, bioavailability and vitamin A activity of β-carotene in vegetable supplements. Within each treatment group, the bioavailability and vitamin A activity of β-carotene in vegetable supplements was compared between those with and without intestinal parasites. For all comparisons independent *t*-tests and Mann-Whitney *U* tests were carried out for

normally and not normally distributed variables, respectively. Within each treatment group, paired *t*-tests and Wilcoxon tests were carried out - for normally and not normally distributed variables, respectively - to compare the following variables between the run-in and treatment periods: intake of retinol and carotenoids from food provided, concentrations in serum of retinol and carotenoids, and degree of isotopic enrichment in serum of retinol and β-carotene.

In our previous study (**Chapter 2**), plateau isotopic enrichment was reached by day 21. To assess the time required for the isotopic enrichment of β-carotene and retinol in serum to reach a plateau, a one-sample *t*-test was used to test whether the regression coefficient of the vitamin A activity of β-carotene in oil compared with that of retinol in oil obtained using data from day 8 and day 21 differed from 0.

The main outcome parameters, bioavailability and bioefficacy were screened for outliers using the 'extreme studentized deviate' statistics to detect many outliers (Rosner, 1995). For these parameters, suitable transformations were used to calculate geometric means if they were not normally distributed. All tests were two-sided and *P* values <0.05 were considered significant. The computer package SPSS (version 10.0.5; SPSS Inc, Chicago) was used for all statistical calculations.

RESULTS

Data are presented for 77 of the 80 children enrolled because two children of the pumpkin group withdrew from the study and one child did not follow the experimental regimen (**Table 3-2** on page 62). Blood was drawn only on days on which a child did not show signs of infection as judged by a physician. The range of leukocyte counts (0.4-1.25 x 10^{10}/L) was within the normal range for children (On-line Medical Dictionary, 1997). Based on these findings and on the absence of signs of an infection at clinical examination, no child was excluded from data analyses. In both periods and in both groups, the compliance to the capsule consumption was >94%, which means that the children followed the experimental regimen very conscientiously.

Energy and nutrient intakes

During the whole study, children obtained >70% of their daily energy intake from the food provided. The remaining daily energy intake derived from food not provided by us. We will only use data on energy and nutrient intakes from food provided (menus of the basal diet, supplements and capsules) to describe the energy and nutrient intakes during this study (**Table 3-3**). Estimates of the energy and nutrient intakes from food not provided by us were based on data from diaries and calculated using a nutrient database. Therefore, these estimates are less accurate and precise than food intake based on food provided (because intake was supervised and recorded by the research team and the nutrient composition was measured in duplicate samples).

No retinol was detected in the duplicate samples (the limit of detection was 3.5 ng/kg food, thus intake was <3 ng/d). During the treatment period, portion sizes of the menus of

Table 3-3. Daily energy and nutrients from food (menus and supplements) and capsules provided during the run-in and treatment period[1]

	Run-in period				Treatment period			
	Spinach group (n = 41)		Pumpkin group (n = 36)		Spinach group (n = 41)		Pumpkin group (n = 36)	
	Long yard beans	Total[2]	Long yard beans	Total	Spinach	Total	Pumpkin	Total
Energy (MJ)	0.3	5.0	0.2	4.8	0.4	4.4	0.6	4.6
	(0.2, 0.3)[3]	(4.8, 5.2)[4]	(0.2, 0.3)	(4.5, 5.1)[4]	(0.4, 0.4)	(4.2, 4.6)[4]	(0.6, 0.6)	(4.3, 4.9)[4]
Fat (g)[5]	5	32	4	31	8	31	2	25
	(4, 5)	(31, 34)[4] [24]	(4, 5)	(28, 33)[4] [24]	(8, 8)	(30, 32)[4] [26]	(2, 2)	(23, 27)[4] [20]
Protein (g)[5]	2	42	2	39	3	37	1	35
	(2, 2)	(40, 44) [14]	(2, 2)	(36, 43) [14]	(3, 3)	(35, 39) [14]	(1, 1)	(32, 38) [13]
Carbohydrates (g)[5]	3	181	3	175	2	152	28	182
	(3, 3)	(175, 187) [62]	(3, 3)	(166, 185) [62]	(2, 2)	(146, 159) [59]	(27, 28)	(172, 193) [67]
β-Carotene (µg)	653	812	621	781	2849	2985	1428	1566
	(609, 697)	(765, 859)[4]	(555, 687)	(713, 848)[4]	(2786, 2912)	(2919, 3051)[4]	(1416, 1440)	(1554, 1578)[4]
α-Carotene (µg)	9	87	8	85	85	133	587	634
	(8, 9)	(84, 90)	(7, 9)	(82, 89)	(84, 87)	(130, 136)	(580, 593)	(628, 640)
β-Cryptoxanthin (µg)	4	4	4	4	38	38	9	9
	(4, 4)	(4, 4)	(3, 4)	(3, 4)	(37, 39)	(37, 39)	(9, 9)	(9, 9)
Lutein (µg)	454	649	431	623	4086	4232	1306	1450
	(423, 484)	(614, 685)	(386, 477)	(572, 675)	(3994, 4178)	(4137, 4327)	(1296, 1316)	(1438, 1461)
Zeaxanthin (µg)	32	32	30	30	276	276	0[6]	0[6]
	(29, 34)	(29, 34)	(27, 34)	(27, 34)	(269, 282)	(269, 282)		
Lycopene (µg)	60	60	57	57	86	86	40	40
	(56, 64)	(56, 64)	(51, 63)	(51, 63)	(84, 88)	(84, 88)	(39, 40)	(39, 40)

[1] Energy and nutrient intakes were calculated by using the records of attendance at meals, the records of leftovers, and a computer program to which the energy and nutrient contents of the foods provided (on the basis of the duplicate analyses) were added. Retinol intake in duplicate samples food provided was below the limit of detection of ≈ 3.5 pg/g food.

[2] Sum of energy and nutrient intakes from the menus, supplements and capsules.

[3] $\bar{x}$; 95% CI.

[4] Small amounts of the total energy and nutrient intake derived from the capsules: during the run-in period, on average 37 kJ energy, 1 g fat, and 88 µg β-carotene per day; and during the treatment periods, on average 37 kJ energy, 1 g fat, and 90 µg β-carotene per day. During the run-in and treatment periods, daily retinol intake from the capsules was on average 34 and 35 µg, respectively.

[5] Proportion of energy (%) is given in brackets.

[6] Below the limit of detection.

Table 3-4. Concentrations (μmol/L) of various carotenoids other than β-carotene in serum during the run-in and treatment period

	Run-in period		Treatment period	
	Spinach group (n = 41)	Pumpkin group (n = 36)	Spinach group (n = 41)	Pumpkin group (n = 36)
α-Carotene	0.08 (0.07, 0.09)[1]	0.07 (0.06, 0.08)[†]	0.08 (0.07, 0.09)[*]	0.25 (0.23, 0.28)[*†]
β-Cryptoxanthin	0.13 (0.11, 0.16)	0.11 (0.08, 0.14)	0.12 (0.08, 0.15)	0.12 (0.09, 0.16)
Lutein	0.25 (0.22, 0.28)[†]	0.24 (0.22, 0.27)[†]	0.46 (0.41, 0.52)[*†]	1.16 (1.05, 1.27)[*†]
Zeaxanthin	0.04 (0.03, 0.05)	0.04 (0.03, 0.05)[†]	0.04 (0.03, 0.04)[*]	0.06 (0.05, 0.07)[*†]
Lycopene	0.02 (0.02, 0.03)	0.02 (0.01, 0.03)[†]	0.02 (0.01, 0.03)	0.02 (0.01, 0.02)[†]

[1] $\bar{x}$; 95% CI.

[*] Values within one row within one period differ significantly between the spinach and pumpkin groups ($P < 0.05$).

[†] Values within one row within one group differ significantly between the run-in and treatment periods ($P < 0.05$).

Table 3-2. Characteristics of the population at baseline[1]

	Spinach group (n = 20 girls, 21 boys)	Pumpkin group (n = 18 girls, 18 boys)
Age (y)	9.4 ± 1.4[2]	9.7 ± 1.4
Weight (kg)	23.9 ± 4.9	24.2 ± 4.6
Height (m)	1.25 ± 0.08	1.25 ± 0.08
MUAC (cm)	18.3 ± 1.7	18.7 ± 1.6
Serum retinol concentration (μmol/L)	0.85 ± 0.25	0.79 ± 0.18
Serum β-carotene concentration (μmol/L)	0.15 ± 0.08	0.13 ± 0.08
Hemoglobin concentration (g/L)	102.1 ± 8.0	99.9 ± 6.8
Hematocrit (L/L)	0.32 ± 0.03	0.31 ± 0.02
Parasitic infestation (% with positive stool)[3]	73	75
Ascaris lumbricoides	10	22
Trichuris trichuria	63	58
Entamoeba hystolitica	29	11
Giardia intestinalis	7	3
Other cysts[4]	5	3
Egg load in infested children (epg)[3]		
Ascaris lumbricoides	770 (25, 8590)[5]	170 (25, 1460)[5]
Trichuris trichuria	413 (113, 1438)[5]	388 (163, 900)[5]

[1] There were no differences between groups.

[2] $\bar{x}$ ± SD.

[3] No stool was available from 3 children in the spinach group (n = 38) and from 1 child in the pumpkin group (n = 35). Parasitic infestation was diagnosed by the Kato Katz and Ridley method (Polderman et al, 1993). For those infested with *Ascaris lumbricoides* and *Trichurius trichuria* egg load was quantified by the Kato Katz method (Polderman et al, 1993). Infestation with hookworm was not found in the feces of these children.

[4] Other cysts as diagnosed by the Ridley method (Polderman et al, 1993) were *Entamoeba coli* and *Endolimax nana*.

[5] Median (25th, 75th percentile).

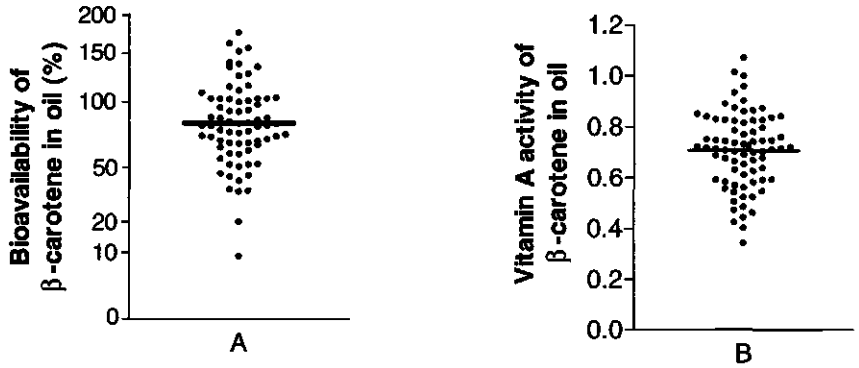

Figure 3-3. Bioavailability (A, n = 73) and vitamin A activity (on a molar basis) (B, n = 77) of β-carotene in oil compared with that of retinol in oil. The vitamin A activity of 2.7 μg β-carotene in oil (mean value) is equivalent to that of 1 μg retinol. Individual data are shown and the solid lines are the geometric mean bioavailability (A) and mean vitamin A activity (B).

the basal diet were approximately 30 g/d lower than during the run-in period, consequently, energy and nutrient intakes were also lower. The differences in energy and nutrient intakes from the vegetable supplements can be explained by the different vegetables and recipes used for the preparation of long yard beans, spinach and pumpkin. Before the study began, the acceptance of different recipes was tested by 5 children. During the treatment period, the intake of carotenoids from the basal diet did not differ between the spinach and pumpkin groups. The intake of carotenoids was, as intended, higher during the treatment period than during the run-in period. Intake of β-carotene from spinach was almost twice that of β-carotene from pumpkin. Intake of α-carotene from pumpkin was more than four times that of α-carotene from spinach. Intake of β-cryptoxanthin was higher from spinach than from pumpkin, but intakes were low. For calculations of the bioavailability and bioefficacy of β-carotene in spinach and pumpkin, the sum of the intake of β-carotene and half of the intake of other provitamin A carotenoids (here, α-carotene and β-cryptoxanthin) was calculated (Table 3-1). During the treatment period, 97 and 82% of the provitamin A intake were derived from β-carotene in spinach and pumpkin, respectively.

Bioavailability and bioefficacy of β-carotene

During the run-in period, the concentrations in serum of retinol and carotenoids did not differ between the spinach and pumpkin groups. During the treatment period, serum concentrations of β-carotene and lutein increased in both groups while in the pumpkin group, serum concentrations of α-carotene and zeaxanthin also increased (**Table 3-4**). During the treatment period, serum concentrations of β-carotene, α-carotene, lutein and zeaxanthin but not of retinol differed significantly between the pumpkin and spinach groups.

The degree of isotopic enrichment in serum of retinol with [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol and of β-carotene with [$^{13}C_{10}$]β-carotene during the run-in period did not differ between the spinach and pumpkin groups (Table 3-1). In both groups, the degree of isotopic enrichment of β-carotene in serum decreased significantly during the treatment period. This decrease was larger in the pumpkin group than in the spinach group. In the pumpkin group, the isotopic enrichment of retinol with [$^{13}C_5$]retinol but not with [$^{13}C_{10}$]retinol decreased significantly during the treatment period but this decrease was small.

The bioavailability and vitamin A activity of β-carotene in oil compared with that of retinol in oil are shown in Table 3-1 for the total group while individual data are shown in **Figure 3-3**. For the determination of vitamin A activity and of bioavailability, data were omitted from one child and 4 children, respectively, because these data were regarded as outliers (Rosner, 1995). Because the bioavailability of β-carotene in oil showed a slightly positively skewed distribution, data were transformed by calculating the square root of each value after which the geometric mean was calculated. The bioavailability of β-carotene in oil compared with that of retinol in oil was 81% (95% CI: 73, 89). On a molar basis, the vitamin A activity of β-carotene in oil compared with that of retinol in oil was 0.707 (95% CI: 0.673, 0.741). This means that, 2.7 μg (95% CI: 2.5, 2.8) β-carotene in oil has the same vitamin A activity as 1 μg retinol. From the results obtained using data from a sub-sample of 6 children, it can be calculated that the coefficient of the regression of the vitamin A activity of β-carotene in oil compared with that of retinol in oil on day 8 and 21 differed significantly

from 0. Thus, in these children isotopic enrichment of retinol and β-carotene in serum reached a plateau after day 8. In our previous study (**Chapter 2**), plateau isotopic enrichment was reached by day 21.

Despite the consumption of large amounts of β-carotene in spinach and pumpkin in the treatment period, the degree of isotopic enrichment in serum of retinol did not change during the treatment period. Therefore, the mathematical model could not be used to obtain absolute data on the bioavailability and bioefficacy of β-carotene in spinach and pumpkin. However, the relative bioavailability and vitamin A activity were estimated as described in Table 3-1. The ratio was calculated using the means of the numerator and denominator. Interval of spread of this ratio was calculated using the upper and lower 95% CI limits of the means of the numerator and denominator (Sokal and Rohlf, 1997). The bioavailability of β-carotene in pumpkin was 1.7 (interval: 1.3, 2.4) times that of β-carotene in spinach. The vitamin A activity, and thus the bioefficacy, of β-carotene in pumpkin also was 1.7 (interval: 1.3, 2.3) times that of β-carotene in spinach.

Parasitic infestation and bioavailability and bioefficacy of β-carotene

Parasite status as assessed in feces samples obtained during the baseline measurements showed that classification of children according to parasite status as assessed during the screening (a month before the start of the study) could not be maintained. At baseline, the prevalence of intestinal parasites was 74%. During the run-in and treatment periods, the prevalence of intestinal parasites further increased to a prevalence of 84 and 81%, respectively. During both periods, the prevalence did not differ between the spinach and pumpkin groups. For 77% of the children infested with *Ascaris lumbricoides*, the intensity of the infection was light, for the remaining children the intensity was medium. For 70% of the children infested with *Trichuris trichuria*, the intensity of the infestation was light, for the remaining children the intensity was medium (WHO, 1987). The bioavailability and vitamin A activity of β-carotene in oil did not differ significantly between those with and without intestinal parasites during the run-in period. During the treatment period, within each group, the bioavailability and vitamin A activity of β-carotene in spinach and pumpkin did not differ significantly between those with and without intestinal parasites. However, because the prevalence of intestinal parasites was much higher than expected, no conclusions can be drawn on the effect of intestinal parasites on bioavailability and bioefficacy.

Anthropometry and hematological parameters

Over the course of the study (compared with baseline), in both groups the children grew 1 cm in length, increased 2 mm in MUAC, and increased almost 1 kg in weight. Throughout the study, hemoglobin concentrations were comparable in both groups. At the end of the run-in and treatment periods, mean concentrations were 111.3 and 119.8 g/L, respectively. Hematocrit values were consisted with hemoglobin concentrations. At the end of the run-in and treatment periods, mean values were 0.357 and 0.365 L/L, respectively.

DISCUSSION

In this study of Indonesian school children, the bioavailability of β-carotene in oil was 81% and the vitamin A activity of 2.7 μg β-carotene in oil was equivalent to that of 1 μg retinol. Furthermore, the bioavailability and bioefficacy of β-carotene in pumpkin were both 1.7 times those of β-carotene in spinach.

The bioavailability and bioefficacy of β-carotene in oil

Much of our knowledge on the bioefficacy of β-carotene in foods comes from a two-step process. Firstly, examining the bioefficacy of β-carotene in oil compared to that of retinol in oil. Secondly, examining the relative bioavailability of β-carotene in food compared to that of β-carotene in oil. Prior to our previous study (**Chapter 2**) and the present study, data for the first step have been derived from five studies in humans (**Table 3-5**). The most influential of the five studies was the 'Sheffield Experiment' conducted during World War II and described by Hume & Krebs in 1949. Dark adaptation was used as a functional indicator of bioefficacy. From this study it was concluded that the functional bioefficacy of β-carotene in oil was 24%. Two other depletion-repletion studies (Booher et al, 1939; Wagner, 1940), also found a functional bioefficacy of 24-27%. In the 1970s, Sauberlich and colleagues (1974) found a functional bioefficacy of 47%. A major disadvantage of these studies on the functional bioefficacy is that they yield only crude estimates because only stepwise increased doses of retinol and β-carotene were tested and compared. The first study to assess the bioefficacy of β-carotene in oil was published in 2000 by Tang and colleagues (Tang et al, 2000). From this study, it was concluded that from a dose of 6 mg and 126 mg β-carotene in oil, 3.8 μg and 55 μg β-carotene, respectively, have the same vitamin A activity as 1 μg retinol. These amounts corresponds to a bioefficacy of β-carotene in oil of 25% (low dose) and 1.7% (high dose). Recently, the Institute of Medicine (IOM) of the National Academy of Sciences of the USA (2001) revised the estimates of the relative efficiency of β-carotene and retinol to meet the recommended vitamin A intakes. In fact, for the bioefficacy of β-carotene in oil (Table 3-5), the IOM drew only on the functional bioefficacy study of Sauberlich and colleagues (1974) which is at variance with the other functional bioefficacy studies (Table 3-5). The IOM did not quote the papers by Booher et al (1939) and Wagner (1940). In addition, the first release of the IOM report (2001) contained two errors. Firstly, the bioefficacy, as calculated from data from the 'Sheffield Experiment', was overestimated by a factor of 2 because it was assumed that 1 IU of retinol and of β-carotene had the same weight. Secondly, from the study of Tang and colleagues (2000), the amount of β-carotene in oil that has the same vitamin A activity as 1 μg retinol was reported to be 2.6 μg based on a dose of 6 mg (published value was 3.8, see above). It would be interesting to know whether the IOM (2001) would have proposed a bioefficacy of β-carotene in oil of 47% if they were aware of this information at the time of their deliberations. At the time this thesis went to press, discussions were being held with the Institute of Medicine to resolve this issue.

In our previous stable isotope study in 35 Indonesian children (**Chapter 2**), we found that 2.4 μg (95% CI: 2.1, 2.7) β-carotene in oil has the same vitamin A activity as 1 μg retinol, which corresponds to a bioefficacy of 39%. The proportion of *all-trans*-β-carotene in

Table 3-5. Estimates of bioefficacy of β-carotene in oil[1]

Reference	Subjects (country)	Study design	Diet/dose/d	Results	Bioefficacy
Studies designed to quantify functional bioefficacy[2]					
Booher et al, 1939	2 men and 3 women, 21-40 y (United States)	Depletion for 16-124 d; repletion for 3-10 d	Low (< 31 μg/d) retinol plus varying doses of retinol and of β-carotene in crystalline form administered to each subject after repletion[3]	Abnormal dark adaptation prevented with 3.5 times (range: 3.2-3.7) as much β-carotene as retinol in each subject, calculated on a weight basis relative to body weight[4]	1:3.5 for prevention of abnormal dark adaptation
Wagner, 1940	10 men, 23-57 y (Germany)	Depletion for 188 d; repletion for 105 d	Low retinol plus doses of 180, 360, 600 and 750 μg retinol (n = 5) or doses of 720, 1,200, 2,520 and 3,000 μg β-carotene in sesame oil (n = 5) administered during repletion	Abnormal dark adaptation reversed with 750 μg retinol (2,500 IU × 0.3 μg/IU)/d[4,5] and with 3,000 μg β-carotene (5,000 IU × 0.6 μg/IU)/d[4].	1:4.0 for reversal of abnormal dark adaptation
Hume and Krebs, 1949	8 men and 1 woman, 19-34 y (United Kingdom)	Depletion for 8.5 to 25 mo; repletion for 3 wk to 6.5 mo	Low (<21 μg/d) retinol plus 370 (or 390) and 750 μg retinol or 750, 1,500 and 3,000 μg β-carotene in arachis oil administered during repletion/prevention	Abnormal dark adaptation reversed with 390 μg retinol (1,300 IU × 0.3 μg/IU)/d[4] (n = 1) and with 1,500 μg β-carotene (2,500 IU × 0.6 μg/IU)/d[4] (n = 2); abnormal dark adaptation prevented with 750 μg retinol (2,500 IU × 0.3 μg/IU)/d[4] (n = 2) and with 3,000 μg β-carotene (5,000 IU/d × 0.6 μg/IU)/d[4] (n = 4[6]).	1:3.8 for reversal and 1:4.0 for prevention of abnormal dark adaptation
Sauberlich et al, 1974	8 men, 31-43 y (United States)	Depletion for 359 to 771 d; repletion for 9 to 455 d	Low (<23 μg) retinol plus varying doses of retinol or β-carotene in corn oil administered during repletion[7]	Abnormal dark adaptation reversed with 150 μg retinol/d (n = 4) and with 300 μg β-carotene/d (n = 2)	1:2 for reversal of abnormal dark adaptation[8]

Studies designed to quantify bioefficacy[9]					
Tang et al, 2000	1 woman, 47 y (United States)	Measurement of [$^2H_4$] and [$^2H_8$]retinol in serum	Administration of single doses of 126 mg (high dose) and 6 mg (low dose) [$^2H_8$]β-carotene in crystalline form and 9 mg [$^2H_8$]retinyl acetate over a 2.5 y period	Amount of 55 µg (high dose) and 3.8 µg (low dose) of β-carotene in crystalline form has the same vitamin A activity as 1 µg retinol	1:55 (high dose) and 1:3.8 (low dose)
Van Lieshout et al, 2001 (**Chapter 2**)	35 children, 8-11 y (Indonesia)	Measurement of [$^{13}C_{10}$] and [$^{13}C_5$]retinol in plasma	Administration of 160 µg [$^{13}C_{10}$]β-carotene and 87 µg [$^{13}C_{10}$]retinol in sunflower oil for ≤10 wk	Amount of 2.4 µg (95% CI: 2.1, 2.7) β-carotene in oil was found to have the same vitamin A activity as 1 µg retinol	1:2.4[10]
Van Lieshout et al, (**this chapter**)	77 children, 7-13 y (Indonesia)	Measurement of [$^{13}C_{10}$] and [$^{13}C_5$]retinol in plasma	Administration of 89 µg [$^{13}C_{10}$]β-carotene and 34 µg [$^{13}C_{10}$]retinol in sunflower oil for 6 wk	Amount of 2.7 µg (95% CI: 2.5, 2.8) β-carotene in oil was found to have the same vitamin A activity as 1 µg retinol	1:2.7[10]

[1] This table also appeared in West et al, 2001.

[2] Functional bioefficacy, which is defined as the proportion of an ingested nutrient that carries out a given metabolic function, such as the ability of ingested β-carotene to reverse or prevent abnormal dark adaptation (Brouwer et al, 2001). Vitamin A was provided as retinyl esters but the amounts are expressed in terms of retinol.

[3] As an example, one subject received 270, 405, 450 and 540 µg retinol, and 1,274 and 1,770 µg β-carotene.

[4] Based on 1 IU β-carotene is 0.6 µg and 1 IU vitamin A is 0.3 µg as mentioned in each paper.

[5] Hume and Krebs (1949) suggested that there was some confusion about the potency of the retinol preparation ('Vogan') used. However, in personal communications to Hume and Krebs both from Wagner himself and from Scheunert in 1947, it was confirmed that the potency quoted by Wagner (1940) was correct.

[6] In two of those four subjects the same amount of β-carotene in margarine was found to prevent impaired dark adaptation.

[7] Three subjects received 2,400 µg; or 150, 300, 600, 1,200 and 2,400 µg; or 150, 300, 600 and 1,200 µg β-carotene and five subjects received 37.5, 75, 150, 300 and 600 µg; or 2,400 and 25,000 µg; or 150, 300, 600, 1,200, 2,400 µg; or 150, 300, 600 and 1,200 µg; or 75, 150, 300 and 8,300 µg retinol.

[8] The ratio of 1:2 was also obtained with other measures: electroretinograms, prevention of cutaneous lesions, and maintenance of plasma retinol concentrations.

[9] Bioefficacy is defined as the fraction of ingested provitamin A carotenoids absorbed and converted to retinol in the body (Chapter 2).

[10] Medians: the median based on all subjects in both studies is 1:2.6

the labeled β-carotene in that study was 25%. However, in our present study, the proportion of *all-trans*-β-carotene in the labeled β-carotene was 90%. In both studies, the isotopic purity of labeled β-carotene was (99%) (Lugtenburg et al, 1999). The bioefficacy of β-carotene in oil in our present study was 35%. The prevalence and intensity of the infestation with intestinal parasites was slightly higher in this study than in our previous study, which might explain the non-significant difference in bioefficacy. If data from both studies are pooled (n = 111), it is estimated that 2.6 μg β-carotene in oil (median value) has the same vitamin A activity as 1 μg retinol. Thus, the best estimate of bioefficacy of β-carotene in oil is 36%.

The IOM based its values on healthy subjects in developed countries. It may well be that the bioefficacy is lower in children from Indonesia compared with those from developed countries. The data in Indonesian children are most probably applicable to other children in developing countries. The bioavailability of β-carotene in oil compared to that of retinol in oil was 81% in the present study, which compares with - but is lower than - the value of 108% geometric mean calculated by natural log transformation; n = 33; 95% CI: 93,122) which we can now calculate (data not previously published) from the data from the earlier study (**Chapter 2**). A bioavailability >100 indicates that the bioavailability of β-carotene was more efficient that of retinol. In our calculations of the bioavailability, we assume a very efficient stoichiometry of the cleavage of β-carotene to retinol. Estimates of the bioavailability would be higher if a less efficient stoichiometry - indicating eccentric cleavage - were used. Information available on the stoichiometry is limited and does not allow a definitive conclusion on the nature of the cleavage (Van Vliet, 1996). We cannot explain the different estimates of bioavailability of β-carotene in oil obtained in the two studies. If data from both studies are pooled (n = 106), the median bioavailability of β-carotene in oil was 86%.

The bioavailability and bioefficacy of β-carotene in fruit and vegetables

For a long time, data on the second step - the relative bioavailability of β-carotene in food compared to that of β-carotene in oil - were as scarce as those on the first step. In a number of studies, differences between the amount of β-carotene in food consumed and that excreted in feces (oral-fecal balance technique) were calculated. These differences were assumed to reflect absorbed β-carotene (apparent absorption). In 1967, FAO/WHO reviewed these studies and proposed that 6 μg β-carotene in food has the same vitamin A activity as 1 μg retinol. A conclusion which was confirmed by FAO/WHO in their guidelines of 1988. Thus, according to FAO/WHO (1967; 1988) the bioefficacy of β-carotene in food is 16%.

It was not until the 1990s that evidence was mounting that the bioefficacy of provitamin A carotenoids in fruit and vegetables was only 20-50% of the FAO/WHO estimates (Micozzi et al, 1992; Törrönen et al, 1996; De Pee et al, 1998; Khan et al, 1998; Castenmiller et al, 1999; Tang et al, 1999; Van het Hof et al, 1999). Therefore in its recent report the IOM (2001) recognized that data on the effective supply of vitamin A from provitamin A carotenoids in foods needed to be revised. Although more studies were available, the IOM used data from one study, conducted by Van het Hof and colleagues (1999), because that was the only study that not only had been conducted in healthy subjects in a developed

country but also used a mixture of vegetables and some fruit. In Van het Hof's study (1999), the increase in serum β-carotene concentration after consumption of β-carotene-rich vegetables and a limited amount of fruit was one-seventh, i.e. 14%, of the increase after consumption of β-carotene in oil. The IOM (2001) adjusted this to one-sixth, i.e. 17%, because of the low fruit content in the diet used in this study. The IOM then calculated the vitamin A activity of β-carotene in food by calculating the product of values from the first and second step. Thus, 6 μg β-carotene in food has the same bioavailability as 1 μg β-carotene in oil (second step), which in turn, according to IOM (2001), has the same vitamin A activity as 0.5 μg retinol (first step; as described above in fact based on a value found only by Sauberlich and colleagues in 1974). Consequently, IOM (2001) proposed that 12 μg β-carotene in food has the same vitamin A activity as 1 μg retinol. Thus, according to IOM the bioefficacy of β-carotene in a mixed diet is 8%. Note that if the IOM had used values of bioefficacy of β-carotene in oil available at the time of their deliberations in the range of 24-27% (Hume and Krebs, 1949; Booher et al, 1939; Wagner, 1940; Tang et al, 2000), the bioefficacy of β-carotene in a mixed diet would range from 4 to 4.5%. Such values would be more in line with values derived from controlled dietary trials in Indonesia (De Pee et al, 1998) and Vietnam (Khan et al, 1998) in which it was found that 26 μg β-carotene in dark green leafy vegetables or 12 μg β-carotene in orange fruits have the same vitamin A activity as 1 μg retinol (a bioefficacy of 3.6% and 7.8%, respectively). In a study in China (Tang et al, 1999), 27 μg β-carotene in green and yellow vegetables was found to have the same vitamin A activity as 1 μg retinol. Based on these three studies it can be estimated that as much as 21 μg β-carotene in a mixed diet (4:1 vegetables to fruit intake) has the same vitamin A activity as 1 μg retinol (West et al, 2001). This corresponds to a bioefficacy of β-carotene in food of 4.5%.

The vitamin A activity of β-carotene in dark green leafy vegetables relative to that of β-carotene in orange fruit in these studies was 26/12 = 2.2. In our present study, the effective supply of vitamin A from β-carotene in pumpkin was 1.7 times that of β-carotene in spinach. In fact, the estimate obtained from our present study (1.7) might be more accurate than the estimate of the earlier studies (2.2) because one of the problems of the methods used in those earlier studies is that the vitamin A activity suffers from the homeostatic control of serum retinol concentrations while the plateau isotopic enrichment technique used in our present study does not.

In our study, the amount of retinol in the basal diet was low and constant. In further studies, we plan to feed small amounts of retinol in a highly bioavailable matrix. We expect this to provide a constant and low degree of isotopic enrichment of retinol in food during the run-in and treatment periods while the degree of isotopic enrichment of β-carotene in food will be diluted in the treatment period (compared with the run-in period) by the large amount of unlabeled β-carotene in vegetable supplements provided. We expect this dilution in food to result in a measurable dilution in the degree of isotopic enrichment in serum of retinol and β-carotene from which the bioavailability and bioefficacy of β-carotene in food can be estimated. In the present study, the vegetable supplement provided during the run-in period contained some unlabeled β-carotene. In further studies, we plan to provide a carotene-free vegetable during the run-in period. We will study whether such studies could

have a cross-over design, thus eliminating possible time-effects. By altering the design of this stable isotope technique in such a way, we expect to be able to observe a change in the degree of isotopic enrichment in serum not only in β-carotene (as seen in this study) but also in retinol, from which the bioavailability and bioefficacy of β-carotene in fruit and vegetables could be quantified.

Conclusions and implications

Many impoverished people depend on provitamin A carotenoids in fruit and vegetables as their major source of vitamin A. However, even if such foods, rich in provitamin A carotenoids, are consumed in large quantities, the effective supply of vitamin A from these foods will remain limited because the bioefficacy of the provitamin A carotenoids is <8%. The method presented here, with the modifications suggested, will allow the bioavailability and bioefficacy of provitamin A carotenoids in possible food sources to be quantified. These data are required before food - including those from genetically modified plants such as 'Golden Rice' (Ye et al, 2000) - can be promoted as an appropriate approach to eliminating vitamin A deficiency.

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Extraction of carotenoids from feces enabling the bioavailability of β-carotene to be studied in Indonesian children

4

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

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INTRODUCTION

Vitamin A deficiency is a problem in developing countries (Beaton et al, 1993; De Pee et al, 1998; West et al, 1999; Dijkhuizen et al, 2001). Supplementation with vitamin A of children under 5 years of age in such countries reduces morbidity and mortality by 23%, probably because of improved immunocompetence (Beaton et al, 1993). Improved vitamin A status can be achieved by reducing the demand for vitamin A or by increasing the effective supply of vitamin A or by combining both these approaches. Three factors determine the effective supply of vitamin A: the consumption of foods and pharmanutrients (i.e. dietary supplements); the content of vitamin A or its precursor in food or pharmanutrients consumed; and the bioefficacy of vitamin A or its precursor in the food or pharmanutrients. Unless the bioefficacy is sufficiently high, the effect of increasing the consumption of provitamin A containing food and the provitamin A content of food consumed will be limited. Bioefficacy of provitamin A carotenoids in plant foods is a particular problem because plant foods are the major source of vitamin A in the diet of a large proportion of the world's population (IVACG, 1999). Thus, such low bioefficacy limits the efficacy and therefore effectiveness of many interventions in controlling vitamin A deficiency. The impact of the bioefficacy on the effective supply of vitamin A has received little attention until the 1990s, mainly because sensitive quantitative techniques were lacking (**Chapter 5**).

Isotope techniques can provide accurate and precise estimates of bioavailability and bioefficacy. We developed a technique to quantify the bioavailability and bioefficacy of β-carotene in oil by using multiple low (< 100 μg) doses of β-carotene and retinol, each specifically labeled with 10 ^{13}C atoms. This technique has been tested in studies involving 35 (**Chapter 2**) and 77 (**Chapter 3**) Indonesian school children. In the latter study, the relative bioavailability and bioefficacy of β-carotene in spinach and pumpkin was also estimated. All results obtained from these studies were based on data from serum samples. Because stool samples can be collected non-invasively, the aim of the present study was to compare estimates of β-carotene bioavailability using data from serum with those based on data from feces. For this purpose, a sub-sample of children from our second study (**Chapter 3**) were asked to collect all their stools for 2 consecutive days, 3 times during the study. In feces we measured the same variables as in serum, which required the development of an extraction technique to use in conjunction with existing HPLC (Hulshof et al, 1997) and liquid chromatography coupled with mass spectrometry (Van Breemen et al, 1998; Wang et al, 2000) methods. We also extended the mathematical model to estimate the bioavailability of β-carotene. It is not possible to quantify the bioefficacy of β-carotene based on measurements in feces because the bioconversion of β-carotene is a metabolic process that occurs in the body.

MATERIALS AND METHODS

Definitions

In this paper, a number of terms are used which need to be defined. Bioavailability is the fraction of an ingested nutrient that is available for utilization in normal physiologic functions and for storage (Jackson, 1997). Bioconversion is the fraction of a bioavailable nutrient (here, absorbed provitamin A carotenoids) that is converted to the active form of a nutrient (retinol). Bioefficacy is the fraction of an ingested nutrients (here, dietary provitamin A carotenoids) that is absorbed and converted to the active form of the nutrient (retinol) (**Chapter 2**).

Subjects

The study was conducted from March to May 1999 in a rural village in Bogor District, West Java. Screening procedures and selection criteria of the study have been reported in detail elsewhere (**Chapter 3**). There were no additional eligibility criteria for inclusion in the sub-sample of children being asked to collect their stools during the study. During the baseline measurements, 20 of the 80 apparently healthy school children (9 boys and 11 girls), aged 7-12 y, participating in the study were randomly selected to collect their stools during the study (**Figure 4-1**). The parents or guardians of these children were informed about the purpose and procedures of the stools collection and gave their written informed consent. At the end of the run-in period of 3 wk, children were randomly allocated to receive either spinach (n = 12) or pumpkin (n = 8) during the subsequent treatment period of 3 wk. The study was approved by the Medical Ethics Committee of the Ministry of Health, Indonesia; the Indonesian Institute of Science; and the Medical Ethical Committee of the Division of Human Nutrition and Epidemiology of Wageningen University, the Netherlands.

Study type and intervention

The study was a randomized controlled dietary trial with two treatment arms - spinach and pumpkin - and a parallel design (Figure 4-1). During the study, comprising a 3-wk run-in period followed by a 3-wk treatment period, each child received capsules (3/d for 7d/wk) containing 31 μg [12,13,14,15,20,12',13',14',15',20'-$^{13}C_{10}$]β-carotene (analyzed value) and 21 μg [8,9,10,11,12,13,14,15,19,20-$^{13}C_{10}$]retinyl palmitate (calculated based on analysis; equivalent to 12 μg retinol). Each capsule also contained 0.35 g highly unsaturated sunflower oil and 0.12 mg vitamin E as an antioxidant for the oil. Preparation of the labeled materials (Lugtenburg et al, 1999) and capsules is described elsewhere (**Chapter 3**). Children consumed the capsules with a low-retinol, low-carotenoid meal 3 times/d. During the run-in period, the lunch and afternoon meals were supplemented with a portion of 77 g long-yard beans, containing 0.4 mg β-carotene (analyzed value). During the treatment period, children received daily either 2 portions of 82 g spinach (n = 41) or 81 g pumpkin (n = 36) containing 1.5 mg and 0.7 mg β-carotene/portion, respectively (analyzed values). Preparation of the meals is described elsewhere (**Chapter 3**). During the study, members of the research team supervised and recorded compliance of consumption of the capsules and meals. Main outcome measures of the study were the dilution of the degree of isotopic enrichment of retinol and β-carotene in serum and in feces during the treatment period (compared with the

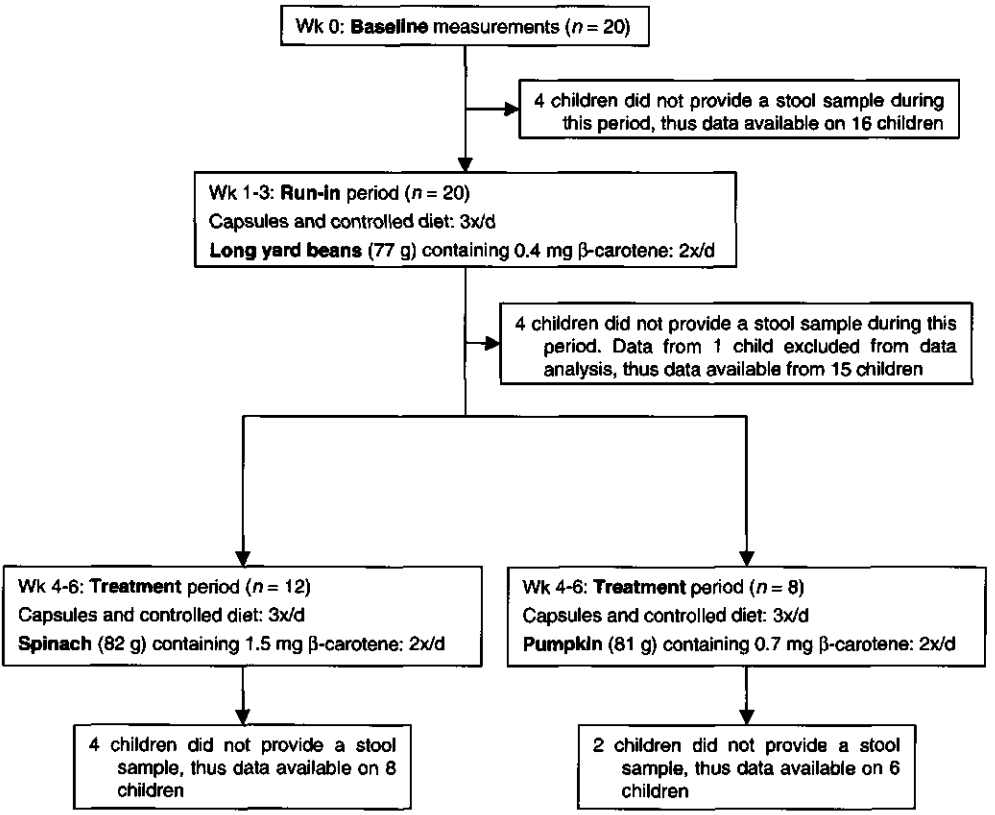

Figure 4-1. Study design. Capsules contained 31 μg [$^{13}C_{10}$]β-carotene and 21 μg [$^{13}C_{10}$]retinyl palmitate. The basal diet was low in retinol and carotenoids. At baseline and at the end of the run-in and treatment periods, blood and stools were collected.

run-in period). Either using data from serum or from feces, the relative bioavailability of β-carotene in spinach and pumpkin was estimated. Samples of feces collected at the end of the run-in period were used for measurement of the bioavailability of β-carotene in oil.

Measurements

During the baseline measurements and at the end of the run-in and treatment periods, a physician examined the children and each child's weight, height, mid-upper-arm-circumference (MUAC) were measured. During the one-week baseline measurements, children were asked to collect all their stool samples for 48 h (day 2 and 3). On day 4 or 5 of the baseline period, blood samples were drawn. Three days before the end of the run-in and of the treatment periods, children were asked to collect all their stools for 48 h. On the second last or last day of each period, blood samples were drawn from apparently healthy children (one sample/child/period).

Blood samples were used to obtain data as described previously (**Chapter 3**): leukocytes counts; hemoglobin concentration, hematocrit values, concentration of retinol and various carotenoids, degree of isotopic enrichment of retinol and β-carotene. Stool samples were

used to obtain data on: prevalence and intensity of parasitic infestation (as described in **Chapter 3**); concentration of retinol, retinyl esters, various carotenoids; degree of isotopic enrichment of retinol and β-carotene.

Estimates of the energy and nutrient intakes were based on a combination of sources: diaries in which children daily recorded, both qualitatively and quantitatively, consumption of foods and drinks not provided by us; records of attendance and of consumption of the meals; and a computer program suite (KOMEET, version 2.0c, and VBS-edit, version 1.0; B-ware Nutrition Software, Wageningen, Netherlands) with a nutrient database (bg95k99t; **Chapter 2**) based on that developed by de Pee and colleagues (1996) to which the energy and nutrient contents of the foods provided (on the basis of duplicate analyses of meals and supplements) were added. Collection of the duplicate samples and analysis of the energy and nutrient intakes in these samples has been described elsewhere (**Chapter 3**). In this paper, we will only report retinol and carotenoid intakes from food provided.

Collection of feces samples

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

Extraction of human feces

For the extraction of human feces, we extended a method, for measuring the concentrations of provitamin A carotenoids in foods (Hulshof et al, 1997). Beginning 16 h prior to analysis, samples were allowed to thaw in the dark at 4 °C. An aliquot (2 g) was extracted in duplicate with 4 g Na_2SO_4, 0.5 g $CaCO_3$, 30 mL tetrahydrofuran (THF) containing 0.01% butylated hydroxytoluene (BHT), and 1 mL of an internal standard in THF/methanol (3:1 v/v) containing a known amount of retinyl acetate (ca. 1 μg) in a 100 mL measuring cylinder using a rod mixer (Polytron PT 20 OD, Kriens/Luzern, Switzerland) at moderate speed (speed 4) 3 times for 1 min. The solution was allowed to stand for 3-5 min after which the extract was filtered through a filter paper (Whatman paper no 54, diameter 11 cm) in a glass funnel. The residue was re-extracted twice with 30 mL THF. The volume of the combined filtrates was reduced to near dryness at 35 °C under nitrogen in a

rotary evaporator (Büchi, Flawil, Switzerland). Using a glass pipette, 25 mL THF/methanol (3:1 v/v) containing 0.01% BHT' was added to the concentrated filtrate. After homogenizing, this sample was portioned over one crimpcap vial (1.5 mL; 403682, Phase Separation LTD, Deeside, UK) closed with a crimpcap (412115, Phase Separation) and three vials (1.8 mL; A4954-010, Thermo Separation Products, San Jose, CA) closed with a screwcap. At the end of each day of sample preparation, the 1.5-mL vials without screwcap were placed into the tray (protected from light, kept at 4 °C) of the HPLC system for duplicate injections of 25 μL. The 1.8-mL vials were stored in containers at -80 °C for ≤2 wk before being packed on dry ice and transferred to the Department of Medicinal Chemistry and Pharmacognosy in Chicago. There the extracts were stored at -80 °C for ≤2 wk before measurement of the degree of isotopic enrichment of β-carotene (Wang et al, 2000) and retinol (Van Breemen et al, 1998; Wang et al, 2000).

Storage of feces extracts

The effect of storage of feces on the concentrations of β-carotene and lutein (the most prevalent carotenoids found in feces in this study) was assessed. For this purpose, 2 samples were extracted in duplicate following the above protocol. The extract was portioned (6 × ca. 4 mL) over tubes (8-mL heavy duty centrifuge tubes with screw cap, Kimble Glass Inc, Vineland, NJ, USA) and stored under nitrogen at -80 °C for periods up to 5 wk. On the day of extraction and once per week during the next 5 wk, one tube from each sample was opened and 1.5 mL was transferred to a crimpcap vial closed with a crimpcap from which 25 μL was injected in duplicate into the HPLC system. The average coefficient of variation (CV) over the 5-wk period for each sample was 5% (range, 4-6%) for β-carotene and 5% (range, 2-8%) for lutein. For each sample, Spearman correlation coefficients were calculated between 'week' and 'β-carotene or lutein' concentration to assess whether there was any change in concentration. Only for one sample was the correlation between 'β-carotene' and 'weeks' significant. Because the mean CV was <15% and because there was no drift with time in concentration, we concluded that extraction of feces in Wageningen and shipment of the extracts to Chicago within 2 weeks - where they would be injected in the LC-MS system within 2 weeks - would not cause significant losses of carotenoids. In fact, small losses would not be a problem because measurement is based on the ratio of labeled to unlabeled compounds provided that such losses affect labeled and unlabeled compounds to a similar extent.

Quality control

Samples from each child were analyzed within one run to minimize analytical variation. In each run, a control sample was extracted in duplicate and injected into the HPLC system for monitoring the stability of the analytical procedure over time. The control sample for the HPLC analysis was homogenized baby food from one lot obtained from Nutricia BV (Zoetermeer, The Netherlands) comprising 42% carrots (*Daucus carota*), 30% peas (*Pisum sativum*), 21% low fat milk, and 1% parsley (*Petroselinum crispum*) as described earlier (Hulshof et al, 1997). For each sample run, a new jar of baby food was opened. The average carotenoid concentrations of duplicate control samples were required to be within 2 SD of

the mean concentrations measured, when extracted and analyzed by HPLC as described by Hulshof and colleagues (1997), over the past 5 y. If not, the sample run as repeated. With each sample run, two blank samples were included. No retinol and carotenoids were detected in these blank samples.

HPLC analysis

The HPLC system, and the source and preparation of internal and external standards have been described elsewhere (Hulshof et al, 1997). A reversed phase column was used and the mobile phase comprised a mixture of methanol, THF and water containing 0.1% triethylamine which was pumped isocratically at a flow of 0.7 mL/min. For the first 15 sec, the solvent ratios were 88:2:10 (v/v/v) followed by 30 sec in which the solvent concentrations changed to 92.5:7.5:0 which was maintained for the remainder of the 25 min runtime. There was a 5 min equilibration period between each run. Samples were injected in duplicate: all samples were injected once when elution of retinol and retinyl palmitate was monitored at 325 nm; after which all samples were injected for a second time when elution of various carotenoids was monitored at 450 nm. Carotenoids, retinol and retinyl palmitate were identified by comparing their retention times with those of standards. All peaks eluting at the same time as retinol standards were further identified by spectral analysis and no retinol and retinyl palmitate appeared to be present in the samples. Quantification was done by internal standard method and calibration was performed in bracketing mode by a 3-level calibration line. A solvent blank was included in each run. Detector responses were linear over the concentration range: Pearson's correlation coefficients were >0.99 for all standards. Total *cis*-β-carotene was quantified from the standard line for *all-trans*-β-carotene, assuming the same detector response for both carotenoids (Hulshof et al, 1997). The quantitation limit was set at 10 times the minimal detectable level, which in turn was defined as the amount of lutein, α-carotene, and β-carotene resulting in a peak-height 3 times the baseline noise. Data from duplicate samples were averaged and expressed as μmol/g feces.

APCI LC-MS analysis

For the LC-MS analysis the feces extracts were analyzed by reversed-phase HPLC with a C_{30} column interfaced to a mass spectrometer equipped with positive ion atmospheric pressure chemical ionisation (APCI). To assess the ratio of labeled to unlabeled β-carotene, selected ion monitoring was carried out at mass-to-charge ratios (m/z) of 537 and 547. These ions corresponded to circulating β-carotene and orally administered [$^{13}C_{10}$]β-carotene, respectively. To assess the ratio of labeled to unlabeled retinol, selected ion monitoring was carried out at m/z 269, 274, and 279. These abundant fragment ions corresponded to the loss of water from the protonated molecule of circulating retinol, [$^{13}C_5$]retinol (metabolically formed from orally administered [$^{13}C_{10}$]β-carotene), and [$^{13}C_{10}$]retinol (formed by hydrolysis of orally administered [$^{13}C_{10}$]retinyl palmitate), respectively. Quality control measures and technical details of the LC-MS methods and equipment did not deviate from the protocol described in the original papers describing these methods (Van Breemen et al, 1998; Wang et al, 2000).

Reagents and standards

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

Calculation of the bioavailability of β-carotene

From the degree of isotopic enrichment of β-carotene with [$^{13}C_{10}$]β-carotene and retinol with [$^{13}C_5$]retinol and [$^{13}C_{10}$]retinol in serum and feces at plateau isotopic enrichment during the run-in period, the bioavailability of β-carotene in oil was calculated using the CarRet PIE mathematical model as described earlier (**Chapter 2**) and in **Table 4-1**, respectively. The plateau can be reached during prolonged consumption of multiple doses of [$^{13}C_{10}$]retinol and [$^{13}C_{10}$]β-carotene. Note that the formula for determining the bioavailability of β-carotene in oil using data from feces is based on an oral fecal balance.

During the run-in period, the intake of retinol and β-carotene from sources other than the capsules was kept constant and low. During the treatment period, the intake of retinol and β-carotene from the capsules was similar to the intake during the run-in period. The intake of retinol from other food sources was similar to the intake during the run-in period. The only difference between the 2 periods was the treatment, i.e. spinach or pumpkin, rich in β-carotene. It is assumed that β-carotene and retinol released from the food matrix and available for absorption mixes completely with labeled β-carotene and retinol. Changes in the degree of isotopic enrichment of retinol and β-carotene in serum and feces (versus the run-in period) reflect the bioavailability of β-carotene in spinach and pumpkin.

However, despite the consumption of large amounts of β-carotene in spinach and pumpkin in the treatment period, the degree of isotopic enrichment in serum did not change during the treatment period. Therefore, the mathematical model could not be used to obtain absolute data on the bioavailability and bioefficacy of β-carotene in spinach and pumpkin. However, the relative bioavailability was estimated as described elsewhere (**Chapter 3**) and for clarity repeated in Table 4-1. We extended the CarRet PIE mathematical model to estimate the relative bioavailability of β-carotene in spinach and pumpkin (Table 4-1).

Statistical evaluation

Because the sample size was relatively small (<20) normality of data was not tested and data are expressed as medians and 25th to 75th percentiles, except for baseline characteristics which are expressed as mean ± SD. As the run-in period serves as a control for the treatment period, data collected during the run-in period are shown separately for each treatment group. Results obtained using data from the run-in and treatment periods were compared between the spinach and pumpkin groups for variables referred to in Table 4-1. For all comparisons, Mann-Whitney *U* tests were used. Within each treatment group, Wilcoxon tests were carried out to compare the variables referred to in Table 4-1 between the run-in and treatment periods. All tests were two-sided and P values < 0.05 were considered significant. The computer package SPSS (version 10.0.5; SPSS Inc, Chicago) was used for all statistical calculations.

RESULTS

Baseline characteristics and experimental regimen

Data are presented for 17 (9 boys, 8 girls) of the 20 children enrolled because 2 girls in the pumpkin group and one in the spinach group provided only one stool sample at baseline. Mean age of the children was 9.1 ± 1.2 y (SD). At baseline, children weighed 24.0 ± 3.8 kg, were 1.25 ± 0.06 m high and had a mid-upper-arm-circumference of 18.2 ± 1.6 cm. At baseline, the mean serum concentration of retinol was 0.78 ± 0.26 μmol/L and of β-carotene 0.14 ± 0.08 μmol/L. In feces, the baseline concentration of β-carotene was 0.011 ± 0.010 μmol/g while no retinol was detected in feces. At baseline, the hemoglobin concentration was 101.6 ± 7.3 g/L and the hematocrit 0.32 ± 0.03 L/L. In stools of 65% of the children, intestinal parasites were prevalent at baseline (no data available on one child). Anthropometric data, hemoglobin concentrations, hematocrit values, and prevalence and intensity of intestinal parasites during the study are reported elsewhere (**Chapter 3**). In both periods and in both groups, the compliance to the capsule consumption was >92%, which means that the children followed the experimental regimen very conscientiously.

During the baseline, run-in and treatment periods, the average weight of feces samples collected over the 48-h per period was 168 ± 94, 130 ± 62, and 151 ± 69 g, respectively. Although, children were instructed to provide all their stools over a 48-h period, some children did not provide a single stool sample in some, one or all periods. In retrospect, children mentioned that their feces collection had often been incomplete. However, we were unable to estimate the proportion of feces collected.

During both periods of the study, children obtained >70% of their daily energy intake from the food provided. Therefore, and because food intake from the foods provided can be estimated more accurately and precisely (**Chapter 3**), we will only use data on nutrient intakes from food (menus, supplements and capsules) provided to describe the nutrient intakes during this study. Daily retinol and provitamin A intakes are shown in Table 4-1. Because during the treatment period >82% of provitamin A intakes was β-carotene, we refer to the bioavailability as the bioavailability of β-carotene. During the run-in period, intake of lutein was approximately 700 μg/d and intake of zeaxanthin and lycopene was <75 μg/d. During the run-in period, retinol and β-carotene intake from capsules was significantly higher in the pumpkin than in the spinach group, but the difference was small. During the treatment period, daily intakes of β-carotene (5.55 vs 2.76 μmol) and β-cryptoxanthin (0.07 vs 0.02 μmol) was significantly higher in the spinach than in the pumpkin group while intake of α-carotene (0.25 vs 1.19) was lower. In both groups, intake of carotenoids differed significantly between the two periods because different recipes were used for the different vegetable supplements (data for provitamin A carotenoids shown in Table 4-1).

Concentrations and degree of isotopic enrichment of retinol and carotenoids in serum and feces

Concentrations of retinol in serum are shown in Table 4-1. Using HPLC and LC-MS, with limits of detection of 3.5 ng/kg feces and ≤1pmol injected on-column, respectively, no retinol was detected in feces samples collected during this study, possibly because intake of retinol from food provided was negligible. Concentrations of retinol in serum did not differ

Table 4-1. Description of the β-carotene and retinol at plateau isotopic enrichment (CarRet PIE) mathematical model (**Chapter 2**) using data from serum and feces

Symbol	Description	Derivation	Run-in period		Treatment period	
			Spinach group ($n = 9$)	Pumpkin group ($n = 6$)	Spinach group ($n = 8$)	Pumpkin group ($n = 6$)
a	Retinol intake from diet (μmol/d)[1]		0	0	0	0
b	Retinol intake from capsules (μmol/d)		0.122 (0.120, 0.124)[2*]	0.126 (0.125, 0.127)[*†]	0.122 (0.121, 0.123)	0.124 (0.121, 0.127)[†]
d	β-Carotene intake from diet (μmol/d)[1]		1.52 (1.29, 1.75)[†]	1.69 (1.38, 2.00)[†]	5.71 (4.86, 5.81)[*†]	3.33 (3.23, 3.39)[*†]
e	β-Carotene intake from capsules (μmol/d)		0.168 (0.164, 0.172)[*]	0.173 (0.172, 0.175)[*†]	0.168 (0.155, 0.181)	0.170 (0.166, 0.175)[†]
C_{sR}	Total retinol concentration in serum (μmol/L)[3]		0.67 (0.56, 1.03)	0.64 (0.52, 0.78)[†]	0.72 (0.69, 1.09)	0.80 (0.72, 0.85)[†]
$E_{10,sR}$	Enrichment of retinol in serum with [$^{13}C_{10}$]retinol	$\frac{M_{279,sR}}{(M_{269,sR} + M_{274,sR} + M_{279,sR})}$[4]	0.0462 (0.0319, 0.0589)[*†]	0.0697 (0.0541, 0.0826)[*]	0.0520 (0.0184, 0.0658)[†]	0.0617 (0.0529, 0.0702)
$E_{5,sR}$	Enrichment of retinol in serum with [$^{13}C_5$]retinol	$\frac{M_{274,sR}}{(M_{269,sR} + M_{274,sR} + M_{279,sR})}$	0.0462 (0.0296, 0.0663)	0.0634 (0.0431, 0.0838)	0.0520 (0.0142, 0.0759)	0.0571 (0.0420, 0.0715)
C_{sC}	Total β-carotene concentration in serum (μmol/L)[3]		0.16 (0.12, 0.22)[†]	0.13 (0.11, 0.20)[†]	0.31 (0.22, 0.37)[*†]	0.51 (0.43, 0.58)[*†]
C_{fC}	Total β-carotene concentration in feces (nmol/g)[3]		4 (2, 12)[†]	5 (1, 12)[†]	21 (1, 34)[†]	10 (2, 24)[†]
$E_{10,sC}$	Enrichment of β-carotene in serum with [$^{13}C_{10}$]β-carotene	$\frac{M_{547,sC}}{(M_{537,sC} + M_{547,sC})}$	0.1425 (0.0940, 0.2343)[†]	0.1643 (0.1129, 0.2280)[†]	0.0118 (0.0071, 0.0268)[*†]	0.0361 (0.0266, 0.0675)[*†]
$E_{10,fC}$	Enrichment of β-carotene in feces with [$^{13}C_{10}$]β-carotene	$\frac{M_{547,fC}}{(M_{537,fC} + M_{547,fC})}$	0.0691 (0.0495,0.1668)[†]	0.0639 (0.0535, 0.0941)	0.0118 (0.0071, 0.0268)[*†]	0.0486 (0.0111, 0.0861)[*]
BV_{dCo}	Bioavailability (%) of β-carotene in oil[5]					
	Using data from serum[6]	$\frac{[(E_{10,sC1} \times C_{sC1}) + (StC \times E_{5,sR1} \times C_{sR1})] \times b_1}{(E_{10,sR1} \times C_{sR1}) \times e_1} \times 100$	114 (67, 136)	75 (52, 85)	-	-

	Using data from feces[7]	$\frac{\left[(2\times e_1)-(E_{10,fC1}\times C_{fC1}\times W_f)\right]}{(2\times e_1)}\times 100$	-	-	-
BV_{dCs_p}	Relative bioavailability of β-carotene in spinach and pumpkin				
	Using data from serum	$\frac{\left\{\frac{\left[(E_{10,sC2}\times C_{sC2})+(StC\times E_{5,sR2}\times C_{sR2})\right]\times d_2}{e_2}\right\}_{\bar{sp}}}{\left\{\frac{\left[(E_{10,sC2}\times C_{sC2})+(StC\times E_{5,sR2}\times C_{sR2})\right]\times d_2}{e_2}\right\}_{\bar{pu}}}$	-	-	1.7 (0.9, 3.1)[8]
	Using data from feces[9]	$\left[\frac{(E_{10,fC2}\times d_2)}{e_2}\right]_{\bar{sp}}\Big/\left[\frac{(E_{10,fC2}\times d_2)}{e_2}\right]_{\bar{pu}}$	-	-	1.8 (0.6, 5.5)[8]

[1] Intake of nutrient is intake from food provided. Retinol in food provided was below the limit of detection (thus, ≤3 ng/d). β-Carotene intake is the sum of the intake of β-carotene and half the intake of other provitamin A carotenoids from food provided. During the run-in period in both groups, α-carotene intake was approximately 0.17 μmol/d and β-cryptoxanthin intake was negligible (<0.01 μmol/d). During the treatment period, α-carotene was higher (1.2 μmol/d) in the pumpkin than in the spinach (0.25 μmol/d) group while in both groups, β-cryptoxanthin intake was negligible (<0.07 μmol/d).

[2] Median with 25th and 75th percentiles. Mann-Whitney *U* tests were carried out to compare values between groups within each period. Wilcoxon tests were carried out to compare values within each group between the run-in and treatment periods.

[3] In feces collected over 48-h, concentrations of retinol were below the limit of detection (thus, excretion of retinol was ≤2 ng/d).

[4] Where $M_{279,sR}$ is the signal measured by liquid chromatography-mass spectrometry at *m/z* 279. Values at baseline were subtracted from values during the run-in and treatment periods. If baseline samples were absent, the mean enrichment at baseline was subtracted from the values during the run-in and treatment periods.

[5] Bioavailability is the fraction of an ingested nutrient that is available for utilization in normal physiologic functions and for storage (Jackson, 1997).

[6] Figures 1 and 2 in the subscripts refer to the period from which data were used (1 = run-in and 2 = treatment period). *StC* refers to the stoichiometry of the cleavage of β-carotene to retinol in the body. We assume the most efficient stoichiometry, i.e. 1 μmol β-carotene yields 2 μmol retinol, thus obtaining minimum estimates for the bioavailability. With this stoichiometry, *StC* = 0.5.

[7] Where W_f is the weight of feces collected. Because complete collection of feces is a prerequisite for adequate use of this formula and feces collection was incomplete, bioavailability of β-carotene in oil was not calculated using data from feces.

[8] Ratio calculated using median values of the numerator and denominator. Interval of spread of ratio calculated using the 25th and 75th percentiles of the median values of the numerator and denominator (Sokal and Rohlf, 1997). A ratio >1 indicates that the bioavailability of β-carotene in spinach is less than that of β-carotene in pumpkin.

[9] In these formulas, the absorption of retinol is not taken into account as is done in the formulas for serum. This cannot be done, because no retinol was detected in feces.

* Values within one row within one period differ significantly ($P < 0.05$).

† Values within one row within one group differ significantly ($P < 0.05$).

between the spinach and pumpkin groups in each period, nor within each group between both periods.

Concentrations of retinol and carotenoids in serum and in feces did not differ between groups during the run-in period (Table 4-1; data for carotenoids other than β-carotene not shown). During the treatment period, concentrations of β-carotene, lutein, α-carotene, zeaxanthin in serum were higher in the pumpkin than in the spinach group while concentrations of lycopene in feces were higher in the spinach than in the pumpkin group. Except for zeaxanthin these differences reflect differences in intake of carotenoids from food provided (see previous section). In the spinach group, concentrations of β-carotene and lutein in feces and in serum differed significantly between the run-in and treatment periods. In the pumpkin group, concentrations of retinol, β-carotene, lutein, α-carotene, zeaxanthin in serum and of β-carotene, lycopene, lutein in feces differed significantly between the run-in and treatment periods.

During the run-in period, the degree of isotopic enrichment of retinol in serum with [$^{13}C_{10}$]retinol differed significantly between the spinach and pumpkin groups (Table 4-1). In the spinach group, the degree of isotopic enrichment of retinol in serum with [$^{13}C_{10}$]retinol increased significantly over the course of the treatment period while in the same group, the isotopic enrichment of β-carotene in feces decreased significantly over the course of the treatment period. In both groups, the degree of isotopic enrichment of β-carotene in serum decreased significantly over the course of the treatment period. During the treatment period, the degree of isotopic enrichment of β-carotene in serum and feces differed significantly between the spinach and pumpkin groups. The degree of isotopic enrichment of β-carotene in serum was lower in the pumpkin than in the spinach group while in feces, the enrichment was higher in the pumpkin than in the spinach group.

Comparison of bioavailability of β-carotene using data from feces with data from serum

The bioavailability of β-carotene in oil compared with that of retinol in oil obtained using data from serum was 82% (25th to 75th percentile: 62, 1.25) (not different between groups; Table 4-1). These calculations were based on data from 15 children instead of 16, because data from one of the children who participated in this feces collection procedure was omitted (Rosner, 1995) from calculations of the bioavailability of β-carotene in oil if calculations were based on the total study population (n = 77; **Chapter 3**). Data on the bioavailability of β-carotene in oil obtained using data from feces could not be calculated, because the calculations reflected a balance study, for which it is important that feces collection is complete.

Using data from serum, the bioavailability of β-carotene in pumpkin was 1.7 (interval: 0.9, 3.1) times that of β-carotene from spinach. The ratio was calculated using median values of the numerator and denominator. Interval of spread of the ratio was calculated using the 25th and 75th percentiles of the median values of the numerator and denominator (Sokal and Rohlf, 1997). Using data from feces, the bioavailability of β-carotene in pumpkin was 1.8 (interval: 0.6, 5.5) times that of β-carotene in spinach. A ratio >1 indicates that the bioavailability of β-carotene in pumpkin is more than that of β-carotene in spinach. No

statistical tests were performed to compare these ratios because of the low quantitative precision of such ratios.

DISCUSSION

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

From the interval of spread around the estimates based on feces (0.6, 5.5) and serum (0.9, 3.1) it can be seen that the precision of data from feces is probably lower, which may be caused by incomplete collection of feces. The bioavailability of β-carotene in oil could not be estimated using data from feces because collection of feces was incomplete. In future studies, the proportion of feces collected could be assessed if radio opaque markers are fed prior and during the 48-h of feces collection and their recovery in feces assessed by X-ray. There are a number of limitations on basing results on analyses in feces. Firstly, because bioconversion is a process which occurs within the body, bioefficacy cannot be quantified directly using data from feces. Secondly, the amount of feces required for analysis is greater than that for serum samples, thus increasing the cost of sample storage and analysis. In addition, sample preparation of feces is much more labor-intensive and more manual steps are involved than for sample preparation of serum.

Nevertheless, results from this study show that this stable isotope technique can be used for the quantification of the relative bioavailability of β-carotene in food using data from feces. In addition, in conjunction with the use of stable isotopically labeled compounds feces can be collected to study the fate of carotenoids in the gastro-intestinal tract. This is a rather unexplored area of research with the degree of carotenoid degradation in the gastric environment or by bacteria (Rao and Rao, 1970) and the extent of endogenous secretion of carotenoids being largely unknown. Such data are necessary in order to interpret data on the bioavailability of carotenoids obtained using oral fecal balance techniques.

For the study described in this paper, we adapted an HPLC method, based on a validated method, to liberate retinol, retinyl esters and carotenoids from human feces to assess the concentration of β-carotene and other carotenoids. The feces extracts can be stored at -80 °C without considerable loss of carotenoids for at least 5 wk. Therefore, for assessing the concentration of retinol and carotenoids in feces using HPLC and the degree of isotopic enrichment of retinol and β-carotene in feces using LC-MS, sample preparation has to be performed only once. This is not only less labor intensive, it also decreases the variation between data from the two measurements. The extraction procedure for feces described in this paper does not require freeze drying of the samples or saponification as in methods described by other authors (Shiau et al, 1994; Dueker et al, 2000). With saponification, all retinyl esters are hydrolised to retinol, thus in studies in which the retinyl ester content of the diet is high, this structural information will be lost.

For the study described in this paper, we also extended the CarRet PIE mathematical model to estimate the relative bioavailability of β-carotene in spinach and pumpkin. In an intervention study in 188 school children in Indonesia by de Pee and colleagues (1998), there were 4 dietary groups: low-retinol, low-carotenoid (negative control); dark-green leafy vegetables and carrots; yellow and orange fruits; and a retinol-containing diet (positive control). The ratio of the bioavailability of β-carotene in orange fruits to that of β-carotene in dark green leafy vegetables was 5.9 (interval: 4.0, 7.9). This was calculated from the changes in serum β-carotene concentrations (corrected for intake of β-carotene from food). In our study, the bioavailability of β-carotene in pumpkin (an orange fruit) was also higher (1.7-1.8) than that of β-carotene in spinach (a dark green leafy vegetable). This ratio is smaller than when based on data from changes in serum concentrations of β-carotene. The stable isotope technique provides more accurate estimates of the relative bioavailability of β-carotene in food both when using data from serum and when using data from feces than techniques which are based on changes in serum β-carotene concentrations. This can be explained by the latter techniques failing to take into account the bioconversion of β-carotene to retinol. The mathematical model using data from feces data provides data on absorption independent of the rate of bioconversion. As described earlier (**Chapter 3**), the design of studies using this isotope technique should be altered by providing moderate amounts of retinol in food thus enabling quantification of the absolute bioavailability and bioefficacy of β-carotene in fruit and vegetables. Data from such studies will enable proper evaluation of the effective supply of vitamin A of various approaches to eliminating vitamin A deficiency.

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Isotopic tracer techniques for studying the bioavailability and bioefficacy of dietary carotenoids in humans: a review

5

Vitamin A deficiency is a serious health problem in many developing countries. Provitamin A carotenoids in fruits and vegetables are the major source of vitamin A for a large proportion of the world's population. However, the contribution of plant foods can only be substantial when not only the consumption and provitamin A content of such food but also the bioefficacy of the provitamin A is high. With respect to provitamin A carotenoids, bioefficacy is the product of the fraction of ingested amount which is absorbed (bioavailability) and the fraction of that which is converted to retinol in the body (bioconversion). Isotopic tracer techniques can meet the need for accurate and precise estimates on bioavailability, bioconversion and bioefficacy of dietary carotenoids in humans. Use of such techniques will enable proper evaluation of food-based approaches for eliminating vitamin A deficiency. In addition, the putative antioxidant capacities of carotenoids can be better understood if their bioavailability is known. Here we discuss how tracer techniques can be applied to obtain reliable and representative data. A step-by-step discussion of aspects related to these techniques is provided including the design of studies, isotopic tracers, dosing regimen, collection of samples, chemical analysis of samples, and method of analysis of data.

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RATIONALE FOR STUDYING BIOAVAILABILITY AND BIOEFFICACY OF DIETARY CAROTENOIDS

Provitamin A carotenoids, in particular β-carotene in fruit and vegetables, are the major source of vitamin A (retinol) for a large proportion of the world's population (IVACG, 1999). In their guidelines on the human requirement for vitamin A in 1967 and 1988, FAO/WHO proposed that 6 μg β-carotene in food has the same vitamin A activity as 1 μg retinol. Official bodies in many countries have followed their guidelines. Recently, the Institute of Medicine (IOM, 2001) of the National Academy of Sciences in the USA proposed that 12 μg β-carotene in food has the same vitamin A activity as 1 μg retinol. A number of studies in Indonesia (De Pee et al, 1998) and Vietnam (Khan et al, 1998) have found that as much as 21 μg β-carotene in a mixed diet (vegetable to fruit 4:1) has the same vitamin A activity as 1 μg retinol. More data are required on the extent of conversion of provitamin A carotenoids to retinol. However, the results of studies carried out up until now would suggest that alternatives to the promotion of the consumption of fruits and vegetables will be required to combat vitamin A deficiency. Data on the extent of absorption of carotenoids are also required when evaluating their putative antioxidant properties. Thus, there is a need for new techniques to quantify the absorption of carotenoids and the conversion of provitamin A carotenoids to retinol.

In this paper, a number of terms are used which need to be defined. Bioavailability is the fraction of an ingested nutrient that is available for utilization in normal physiologic functions and for storage (Jackson, 1997). Bioconversion is the fraction of a bioavailable nutrient (here, absorbed provitamin A carotenoids) that is converted to the active form of a nutrient (retinol). Bioefficacy is the fraction of an ingested nutrient (here, dietary provitamin A carotenoids) that is absorbed and converted to the active form of the nutrient (retinol) in the body (**Chapter 2**). Functional bioefficacy is the fraction of an ingested nutrient that performs a certain metabolic function (Brouwer et al, 2001), such as the ability of ingested provitamin A carotenoids to reverse or prevent abnormal dark adaptation.

Efforts to estimate or quantify the bioefficacy of dietary carotenoids in humans (**Table 5-1**) have included animal models, depletion-repletion techniques (to estimate functional bioefficacy), oral-fecal balance techniques, serum/plasma or chylomicron response, and the use of isotopic tracers. Much emphasis has been placed on human studies in which changes in the concentration of retinol in plasma/serum are measured in response to various dietary sources of β-carotene. Since techniques using isotopic tracers can provide the most accurate data on the bioavailability and bioefficacy of dietary carotenoids in humans, these will be discussed in detail. As outlined in Table 5-1, considerations that need to be taken into account in the design of such studies will be discussed.

Table 5-1. Considerations that need to be taken into account in the design of studies on the bioavailability and bioefficacy of dietary carotenoids with emphasis on techniques using isotopic tracers

CHOICE OF THE MODEL: TECHNIQUES NOT INVOLVING TRACERS

Animal models

Animal models can be very useful for studying mechanisms (West and Beynen, 1988) because they allow the use of procedures which cannot readily be used with humans. Such procedures include the use of radioisotopes, removal of tissues and feeding diets which result in deficiency. However, the results obtained from the use of animal models cannot always be extrapolated directly to humans, especially with regard to making quantitative estimates. With respect to carotenoid metabolism, animal models have a number of limitations. In their extensive reviews of animal models used for studying carotenoid metabolism Van Vliet (1996) and Lee and colleagues (1999), concluded that no animal model completely mimics human absorption and metabolism of carotenoids. For example, cats can absorb β-carotene (Chew et al, 2000) but they cannot convert β-carotene, whereas rats and chickens convert >90% of absorbed β-carotene to retinol (Van Vliet, 1996; Lee et al, 1999). Ferrets, preruminant calves, and possibly also monkeys absorb β-carotene and convert some to retinol as do humans (Van Vliet, 1996). However, ferrets are very inefficient converters of absorbed β-carotene, thus making them an inappropriate model for studying the bioefficacy of carotenoids (Lederman et al, 1998) while the cost of housing (Lee et al, 1999) calves and monkeys limits their use.

Human studies

Depletion-repletion studies and oral-fecal balance techniques

In the 'Sheffield experiment' (Hume and Krebs, 1949), conducted in the 1940s, in which male participants were conscientious objectors to military service, 16 (2 women and 14 men) subjects were asked to consume a vitamin A deficient diet and 7 subjects (1 woman and 6 men) served as controls consuming the same diet but with additional supplements of retinol or β-carotene, for periods ranging from 8.5 to 25 mo. Dark adaptation was used as a functional indicator of the bioefficacy. In the control group, either 750 μg retinol/d (n = 2) or 3,000 μg β-carotene/d in oil (n = 4) or in margarine (n = 2) prevented impaired dark adaptation. From the other 16 subjects, only three men, who also had the lowest plasma retinol concentrations, developed impaired dark adaptation (depletion phase of the study). Either 390 μg retinol/d (n = 1) or 1,500 μg β-carotene/d in oil were required to reverse this impaired dark adaptation (repletion phase). Other depletion-repletion studies conducted by Booher and colleagues (n = 5) (1939) and by Wagner (1940) shortly before World War II (n = 10) reached similar conclusions while Sauberlich and colleagues (1974) in the 1970s (n = 8) concluded that, on a weight basis, twice as much β-carotene as retinol was required to reverse impaired dark adaptation. However, depletion-repletion techniques not only require a very long study period (up to 2 y) but medical ethics committees may be reluctant to approve such studies. In addition, depletion-repletion studies yield only crude estimates of the bioefficacy because only stepwise increased doses of retinol and β-carotene can be tested and compared.

In oral-fecal balance techniques, the difference between the amount of β-carotene in food consumed and that excreted in feces is assumed to represent the amount of β-carotene

absorbed (Bowen et al, 1993). With these techniques, gastric or bacterial degradation of unabsorbed carotenoids may contribute to overestimation of absorption. Rao and Rao (1970) found indications that such bacterial degradation occurs. On the other hand, endogenously secreted carotenoids might be excreted in feces, thus leading to an underestimate of bioavailability. Thus, data on the bioavailability of carotenoids obtained with balance techniques should be interpreted with care. Early studies, carried out before carotenoids could be analyzed by HPLC, have been reviewed by Hume and Krebs (1949) and by Rodriguez and Irwin (1972).

Serum/plasma or chylomicron response

Since the 1980s, various studies including many with limitations - small sample sizes or the absence of a control group - have been carried out in which changes in the concentration of carotenoids and retinol in serum or plasma were measured after feeding foods or pharmanutrients (dietary supplements) containing carotenoids and/or retinol once or more often over a fixed period (De Pee and West, 1996; Castenmiller and West, 1998; IVACG, 1999). Inclusion of a group fed a diet containing synthetic β-carotene enables quantification of relative bioavailability while inclusion of a group fed a diet containing retinol enables quantification of relative bioefficacy. After consumption of foods containing β-carotene, β-carotene is released by mechanical and chemical disruption of the food matrix, and solubilized with bile salts. β-Carotene becomes incorporated in micellar particles, which cross the unstirred water layer, after which it is absorbed into the enterocytes, and together with its metabolites retinol or retinyl esters, is incorporated into chylomicrons. Chylomicrons are transported via the lymph to the blood stream from where they deliver β-carotene or its metabolites to other tissues and the liver (Van Vliet, 1996; Furr and Clark, 1997; Parker et al, 1999). Therefore, a modification of the serum-response technique has been to measure the response in chylomicrons which reflects newly absorbed β-carotene (Van Vliet et al, 1995). In general, both techniques have low precision, thus requiring large numbers of subjects in order to yield reliable quantitative data.

ISOTOPIC TRACER STUDIES CONDUCTED UP UNTIL NOW

The need for techniques using isotopic tracers for studying the bioavailability and bioefficacy of dietary carotenoids in humans was stressed by a Task Force of the International Vitamin A Consultative Group in 1999 because such techniques can probably provide the most reliable estimates of carotenoid bioavailability and bioefficacy. Up until now, a number of studies have been conducted using both radio- and stable isotopes techniques, and their design and results are briefly discussed below.

Radioisotope studies

In the 1960s, Goodman, Blomstrand and their colleagues (Goodman et al, 1966; Blomstrand and Werner, 1967) measured the concentrations in lymph of single orally administered doses of 0.4 mg or 1.3 mg [^{14}C]β-carotene in oil (n = 2) or 47 μg [^{3}H]β-carotene

in oil (n = 2). Recoveries of β-carotene of 52, 15, 9 and 17% were calculated, respectively. The value of 52% was regarded as an exception, possibly due to a metabolic disorder because 90% of the radioactivity recovered in the lymph was in the β-carotene fraction. In the other 3 patients 69-88% of the radioactivity recovered in the lymph was in the retinyl ester fraction. Three decades later, Dueker and colleagues (2000) determined the recovery of a single oral dose of 306 μg [^{14}C]β-carotene in β-carotene and retinol fractions in plasma, feces and urine of one subject. In this study, 57% of the dose was recovered in the stool within 48-h post-dosing. From the measurements made, it was also concluded that 3.5 μg β-carotene in oil has the same vitamin A activity as 1 μg retinol. As explained earlier, balance studies might overestimate or underestimate bioavailability, and thus bioefficacy.

Stable isotope studies

The potential risk and increased reluctance to use radioisotopes has stimulated the use of stable isotopes. Thus in recent years, stable isotopes and compounds labeled with stable isotopes have become increasingly available. In the 1930s, Rudolf Schönheimer and David Rittenberg conducted the first tracer studies involving stable isotopes for studying the metabolism of macronutrients (Schönheimer, 1942). Later, stable isotopic tracer techniques have been developed successfully for studying the metabolism of minerals such as iron, magnesium, and zinc (Patterson and Veillon, 2001; Abrams, 1999); and vitamins and their precursors such as folate (Gregory et al, 1990) and provitamin A carotenoids (Olson, 1999; Russell, 2000). Because of the limited availability of organic nutrients labeled with stable isotopes and the difficulty in their quantification, few studies have been carried out so far.

Studies designed to estimate body stores of vitamin A

In a healthy individual, the liver contains approximately 80-90% of the total body stores of vitamin A, most in the form of retinyl esters. However, live biopsies of humans to assess the body stores are justifiable only under certain instances and therefore indirect techniques to estimate liver reserves of vitamin A have been developed (Tanumihardjo et al, 1994; Ribaya-Mercado et al, 1999). These techniques include the relative dose response and modified relative dose response methods which provide an indication of when vitamin A liver stores are depleted (Tanumihardjo et al, 1994). During the last few decades, isotope dilution techniques have been developed to estimate body stores of vitamin A in humans (Furr et al, 1989; Haskell et al, 1997; Haskell et al, 1998; Tang et al, 1999a; Ribaya-Mercado et al, 1999; Ribaya-Mercado et al, 2000). All isotope dilution techniques measure the degree of isotopic enrichment of retinol after a challenge dose of labeled retinyl ester. It is assumed that measurements are performed after the isotope has equilibrated with the body's vitamin A pool. A mathematical formula (Furr et al, 1989) based on estimates of the absorption and storage of retinol derived from isotope dilution studies in rats (Bausch and Rietz, 1977) is used for the interpretation of data to enable estimation of the body stores of vitamin A. Since most detection methods are relatively insensitive (see section on methods of detection), the first deuterated retinol dilution tests required large doses of labeled retinol [1.35 μmol/kg body weight (Furr et al, 1989), 0.70 μmol/kg (Ribaya-Mercado et al, 2000), 0.75 μmol/kg (Haskell et al, 1997), 0.75 μmol/kg and 0.85 μmol/kg (Haskell et al, 1998)]. However, more

sensitive detection methods have enabled slightly lower doses of retinol to be used [0.61 μmol/kg body weight (Ribaya-Mercado et al, 1999), and 0.45 μmol/kg (Tang et al, 1999a)]. Since these techniques are primarily developed to assess body stores of retinol in retinol-depleted subjects, large doses might bias the estimates that are obtained. These techniques have been validated against concentrations of retinol in biopsies of liver which is the primary storage site of retinol (Furr et al, 1989; Haskell et al, 1997). Another technique involving administration of 52 nmol [$^{13}C_4$]retinol to weaning rats with an average weight of 60 g at onset has been reported (Tanumihardjo, 2000). Although the author suggested that this method was more sensitive than others, the dose administered to rats corresponded to 0.87 μmol/kg body weight which was similar to the other methods. In fact, if related to body surface area, the dose would be relatively greater in humans than that which has been used up until now.

Studies designed to obtain qualitative information on bioavailability and bioefficacy of carotenoids

To our knowledge, eleven studies have been published since 1990 using compounds labeled with stable isotopes for studying carotenoid bioavailability and/or bioefficacy in humans. Some of these investigations have been reviewed previously (Swanson et al, 1996; Parker, 1997). Based on their design and data published (**Table 5-2** on page 96-99), 5 studies provide only qualitative information while 6 provide quantitative data. In the first of the qualitative studies, which involved one man, Parker and colleagues (1993) administered a single physiological dose of 1 mg of uniformly labeled [^{13}C]β-carotene which had been biosynthesized in green algae grown with ^{13}C as the sole carbon source. From this experiment, the only conclusion which can be drawn is that β-carotene is absorbed and partially converted to retinol in the body. Yao and colleagues (2000) administered a single physiological dose of 3 mg of uniformly labeled biosynthesized [^{13}C]lutein to each of four women and could measure the appearance and disappearance in plasma. You and colleagues (1996) administered a single physiological dose of 992-994 μg uniformly labeled [^{13}C]9-*cis*-β-carotene and 6-8 μg [^{13}C]*all-trans*-β-carotene (biosynthesized by green algea) to each of 3 subjects. At least 8 mo later, each subjects also consumed a single dose of 992-994 μg unlabeled 9-*cis*-β-carotene and 6-8 μg [^{13}C]*all-trans*-β-carotene. From this experiment it could be concluded that *cis*-β-carotene in part is isomerized to *all-trans*-β-carotene before or during its absorption. Burri and Park (1998) fed a pharmacological dose of 40 mg [$^2H_8$]β-carotene to each of eight women. Although the data obtained from this study were analyzed using a mathematical compartmental model designed to quantify the bioavailability of β-carotene, the only conclusion drawn by the authors was that β-carotene is converted to retinol in the body with high interindividual variation. Finally, Pawlosky and colleagues (2000) fed one woman a single physiological dose of 5 mg [$^2H_8$]β-carotene and concluded that the appearance and disappearance in plasma of labeled β-carotene could be measured.

Studies designed to quantify bioavailability and bioefficacy of carotenoids

Novotny and colleagues (1995) administered a single pharmacological dose of 40 mg [$^2H_8$]β-carotene to one man. Although they estimated the bioavailability at 22%, assumptions

Table 5-2. Human studies using stable isotopic tracers for studying bioavailability and bioefficacy of dietary carotenoids qualitatively or quantitatively

Reference	Intervention: a) Description of subject(s); b) Isotopic tracer and dosing regimen[1]; c) Number and timing of blood sampling[2]	Analysis: a) Sample preparation and measurement of isotopic enrichtment[3]; b) Method of data analysis	Conclusion: a) results (mean with CV)[4]; b) comments
Studies designed to obtain qualitative information on carotenoid bioavailability and bioefficacy			
Parker et al, 1993	a) 1 man, 41 y; b) Single physiological dose of 1 mg intrinsically uniformly labeled [^{13}C]β-carotene in oil; c) 12 samples within 22 h	a) Plasma saponified, purified by using HPLC, for β-carotene hydrogenated overnight, and then analyzed by GC-IRMS[5] (Goodman and Brenna, 1992); b) Estimation of maximal ^{13}C degree of isotopic enrichment in retinol, retinyl esters, and β-carotene in plasma	a) Maximal degree of isotopic enrichment in retinol, retinyl esters and β-carotene in plasma with ^{13}C was 0.8%, 9.2% and 1.2%, respectively; b) β-Carotene is converted to retinol in the body
You et al, 1996	a) 2 men and 1 woman, 23-38 y; b) Single dose of 992 or 994 µg [^{13}C]9-*cis*-β-carotene and 6-8 µg [^{13}C]*all-trans*-β-carotene min. 8 mo later followed by a single dose of 992 or 994 µg unlabeled 9-*cis*-β-carotene and 6-8 µg [^{13}C]-*all-trans*-β-carotene (each preparation was dissolved in safflower oil); c) ≤17 samples within 33 h using a catheter, then several samples by venipuncture up to 10 d	a) Plasma saponified, purified by using HPLC, for β-carotene hydrogenated overnight, and then analyzed by GC-IRMS (Parker et al, 1993; You et al, 1996); b) Plasma concentration versus time curve of [^{13}C]retinol, [^{13}C]*all-trans*-β-carotene and [^{13}C]9-*cis*-β-carotene in plasma	a) Substantial amounts of [^{13}C]*all-trans*-β-carotene and [^{13}C]retinol appear in plasma after ingestion of an oral dose consisting of >99% [^{13}C]9-*cis*-β-carotene while only small amounts of [^{13}C]9-*cis*-β-carotene appear to be secreted in the bloodstream; b) *cis*-β-carotene in part is isomerized to *all-trans*-β-carotene before or during its absorption
Burri and Park, 1998	a) 5 adult women, 21-28 y; b) Single pharmacological dose of 40 mg [$^{2}H_8$]β-carotene in oil; c) 5 samples within 15 h and 7 up to 21 d	a) Plasma extracted, saponified, extracted by solid phase, derivatizated, and then analyzed by GC-MS (Dueker et al, 1993) for retinol and by HPLC for β-carotene (Dixon et al, 1994, modified after Dueker et al, 1994; b) Compartmental model developed by Novotny et al (1995)	a) A working compartmental model for β-carotene metabolism was developed, which suggests that [$^{2}H_8$]β-carotene converts to [$^{2}H_4$]retinol with high inter-individual variation; b) β-Carotene is converted to retinol in the body
Yao et al, 2000	a) 4 women, 25-38 y; b) Single physiological dose of 3 mg intrinsically uniformly labeled [^{13}C]lutein in oil; c) 13 samples within 16 h and 7 samples up to 22 d	a) Plasma extracted, saponified, lutein purified by reversed-phase and normal phase HPLC, hydrolysed and hydrogenated, and then analyzed by GC-IRMS; b) Plasma concentration versus time curve of [^{13}C]lutein	a) Peak enrichment was measured at 16 h, enrichment still measurable at day 22; b) Appearance and disappearance of [^{13}C]lutein in plasma after a single dose can be measured

Pawlosky et al, 2000	a) 1 woman, 32 y; b) Single physiological dose of 5 mg [$^2H_8$]β-carotene mixed into a breakfast drink; c) 1 sample at 8 h and 4 samples up to 29 d	a) Plasma extracted, and then analyzed by particle beam LC-MS[5] for β-carotene; b) Plasma concentration versus time curve of [$^2H_8$]β-carotene	a) Peak enrichment was measured at 8 h, enrichment still measurable at day 29; b) Appearance and disappearance of [$^2H_8$]β-carotene in plasma after a single dose can be measured. Degree of isotopic enrichment of retinol in plasma was not measured
Studies designed to quantify carotenoid bioavailability and bioefficacy			
Novotny et al, 1995	a) 1 man, 53 y; b) Single pharmacological dose of 40 mg [$^2H_8$]β-carotene in oil; c) 7 samples within 12 h period and 18 samples up to 113 d	a) Plasma extracted, saponified, extracted by solid phase, and then analyzed by HPLC for β-carotene (Dueker et al, 1994) and by GC-MS for retinol (Handelman et al, 1993); b) 5-term and a 3-term polyexponential equation, to describe plasma concentration versus time curves of [$^2H_8$]β-carotene and of [$^2H_4$]retinol, respectively[6]	a) 22% of labeled β-carotene was absorbed: 17.8% as intact β-carotene and 4.2% as retinoid; In liver and enterocyte 43% and 57% of β-carotene was converted to retinoid, respectively; Mean residence time of β-carotene in body was 51 d; b) Outcome is heavily affected by assumptions underlying the compartmental model[7]
Tang et al, 2000	a) 1 woman, 47 y; b) Single doses of 126 mg (pharmacological) and 6 mg (physiological) [$^2H_8$]β-carotene in crystalline form dissolved in oil and 9 mg [$^2H_8$]retinyl acetate on separate occasions up to 2.5 y apart; c) 2 (3) samples within 6 (9) h and 6 up to 21 d after consumption of [$^2H_8$]β-carotene ([$^2H_8$]retinyl acetate)	a) Serum extracted, purified by HPLC, derivatized, and then analyzed by electron capture negative chemical ionization GC-MS for retinol (Tang et al, 1997; Tang et al, 1998); b) Area under serum concentration versus time curve of [$^2H_4$]retinol[7] compared to that of [$^2H_8$]retinol[7]	a) Vitamin A activity of 3.8 and 55 μg β-carotene in oil was equivalent to that of 1 μg retinol after doses of 6 and 126 mg β-carotene, respectively; b) Degree of isotopic enrichment of β-carotene in serum was not measured

Table 5-2. (continued)

Reference	Intervention: a) Description of subject(s); b) Isotopic tracer and dosing regimen[1]; c) Number and timing of blood sampling[2]	Analysis: a) Sample preparation and measurement of isotopic enrichtment[3]; b) Method of data analysis	Conclusion: a) results (mean with CV)[4]; b) comments
Lin et al, 2000	a) 11 women, 19-39 y; b) Single pharmacolgical doses of 10 mg [$^2H_6$]retinyl acetate and 20 mg [$^2H_6$]β-carotene in 1 wk apart; c) 5 samples within 20 h and 19 samples up to 28 d after consumption of [$^2H_6$]retinyl acetate, of which 5 samples within 20 h and 8 samples up to 21 d after consumption of [$^2H_6$]β-carotene	a) Plasma extracted, saponified, extracted by solid phase, and then analyzed by GC-MS for retinol (Furr et al, 1989; Dueker et al, 1998b) and by HPLC for β-carotene (modified after Dueker et al, 1998b and Van Kuijk et al, 1985); b) Area under plasma concentration versus time curve of [$^2H_6$]retinol, from 0 to 4 d, and of [$^2H_3$]retinol, from 0 to 4 d, and of [$^2H_6$]β-carotene, from 0 to 21 d; Dose normalised ratios of the AUCs of [$^2H_3$]retinol to [$^2H_6$]retinol were used for calculation of vitamin A activity; Summation of the AUCs of [$^2H_3$]retinol and [$^2H_6$]β-carotene, and a number of assumptions were used for calculation of total absorption (i.e. bioavailability)	a) Vitamin A activity of 2.3 µg (CV, 140%) β-carotene in oil was equivalent to that of 1 µg retinol. Five of 11 women were designated as non-responders; Reported absorption of β-carotene was 3.3% (range, 0 -14.4%; CV 135%); b) Bioefficacy of β-carotene in oil is 41% while bioavailability is 3.3%. Obviously, these conflicting results cannot be true and the assumptions need to be addressed. CV is rather high
Van Lieshout et al, 2001 (**Chapter 2**)	a) 16 girls and 19 boys, 8-11 y; b) 2 doses of 80 µg [$^{13}C_{10}$]β-carotene in oil and 80 µg [$^{13}C_{10}$]retinyl palmitate per d over a period of ≤10 wk; c) 3 samples per child over a period ≤10 wk[8]	a) Serum extracted, and then analyzed by APCI LC-MS[5] for retinol (Van Breemen et al, 1998; Wang et al, 2000) and for β-carotene (Wang et al, 2000); b) Bioefficacy and bioavailability of β-carotene in oil quantified using CarRet PIE mathematical model of β-**Car**otene and **Ret**inol at **P**lateau **I**sotopic **E**nrichment (**Chapter 2**)	a) Plateau isotopic enrichment was reached within 21 days; An amount of 2.4 µg β-carotene in oil (between subjects CV, 36%; within subjects CV, 22%) has the same vitamin A activity equivalent as 1 µg retinol; The bioavailability of β-carotene in oil was 108% (*n* = 33; geometric mean; between subjects CV, 36%; within subjects CV 23%); b) Bioavailability and bioefficacy of β-carotene in oil can be quantified with high precision
Tang et al, 1999b	a) 1 man, 45 y, and 1 woman, 51 y; b) Single dose of 200 g spinach, grown on 30% ^2H_2O, containing 13.8 mg uniformly ^2H labeled *all-trans*-β-carotene[9] and 3.0 mg [$^2H_8$]retinyl acetate 3 d apart; c) Several samples up to 8 d	a) Serum extracted, purified by HPLC, derivatized, and then analyzed by electron capture negative chemical ionization GC-MS for retinol (Tang et al, 1998); b) Area under serum concentration versus time curve of uniformly labeled retinol compared to that of [$^2H_8$]retinol	a) Vitamin A activity of 27-72 µg (range; CV 73%) β-carotene in spinach was equivalent to that of 1 µg retinol; b) Relatively high CV. Degree of isotopic enrichment of β-carotene in serum was not measured.

Van Lieshout et al, 2001 (**Chapter 3**)	a) 38 girls and 39 boys, 7-13 y; b) 3 doses of 31 μg [$^{13}C_{10}$]β-carotene in oil and 21 μg [$^{13}C_{10}$]retinyl palmitate per d for 6 wk, in wk 3-6, 2 portions of either 164 g spinach or 162 g pumpkin were consumed per d, containing 2.8 and 1.4 mg β-carotene, respectively[10]; c) 1 sample at the end of each 3-wk period	a) Serum extracted, and then analyzed by APCI LC-MS[5] for retinol (Van Breemen et al, 1998; Wang et al, 2000) and for β-carotene (Wang et al, 2000); b) Bioefficacy and bioavailability of β-carotene in oil quantified and relative bioefficacy and bioavailability of β-carotene in spinach and pumpkin estimated using CarRet PIE mathematical model of β-**Car**otene and **Ret**inol at **P**lateau **I**sotopic **E**nrichment (**Chapter 2 and 3**)	a) An amount of 2.7 μg β-carotene in oil (CV, 21%) has the same vitamin A activity as 1 μg retinol[11]; Bioavailability of β-carotene in oil was 81% (n = 73; geometric mean; CV, 42%); Bioavailability and bioefficacy of β-carotene in pumpkin was 1.7 (interval: 1.3, 2.4) times that of β-carotene in spinach; b) Findings on bioavailability and bioefficacy of β-carotene confirm earlier studies (**Chapter 2**; De Pee et al, 1998; Khan et al, 1998).

[1] Unless otherwise specified, isotope tracers were specifically labeled.

[2] A baseline sample was collected shortly before consumption of the labeled compound(s) in all studies (see also footnote 8).

[3] Concentration of retinol and β-carotene in serum/plasma was measured by HPLC in all studies.

[4] If more than one subject was studied, values are expressed as mean with coefficient of variation (CV). The CV (SD/$\bar{x}$) comprises the biological variation within and between subjects and the variation in the chemical analysis. All studies investigate the same metabolic processes (bioavailability and bioefficacy). Therefore, it can be expected that the biological variation within and between subjects is somewhat comparable between the studies. Thus, in our opinion, differences in CV's mentioned in this table indicate differences in precision of the techniques used for studying bioavailability and bioefficacy and are related to the design of the studies more than to the subjects studied.

[5] GC-IRMS refers to gas chromatography-isotope ratio mass spectrometry. LC-MS refers to liquid chromatography-mass spectrometry. APCI LC-MS refers to atmospheric pressure chemical ionization LC-MS.

[6] Model intestinal absorption was constrained to be inside the range of two SD of 15% ± 4.5% referring to a publication of Bowen and colleagues (1993). In the original publication of this study (Shiau et al, 1994), 2.6 mg (CV, 14%) of a 15 mg dose of β-carotene was absorbed in 11 healthy men (mean age of 26 y).

[7] [$^{2}H_4$]Retinol was sum of [$^{2}H_3$]retinol, [$^{2}H_4$]retinol + [^{13}C]-[$^{2}H_3$]retinol, and [^{13}C]-[$^{2}H_4$]retinol. [$^{2}H_8$]Retinol was sum of [$^{2}H_6$]retinol, [$^{2}H_7$]retinol, [$^{2}H_8$]retinol, [$^{2}H_8$]retinol + [^{13}C]-[$^{2}H_6$]retinol, and [$^{13}C_2$]-[$^{2}H_8$]retinol.

[8] In a subgroup of 12 children the first blood sample was collected at baseline, for the others, the first blood sample was collected on day 21.

[9] Spinach also contained 2.7 mg other provitamin A carotenoids (1.9 mg 9-*cis*-β-carotene, 0.6 mg α-carotene, and 0.2 mg 13-*cis*-β-carotene). In our calculations we assumed that the vitamin A activity of other provitamin A carotenoids was half that of β-carotene.

[10] Spinach and pumpkin also contained 128 and 604 μg other provitamin A carotenoids, respectively. In our calculations we assumed that the vitamin A activity of other provitamin A carotenoids was half that of β-carotene.

[11] If data from the two studies are pooled, an amount of 2.6 μg β-carotene in oil (median) has the same vitamin A activity as 1 μg retinol and the (median) bioavailability of β-carotene in oil was 86%.

underlying the compartmental model used limited its validity (see section on method of analysis of data). With such a study design, it is not possible to assess the bioefficacy of β-carotene.

To date, five studies have been conducted in which both bioavailability and bioefficacy were estimated. In an investigation involving two adults, Tang and colleagues (1999b) administered a single dose of 3 mg [$^2H_8$]retinyl acetate and 200 g spinach grown on 30% ^2H-labeled H_2O. The spinach contained 13.8 mg uniformly labeled [^2H]β-carotene, 2.7 mg uniformly deuterated other provitamin A carotenoids, and an unstated amount of uniformly labeled carotenoids without vitamin A activity. It was concluded that the vitamin A activity of 27-72 μg β-carotene in spinach was equivalent to that of 1 μg retinol. In another study (Tang et al, 2000), the same research group administered to one woman single doses of 126 mg (pharmacological dose) and 6 mg (physiological dose) of [$^2H_8$]β-carotene and 9 mg [$^2H_8$]retinyl acetate on separate occasions up to 2.5 y apart. From this study, they concluded that an amount of 3.8 or 55 μg β-carotene in oil has the same vitamin A activity as 1 μg retinol after doses of 6 or 126 mg, respectively. The degree of isotopic enrichment of β-carotene in serum was not measured, thus the bioavailability of carotene could not be assessed. From this study, it can be concluded that high pharmacological doses of β-carotene are not absorbed to the same extent as physiological doses. Thus estimates derived from such high doses cannot be readily extrapolated to the bioefficacy of dietary carotenoids.

Lin and colleagues (2000) administered single pharmacological doses of 10 mg [$^2H_6$]retinyl acetate and of 20 mg [$^2H_6$]β-carotene to 11 women one week apart. From the areas under the curve of the concentrations in plasma of [$^2H_3$]retinol, [$^2H_6$]retinol and [$^2H_6$]β-carotene, and using a number of assumptions which are discussed later (see section on mathematical modeling), bioavailability and bioefficacy of carotene were calculated. Five of the 11 women participating in this study were designated as non-responders because they did not absorb β-carotene. In our opinion, there are two reasons for the low isotopic enrichment of retinol in serum in these subjects. Firstly, the matrix of β-carotene probably limited its absorption and thus bioconversion. Secondly, the enrichment being measured approached the limit of quantitation or even detection (see section on methods of detection). Be that as it may, based on the results of the remaining 6 subjects, the investigators reported that the bioefficacy of dietary β-carotene was high, 74% (CV 81%) while the bioavailability was only 6.1% (SE, 1.8; range, 1.1 - 14.4). A bioefficacy of 74% means that 74% of β-carotene is absorbed and converted to retinol (i.e., 1.25 μg β-carotene has the same vitamin A activity as 1 μg retinol) (**Chapter 2**). Since these results are inconsistent with each other, the assumptions underlying the model will need to be addressed (see section on mathematical modeling).

We have conducted two controlled dietary studies involving the daily administration of <200 μg β-carotene and <90 μg retinol, each specifically labeled with 10 ^{13}C atoms. The degree of isotopic enrichment of β-**CAR**otene and **RET**inol in serum at **P**lateau **I**sotopic **E**nrichment were measured by liquid chromatography-mass spectrometry (LC-MS), and data were analyzed using the CarRet PIE mathematical model (**Chapter 2-4**). In the first study lasting ≤10 wk with 35 children, 2.4 μg (mean; 95% CI: 2.1, 2.7) β-carotene in oil was found the have the same vitamin A activity as 1 μg retinol, while in the second study lasting 6 wk

with 77 children, 2.7 μg (mean; 95% CI: 2.5, 2.8) β-carotene in oil had the same vitamin A activity as 1 μg retinol. If data from both studies are pooled (n = 111), it is estimated that 2.6 μg β-carotene in oil (median value) has the same vitamin A activity as 1 μg retinol. Thus, the best estimate of bioefficacy of β-carotene in oil is 36%. If data from both studies are pooled (n = 106), the median bioavailability of β-carotene in oil was 86%. The labeled β-carotene fed in the first study had a *cis-trans* ratio of 3:1. Since β-carotene occurs in foods mainly in the *all-trans* configuration, for the second study care was taken that 90% of the labeled β-carotene was in the *all-trans* configuration. In the second study, comprising a 3-wk run-in period, followed by a 3-wk treatment period, children were randomly allocated to daily receive either 164 g spinach or 162 g pumpkin, containing 2.8 and 1.4 mg β-carotene and 0.12 and 0.60 mg other provitamin A carotenoids, respectively. From this study, it can be concluded that the bioefficacy and the bioavailability of β-carotene in pumpkin were both 1.7 (95% CI: 1.3, 2.4) times that of β-carotene in spinach.

STUDY DESIGN

In designing intervention studies to quantify the bioavailability and bioefficacy of dietary carotenoids in humans using tracer techniques, decisions need to be made at different levels (Table 5-1). Often decisions made at one level will limit decisions which can be made at other levels. Such decisions will be illustrated on the basis of the studies presented above (Table 5-2).

Defining the aim and hypothesis

In general terms, three types of studies can be envisaged in which isotopically labeled carotenoids and/or retinol can be used. These include qualitative studies and two types of quantitative studies: efficacy studies which provide information under closely controlled conditions and effectiveness studies which measure the impact of an intervention under field conditions. As discussed above, many of the initial studies were qualitative in nature and demonstrated that β-carotene was converted to retinol in the body. More recently, attention has been directed towards measuring the extent of conversion of β-carotene to retinol within the framework of efficacy. As discussed later, mathematical models are required which allow such data to be obtained. Studies in which body pools of retinol are estimated can be used in both efficacy and effectiveness studies. Regardless of the type of study, clearly defined hypotheses should be established, which can be evaluated using appropriate statistical tests.

Defining the study population and estimating the sample size

There are two prior considerations in the selection of the study population. The first is to choose a population in which results are most likely to be obtained. Thus, when examining serum retinol response to feeding β-carotene or retinol, vitamin A-depleted subjects are more responsive, thus providing data which could not be obtained in a replete population. The second is to choose a population for which the results are relevant: for example, developed versus developing countries, men versus women versus children. Thus, in

developing countries, the question arises whether subjects should be treated to eliminate intestinal parasites. We have taken the view that untreated subjects should be studied because they are representative of the population. The US Institute of Medicine (IOM, 2001) has taken the view that data from subjects in developing countries cannot be used in establishing bioefficacy or nutrient requirements because such subjects are not representative of the population of the USA.

Considering sample size, many studies conducted up until now have not used sufficient subjects (see Table 5-2) to provide representative data of any population. To a large extent, this has been due to the developmental nature of the work being carried out and the costs involved. However in the future, more attention will need to be addressed to power. Based on the intra- and interindividual variation observed in studies conducted up until now, data are available to perform power calculations.

CHOICE OF ISOTOPIC TRACER

Isotopes

Elements (atoms with the same number of protons) containing a different number of neutrons are referred to as isotopes which can be either stable or radioactive. Although for retinol and some carotenoids (but not cyclic and acyclic carotenes) it would be possible to use isotopes of oxygen, all studies up until now have used isotopes of carbon or hydrogen (see Table 5-2). Isotope effects, such as changes in the reaction rates in biological systems, are most likely to occur when using isotopes of hydrogen (Wolfe, 1992; Dueker et al, 1998a), because the mass of ^{2}H or ^{3}H is double or triple that of ^{1}H, whereas the mass of ^{13}C or ^{14}C is only 8 or 17% higher than that of ^{12}C. Isotope effects are more likely with radioisotopes because their atomic weights are typically higher than those of their stable counterparts. One problem which does not arise when using radioisotopes in studies of carotenoid metabolism is short half-life. Both ^{14}C and ^{3}H have relatively long half-lives, 5760 y and 12.3 y, respectively.

Other reasons dictate whether radio- or stable isotopes are used in human intervention studies (Wolfe, 1992; Patterson and Veillon, 2001). Firstly, since methods for detecting radioactivity are very sensitive (see section on method of detection), much lower doses of labeled compounds can be administered in studies using compounds labeled with radioisotopes - thus avoiding perturbation of metabolism (see section on size of dose). Secondly, after the administration of a compound labeled with a radioisotope, the compound and its metabolites can be traced at low concentrations by measuring the radioactivity. With compounds labeled with stable isotopes the degree of isotopic enrichment might be too low to measure precisely. Thirdly, when using radioisotopes, whole body measurements of radioactivity can be made. With stable isotopes, the tissue and compound in which the isotope is incorporated must be known before the tissue can be collected and the compound isolated in order to measure the degree of isotopic enrichment. However, for many studies in humans involving administration of radioisotopes, prior knowledge of the metabolism is also required because not all tissues can be collected from healthy volunteers (see section on

specimen collection). Fourthly, in the past, organic compounds labeled with stable isotopes were relatively expensive but prices are coming down as supplies increase. In spite of the advantages of using radioisotopes, the reluctance against their use still exists because health risks of using radioisotopes need to be considered as high doses of radioisotopes might be harmful. For the future, we suggest that more discussion takes place on the risks of using radioisotopes in human nutrition research. Hence, we would like to stress that Dueker's radioistope study (2000) is a welcome approach after decades during which the reluctance to use radioisotopes has increased. Until there is more general acceptance of their use, compounds labeled with stable isotopes will continue to be used in studies of carotenoid bioavailability and bioefficacy.

Method and degree of labeling

The method of isotopic labeling of compounds is very important because this has major implications for further aspects of study design such as preparation and analysis of samples, and subsequent analysis of data. Either intrinsic or extrinsic labeling can be used. Intrinsic labeling involves biological incorporation of isotope into compounds so that the compounds are in the same matrix as the foods consumed. Extrinsic labeling refers to the chemical incorporation of isotopes into a compound. Thus for intrinsic labeling, spinach has been grown on 30% ^{2}H-labeled H_2O to produce intrinsically labeled β-carotene in a spinach matrix (Tang et al, 1999b). Although much effort has been spent on intrinsically labeling vegetables such as spinach, carrots and tomatoes, it has been difficult to produce enough vegetable to achieve enrichment of carotenoids in serum at a sufficient level to obtain reliable data. In fact, just one abstract reporting such studies has been published up until now (Tang et al, 1999b).

In our opinion, the disadvantages of intrinsic labeling outweigh the advantages for studies aimed at quantifying carotenoid bioavailability and bioefficacy. Firstly, in intrinsically labeled plants, isotopes are incorporated in all molecules instead of only the compounds of interest. Thus, spinach or tomatoes grown on ^{2}H-labeled H_2O or on $^{13}CO_2$ will also contain other labeled carotenoids (with and without provitamin A activity) as well as other labeled compounds, which might bias the results. Secondly, if the duration of exposure to the label is insufficient, the isotope will not be incorporated into all positions in the molecule to the same extent. This can lead to problems in the interpretation of analytical data and, together with the use of parent compounds labeled to an extent less than 100%, might lead to a low degree of isotopic enrichment of compounds of interest. As a result, the amounts of isotopic compound and plant material which need to be administered to enable adequate measurements must be increased. A third problem is producing sufficient quantities of labeled vegetables to carry out a complete study or a series of studies. Due to limitations in the size of the plant and the length of the growing season, it will prove very difficult to obtain intrinsically labeled fruit. To some extent, these problems can be overcome by not using fruit and vegetables normally consumed but using other biological sources such as green algae. Green algae have been grown on $^{13}CO_2$ as the sole carbon source to provide labeled *all-trans*-β-carotene (Parker et al, 1993), 9-*cis*-β-carotene (You et al, 1996) and lutein (Yao et al, 2000). However, the algae matrix is probably different to that of most fruits and

vegetables. It should be noted that in their studies, Parker (1993), You (1996) and Yao (2000) extracted β-carotene and lutein from the algae and dissolved the compounds in oil. Thus the advantage of intrinsic labeling with respect to the matrix was lost. Fourthly, isotope effects may be enhanced with intrinsic labeling which results in labeling throughout the molecule. As discussed above, such effect would be greater with hydrogen than for carbon.

Although extrinsic labeling can also be used to synthesize uniformly labeled compounds, its greatest advantage is in labeling compounds specifically at a limited number of sites. It is necessary to decide upon the number of atoms which need to be labeled in a molecule. This number should be sufficient to provide enrichment above the natural abundance (1.1% for ^{13}C and 0.016% for ^{2}H). Thus, for retinol and carotenoids, at least 3 ^{13}C atoms need to be incorporated. Since absorbed labeled β-carotene is in part converted to retinol, studies on the bioefficacy of β-carotene should use β-carotene containing at least 6 ^{13}C atoms located at designated sites on each half of the molecule. In order to be able to distinguish labeled retinol which is administered from that synthesized in vivo from β-carotene, the administered retinol should contain at least 3 ^{13}C atoms more than the retinol derived from the labeled β-carotene. We carried out pilot studies using β-carotene and retinol each labeled with 6 ^{13}C atoms. However, we decided to increase the number of ^{13}C atoms in both retinol and β-carotene to 10 in order to improve the performance of the measurements of isotopic enrichment (Van Breemen et al, 1998). Use of different stable isotopes, such as ^{13}C and ^{2}H, is of limited value for this purpose because it is necessary to create molecules differing in molecular weight. When using radioisotopically labeled β-carotene and retinol, it is possible to carry out studies using different radioisotopes, such as ^{14}C and ^{3}H, for each compound (Goodman et al, 1966; Blomstrand and Werner, 1967).

Apart from the number of isotopic atoms incorporated into each molecule, the positions at which isotopes are incorporated should be chosen very carefully. The isotopes should be placed at chemically inert sites in order to minimize loss or rearrangement during metabolism, sample preparation or analysis. This is a major concern with ^{2}H atoms especially when labeled compounds are prepared using reversible reactions (Dueker et al, 1998a). When using ^{2}H atoms for extrinsic labeling at specific positions, scrambling (i.e., rearrangement of isotopes to positions in the molecule other than those in which they were initially incorporated) occurs more often than when using ^{13}C atoms (**Chapter 2**). If scrambling occurs, adjustments in the mass spectrometry method may be required.

Isotopic purity of both intrinsically and extrinsically labeled compounds can be very high, > 95% (Parker et al, 1993; Pawlosky et al, 2000), > 98% (You et al, 1996), or > 99% (Lugtenburg et al, 1999; **Chapter 2 and 3**; Yao et al, 2000). Unfortunately, not all studies report the isotopic purity of compounds used (Burri and Park, 1998; Tang et al, 1999b) while some studies have used compounds with a lower isotopic purity, 91% (Lin et al, 2000), 80-81% (Novotny et al, 1995; Tang et al, 2000). High isotopic purity facilitates detection of labeled compounds by mass spectrometry and subsequent quantification of the bioavailability and bioefficacy by mathematical modeling. Low isotopic purity reduces the sensitivity and precision of mass spectrometric quantification. If multiple ions must be measured as a result, the number of variables increases and consequently the error of the

method also increases (see section on chemical analysis of samples and section method of analysis of data).

Matrix of tracers

When studying carotenoid bioavailability and bioefficacy, two types of matrices are important, namely oil and that of fruit/vegetables. Oil as a matrix is important for two reasons. Firstly, because it is a matrix in which carotenoids are consumed, e.g. β-carotene in palm oil. Secondly, it is expected that bioavailability of carotenoids will be maximal in this matrix. Studies on the bioavailability and bioefficacy of carotenoids in fruit and vegetables are important because there is controversy about the role of the matrix of fruit and vegetables in bioavailability and thus bioefficacy of carotenoids. Therefore, much emphasis has been put on how bioavailability and bioefficacy in such matrices can be measured. Because intrinsically labeled vegetables have the same matrix there has been interest in their use for studying carotenoid bioavailability and bioefficacy. Although, as discussed above, there are a number of problems with intrinsic labeling.

In a study by Tang and colleagues (1999b), [$^2H_8$]retinyl acetate and spinach containing uniformly ^2H labeled carotenoids were administered to two subjects. In our recent study in Indonesia (**Chapter 3**), we used extrinsically labeled β-carotene to quantify the bioavailability and bioefficacy of β-carotene in spinach and pumpkin. The extent of dilution of the isotopic enrichment of retinol and β-carotene were used as the basis for calculating the bioavailability and bioefficacy of β-carotene in spinach and pumpkin.

In one of the investigations by Tang and colleagues (2000), β-carotene was administered in crystalline form. The β-carotene bioefficacy of 25% reported by Tang and colleagues (2000) was lower than that of 36% reported by us (**Chapter 3**). One explanation for this finding might be the lower bioavailability and thus lower bioefficacy of β-carotene in large crystals form compared with that dissolved in oil as shown in studies in rats (Zhou et al, 1996). Thus, in future studies, more attention should be given to ensuring that β-carotene is in fact dissolved, for example by examining the preparation microscopically.

CHOICE OF DOSING REGIMEN

Route of administration

Although compounds are administered by a variety of routes in pharmacological investigations of bioavailability, in studies of carotenoid bioavailability and thus bioefficacy, most attention is directed towards the intravenous and oral routes of administration. In pharmacology, bioavailability is defined as the ratio of the area under the curve of the concentration of the compound of interest after oral administration to that after intravenous administration. However, we define bioavailability differently (see section on rationale), which is necessary because a fraction of the ingested and absorbed provitamin A carotenoids will be converted to retinol in the body (bioconversion). As a result, serum concentrations of provitamin A carotenoids do not reflect the total response to ingested carotenoids as part of the ingested carotenoids will be converted to retinol.

Unlike the administration of hydrophilic compounds, it is impractical to administer retinol and carotenoids parenterally because of the difficulty of formulating them in a physiological form such as chylomicrons or, in the case of retinol, bound to retinol-binding protein and transthyretin.

Frequency of dosing

Single doses of labeled compounds can be administered to provide qualitative information on the kinetics, appearance and disappearance of labeled compounds and their metabolites in the body (Parker et al, 1993; Novotny et al, 1995; You et al, 1996; Burri and Park, 1998; Tang et al, 1999b; Lin et al, 2000; Pawlosky et al, 2000; Tang et al, 2000; Yao et al, 2000). All studies of carotenoid metabolism using radio-labeled compounds, have administered single doses (Goodman et al, 1966; Blomstrand and Werner, 1967; Dueker et al, 2000). There are some limitations using single doses for studying the bioefficacy of carotenoids. Firstly, single doses do not represent habitual intakes and thus their metabolism might differ from that of nutrients consumed over a longer period. Such differences have been found in studies on the absorption of iron and on factors affecting iron absorption. In their review on iron absorption and bioavailability, Hallberg and Hulthen (2000) explain why the variation in iron absorption from the whole diet is lower than from single meals. Almost all studies examining the effect of food components - termed 'effectors' (Castenmiller and West, 1998) - which modulate iron absorption have used single meals containing or not containing the effector under study. The variation in iron absorption between single meals of different compositions may be much greater than the variation in iron absorption from whole diets composed of several single meals because the latter is the mean absorption of several single meals. This does not mean that the absorption of iron from single meals per se would be falsely high or low. Similarly, single dose studies for studying carotenoid bioavailability and bioefficacy might not result in false estimates per se but the estimates cannot be readily extrapolated to the bioavailability and bioefficacy of carotenoids in the habitual diet. In addition, the variation in the estimates obtained using single doses might be relatively high, thus requiring a large number of subjects in a study.

Secondly, at some time-point after administration of a single dose of isotope, enrichment approaches baseline values. Thus, many measurements are often made when the signal-to-noise ratio is low or, in other words, when the limit of quantitation is approached or even exceeded (see section on method of detection). Thirdly, data analysis of single dose studies involves the use of complicated mathematical models often based on assumptions that are difficult to justify (see section on method of analysis of data).

In studies in which both labeled β-carotene and retinol are administered, both should be administered at the same time (**Chapter 2 and 3**). In some studies intervals of one week (Lin et al, 2000) or over six months (Tang et al, 2000) have been used instead of simultaneous dosing. Such dosing regimen might result in bias due to time effects.

The use of multiple doses results in a plateau of isotopic enrichment (Wolfe, 1992). Such a plateau can be obtained earlier if an initial priming dose is used. Because a plateau is reached after a prolonged period of dosing, the degree of isotopic enrichment of nutrients in serum is generally higher than that reached after a single dose, thus facilitating detection.

Thus, not only can repeat measurements be made to establish the level of enrichment but individual measurements will be more reliable because they will not be near or below the limit of quantitation.

Multiple doses may be administered at different intervals. In the studies conducted by our laboratories, a dosing regimen of labeled carotenoids and retinol of 2 or 3 times per day at mealtime was chosen, because these nutrients were normally consumed at mealtime. The aim of this was for the label to mix with the foods ingested. Thus at plateau isotopic enrichment, the ratio of isotopic enrichment in plasma represents the ratio of the flow of labeled and unlabeled nutrients from the gut. In our studies, plateau isotopic enrichment was reached within 21 d of the start of multiple dosing (**Chapter 2**) and might have occurred between 8 and 21 d (**Chapter 3**). As will be discussed later (see section on mathematical modeling), plateau values can be used readily in mathematical models.

Size of dose

It is necessary to decide not only on the frequency of dosing but also on dose size. In the study by Dueker and colleagues (2000), the limit of quantification of [^{14}C]β-carotene in plasma, urine and feces was in the attomole range (10^{-18} mol). This level of sensitivity enabled a low dose (306 μg) of [^{14}C]β-carotene to be administered.

Detection methods for stable isotopes are less sensitive than those for radioisotopes. Thus, the dose administered needs to be relatively high to reach measurable levels of isotopic enrichment. Four single dose studies (Table 5-2; Novotny et al, 1995; Burri and Park, 1998; Lin et al, 2000; Tang et al, 2000) involved the administration of pharmacological doses (> 10 mg) which might be inappropriate for quantifying bioavailability and bioefficacy. The metabolism of retinol and β-carotene might be perturbed as was shown by Von Reinersdorff and colleagues (1996) who administered 55 mg [$^{13}C_3$]retinyl palmitate to 11 healthy men. Although β-carotene is absorbed by passive diffusion, the absorption and metabolism of β-carotene is dose-dependent. Thus, from their own studies in rats and those of others in experimental animals and humans, Brubacher and Weiser concluded that the bioefficacy of β-carotene in humans was dose dependent when more than 4 mg was fed in one meal (Brubacher and Weiser, 1985). Such an effect may explain, at least in part, the results of Tang and colleagues (2000) who administered 6 mg and 126 mg [$^{2}H_8$]β-carotene to the same woman, 2.5 y apart. They reported that carotenoid bioefficacy was 25 and 1.7%, with the respective doses.

To minimize the effects which can be observed when large doses are fed (Wolfe, 1992), studies have been carried out in which amounts normally consumed in a meal have been fed (Table 5-2; Parker et al, 1993; You et al, 1996; Tang et al, 1999b; Pawlosky et al, 2000; Yao et al, 2000). However, with these physiological doses the problem of using relatively insensitive methods of detection of stable isotope labeled compounds may arise. Thus, some of the variation in the results obtained in these studies might be explained through experimental error resulting from attempting to measure isotopic enrichment beyond the limit of quantitation. These limitations were successfully overcome in studies using multiple low doses (<200 μg β-carotene/d and <90 μg retinol/d) of stable isotope labeled compounds for a sufficiently long period to enable isotopic enrichment to reach a plateau (**Chapter 2 and 3**).

CHOICES RELATED TO THE COLLECTION OF SAMPLES

Specimen

An advantage of radioisotopes (except for very weak β-emitters such as tritium) is that whole-body measurements are possible to determine retention of administered compounds (Patterson and Veillon, 2001), irrespective of whether the compounds have been metabolized or not. In addition, the route of labeled compounds through specific body tissues can be followed if such tissues can be sampled. In the radioisotope studies of Goodman, Blomstrand and their colleagues (Goodman et al, 1966; Blomstrand and Werner, 1967) lymph was collected in order to measure β-carotene absorption. However, cannulation of lymph ducts in humans can only be carried out in a clinical setting. In studies using stable isotopes to assess total body stores of retinol, liver biopsies have been collected to evaluate the usefulness of plasma samples in assessing body stores (Bausch and Rietz, 1977; Furr et al, 1989). As for lymph cannulation, liver biopsies can only be taken in a clinical setting.

Since retinol excretion in urine is increased in infection, isotopically labeled compounds can be used to assess the extent of such excretion. Under normal physiological circumstances, only small amounts of retinol are excreted in feces but the amount of β-carotene excreted can be quite large (**Chapter 4**; Dueker et al, 2000) representing β-carotene which has not been absorbed. We measured the isotopic enrichment of β-carotene in feces during multiple low doses of [$^{13}C_{10}$]β-carotene to assess whether the bioavailability of carotenoids could be studied in this way (**Chapter 4**). Bioefficacy cannot be assessed using data from feces because bioconversion occurs in the body while complete collection of feces is necessary to assess the bioavailability of β-carotene in oil using feces. Using data from feces it was assessed that the bioavailability of β-carotene from pumpkin (n = 6) was 1.8 times more efficiently than from spinach (n = 8), which is comparable to the ratio of 1.7 observed in the same subjects using data from serum. Collection of tissues other than serum or feces might be useful for gaining further insights into the metabolism of carotenoids.

Number and timing of collection of samples

In single dose studies, the extent of isotopic enrichment of retinol and β-carotene in plasma is measured to enable the area under the curve of isotopic enrichment to be calculated. Since the accuracy of the estimate of the bioavailability and bioefficacy depends on the frequency and timing of sampling of blood samples (Gibaldi, 1991), a sufficient number of samples at representative time-points (during the absorption and during the elimination phase) need to be collected. In a study, on one adult, by Parker and colleagues (1993) samples of blood were collected up to 22 h after dosing, which is a relatively short period in order to obtain a full picture of the appearance and disappearance of labeled compounds. On the other hand, in another study also involving one adult, Novotny and colleagues (1995) collected samples of blood up to 113 d, which is an extremely long time. As discussed earlier, when isotopic enrichment approaches baseline values, the

signal-to-noise ratio will decrease significantly, therefore dramatically increasing the analytical variation.

In multiple dose studies, the length of time over which samples are collected is determined by the time required to reach plateau isotopic enrichment. Because a plateau is attained, less samples have to be collected than in single dose studies. However, because the intraindividual variation in bioavailability and bioefficacy of dietary β-carotene is >20%, collection of more than one sample would dramatically increase the power of the study, or enable fewer subjects to be required (**Chapter 2**).

CHOICES RELATED TO THE CHEMICAL ANALYSIS OF SAMPLES

Detection methods for radioisotopes

As discussed earlier, radio-isotopically labeled compounds can be detected with high sensitivity (Patterson and Veillon, 2001). Another advantage is that it is not necessary to know in which metabolite the label is incorporated. This might save time in tracking which metabolite has been formed. Activity of isotopes emitting β-radiation can be measured by a liquid scintillation counter while a crystal scintillation counter can be used to measure isotopes emitting γ-radiation. In the study by Dueker and colleagues (2000), activity of β-carotene in serum, feces and urine was assessed by accelerator mass spectrometry (AMS). According to them, combustion of the compound of interest prior to analysis is an advantage because complex matrices such as feces and urine can be studied. However, with combustion structural information is lost. In addition, sample preparation for AMS is labor-intensive since it requires extraction, saponification, reversed phase HPLC fractionation, graphitization and combustion. It should be noted that AMS does not require the use of radioisotopes. Instead, ions containing ^{14}C are separated from those containing ^{12}C and ^{13}C according to their *mass-to-charge* ratio (m/z) in the mass spectrometer. The ^{14}C isotope is preferred for isotope enrichment studies simply because it has a much lower background level than ^{13}C.

Detection methods for stable isotopes

Nuclear magnetic resonance and HPLC

The sensitivity of detection methods to assess isotopic enrichment is generally lower than that of methods to assess radioactivity (Patterson and Veillon, 2001), and there is considerable variation in sensitivity between the various methods. Nuclear magnetic resonance (NMR) is useful to measure the degree of isotopic enrichment of macronutrients. However, serum concentrations of micronutrients such as β-carotene and retinol are very low. Unless plasma samples are of the order of 1 L, NMR is not a useful technique for measuring isotopic enrichment of micronutrients.

In three studies (Table 5-2; Novotny et al, 1995; Burri and Park, 1998; Lin et al, 2000) hexane extraction, saponification and solid phase extraction of plasma was followed by high performance liquid chromatography (HPLC) with a UV detector to baseline separate [$^2H_8$]β-carotene from β-carotene. Development of this method was described by Dueker and colleagues (1994). In another paper (Dueker et al, 1998a), they noted that [$^2H_6$]β-carotene

could not be baseline separated from β-carotene. Lin and colleagues (2000) used a computer program to integrate these peaks and to simulate baseline separation. Since [$^2H_8$]β-carotene can be baseline separated from unlabeled β-carotene, the rate of metabolism of these compounds would differ in biological systems such as humans. The shorter retention time of [$^2H_8$]β-carotene in a reversed-phase HPLC system would suggests that it is more polar than unlabeled β-carotene, probably because the ^2H atoms are located to a large extent on methyl side chains of the β-carotene molecule (Dueker et al, 1998a). This could influence the rate of incorporation of β-carotene into mixed micelles and subsequently absorption from the gut. It is known that polarity affects absorption of carotenoids. For example, lutein is more polar than β-carotene and has been estimated to be absorbed 5 times more efficiently (Van het Hof et al, 1999).

Mass spectrometry

Compared to the NMR instruments discussed above, mass spectrometers are many orders of magnitude more sensitive. The limits of quantification are in the attomole range (10^{-18} mol) for accelerator mass spectrometers and in the femtomole range (10^{-15} mol) for organic gas chromatography-mass spectrometry (GC-MS) and LC-MS instruments. Organic mass spectrometers are also highly selective detectors that may be used to distinguish carotenoids and retinoids based on their molecular weight and fragmentation patterns. Furthermore, tandem mass spectrometry combines mass selectivity with fragmentation pattern selectivity resulting in an even more selective detector of organic ions. These features allow mass spectrometers to be more selective and specific than UV, visible or IR spectrophotometers. In addition, mass spectrometers are more sensitive than absorbance-based detectors. For example, Van Breemen (1996a; 1997) compared visible absorbance detection, electrospray and atmospheric pressure chemical ionization (APCI) mass spectrometry during on-line LC-UV/VIS-MS for the measurement of β-carotene and α-carotene and showed that electrospray and APCI mass spectrometry were up to 100-fold more sensitive than absorbance detection. Since mass spectrometers separate ions of molecules based on their *mass-to-charge* ratio (m/z), this analytical instrument is ideal for the sensitive and selective detection and quantification of isotopically labeled carotenoids and retinoids. Furthermore, stable isotope labeling is preferred over the potentially more hazardous radio-labeling.

Mass spectrometers designed for the analysis of organic molecules are widely available and permit the determination of molecular weights as well as fragmentation patterns. These instruments include GC-MS, LC-MS, MS-MS and LC-MS-MS. Some specialized instruments used for the measurement of isotopic enrichment include accelerator mass spectrometers (AMS), which are expensive and only available at a few facilities, and isotope ratio mass spectrometers (IRMS), which are more widely available than AMS but still less common and more expensive than organic mass spectrometers. Both of these types of specialized mass spectrometers require highly purified samples and involve the combustion of organic compounds into CO_2 and water, thus losing structural information. The abundance of isotopically labeled C, O or H in these products may be measured at high precision. Accelerator mass spectrometers require the most careful and demanding sample preparation

of any mass spectrometer and are intolerant of highly isotopically enriched samples since these tend to contaminate the laboratory and the instrument. IRMS may be used for a wider range of isotopic enrichment and unlike AMS can be interfaced to a GC for on-line sample purification.

Since on-line GC-IRMS is more convenient than purifying a compound prior to IRMS, most studies involving isotopic enrichment of carotenoids and retinoids have used the former approach. Another advantage of GC-IRMS is that it is not necessary to know in advance which GC peak will contain the labeled compound. However, the selectivity of this approach is provided solely by GC since all structural information is lost during combustion. Even though GC provides some separation and selectivity for GC-IRMS, the high temperatures during GC induce isomerization and extensive band broadening of carotenoids such as β-carotene which results in loss of sensitivity (Dueker et al, 1994). Since carotenoids and retinoids do not separate efficiently during GC, HPLC is usually used to purify the peak of interest prior to GC-IRMS. It should be noted that LC cannot be coupled to IRMS because the combustion products from the mobile phase would interfere with the analysis.

In spite of the limitations of GC-IRMS, this technique has been applied to the measurement of the isotopic enrichment of carotenoids and retinoids. For example, Parker and colleagues (1993) developed a labor-intensive and time-consuming method for carotenoids in which the *all-trans*-β-carotene fraction was saponified, purified by using HPLC, hydrogenated overnight to perhydro-β-carotene, and then was analyzed using GC-IRMS. This method has been used by You and colleagues (1996) to measure the degree of isotopic enrichment of 9-*cis*-β-carotene. In addition, this method was modified by Yao and colleagues (2000) by the addition of a hydrogenolysis step and used for the measurement of the degree of isotopic enrichment of lutein. In a variation of the GC-IRMS approach for retinol analysis, Tang and colleagues (1997; 1998) replaced the IRMS with an organic mass spectrometer equipped with electron capture negative ion chemical. Although this type of mass spectrometer is much more widely available than the IRMS, sample preparation was still complex and laborious consisting of liquid/liquid extraction of the serum samples followed by HPLC purification, and then derivatization of retinol to form the trimethylsilyl (TMS) ether. Finally, derivatized retinol and its octadeuterated analog were monitored during GC-MS as an abundant fragment ion formed by elimination of the TMS group. This method permitted the detection of 0.01% [$^2H_8$]TMS-retinol in the presence of unlabeled TMS-retinol. In addition to the low throughput and labor intensive sample preparation, this approach raises the concern of the possibility of deuterium exchange and loss during ionization and fragmentation in the chemical ionization source of the mass spectrometer. In a similar study of retinol, Furr and colleagues (1989) developed a protocol in which retinol and retinyl esters were extracted from serum samples using hexane, purified using semi-preparative HPLC, saponified, recombined, purified again using another HPLC system, and then analyzed using GC-MS. Handelman and colleagues (1993) reported a more selective method in which a derivatization step was added.

Dueker and colleagues (1994) developed a procedure to measure the isotopic enrichment of β-carotene in serum that avoided GC-MS and eliminated derivatization. However, serum had to be extracted twice, first using organic solvent and then using solid phase extraction.

Then, two HPLC purification steps, one reversed phase and the second normal phase, were necessary to remove interfering compounds prior to off-line MS-MS analysis using positive ion electron impact ionization.

A logical simplification of the procedure used by Dueker and colleagues (1994) would be to carry out HPLC separation on-line with mass spectrometric detection and quantification, and at least two research groups have developed such LC-MS solutions. For example, we used reversed phase HPLC with on-line atmospheric pressure chemical ionization (APCI) for the analysis of the degree of isotopic enrichment in β-carotene and its metabolite isotopically labeled retinol (Wang et al, 2000). Also, Pawlosky and coworkers (2000) used HPLC with a particle beam mass spectrometric interface and negative ion electron capture ionization for the measurement of the degree of isotopic enrichment of β-carotene. Both of these methods used solvent extraction of serum without any derivatization followed by on-line LC-MS quantification. Furthermore, these LC-MS approaches used widely available organic mass spectrometers interfaced directly to the HPLC, avoided the thermal degradation problems of gas chromatography, and prevented the loss of structural information and selectivity that would occur upon sample combustion during GC-IRMS. Unlike the method of Dueker and colleagues (1994), only a single extraction step and a single HPLC step were necessary, mass spectrometric quantitation was carried out on-line during HPLC, and ^{13}C labeling instead of ^{2}H labeling was used so that problems associated with loss or scrambling of the label were minimized.

In addition to APCI and particle beam LC-MS, we evaluated electrospray LC-MS for the quantative analysis of isotopically enriched β-carotene and retinol (Van Breemen et al, 1998). Although electrospray LC-MS is highly sensitive for the detection of both β-carotene (Van Breemen et al, 1996b) and retinol (Van Breemen and Huang, 1996c) with detection limits similar to APCI and particle beam (≤1 pmol), we found that the dynamic range of this technique was insufficient for studies involving trace levels of isotope enrichment (Van Breemen and Huang, 1996c). For example, in studies of [$^{13}C_5$] and [$^{13}C_{10}$]retinol in human serum, we found that labeled and unlabeled retinol coeluted during reversed phase HPLC, and the high proportion of unlabeled retinol suppressed the ionization of the labeled form. Furthermore, we found that the electrospray standard curves for retinol (Van Breemen et al, 1998) and β-carotene (Wang et al, 2000) were non-linear.

In contrast to electrospray, APCI produced a linear detector response over at least four orders of magnitude for both retinol and β-carotene, and there was no evidence of ion suppression when measuring trace amounts of labeled compounds in the presence of a large excess of unlabeled material (Wang et al, 2000). For comparison, particle beam with negative ion electron capture detection was reported to show a linear detector response for β-carotene over a range of only two orders of magnitude. Thus far, the use of particle beam LC-MS has not been reported for the analysis of studies of isotopically enriched retinol in human serum so that its application to the study of the bioconversion of β-carotene to retinol remains untested.

In summary, among the methods that have been reported for the determination of bioavailability, bioconversion and bioefficacy of β-carotene, organic mass spectrometry offers higher selectivity and greater availability than either AMS or IRMS. Additional

advantages include faster, simpler and less expensive sample preparation. Comparing the organic mass spectrometry techniques currently available, the on-line chromatographic techniques of GC-MS and LC-MS offer the greatest convenience, sensitivity and speed. In particular, LC-MS stands out as the most practical technique because it eliminates the need for sample derivatization, avoids the possibility of thermal degradation of the sample, and requires only a single serum extraction step prior to analysis. By minimizing sample manipulation, the probability of introducing experimental error is also minimized. In addition, the efficiency and speed of sample preparation and subsequent LC-MS analysis are ideal for large numbers of samples from representative groups. Further refinements and improvements in this approach should involve the incorporation of MS-MS, i.e., LC-MS-MS, to improve the selectivity of this APCI-based method. The use of more selective LC-MS-MS methods should also enable multiple carotenoids and their *cis* and *trans* isomers to be monitored during a single analysis

Method validation and quality control

As outlined by Rodriguez-Amaya (1999), the acquisition of reliable analytical data requires: representative samples, validated analytical methods, quality assurance, adequately trained personnel and ancillary support staff and facilities. The question is not only how good the method is, but also how well it is being used in the laboratory.

The main performance parameters that should be taken into account in assessing any analytic method are: accuracy, closeness of the measured value with the true value; precision, a measure of the repeatability and reproducibility; specificity, the ability of a method to measure the compound of interest exclusively; the limit of detection, the lowest concentration of a compound that the analytic process can differentiate reliably from background levels (generally defined as three times the standard deviation (SD) of the level measured in substrate blank); the limit of quantification, the lowest concentration of a compound that can be measured with a stated degree of confidence (generally defined as ten times the SD of the substrate blank); linearity, the range of concentrations over which the method has been demonstrated to give a linear response; the scope of the method, the number of different substrates to which the method can be successfully applied (Huber, 2001).

Quality control consists of obtaining data of the appropriate fitness for their intended use. The quality of data is affected (in random order) by: man, material, manipulation (in the sense of interpretation), machine, method. The quality of data can be monitored by an effective quality control program to prevent, identify and correct errors. It is outside the scope of the paper to explain this in much detail, but we would like to stress the importance of using in-house control samples, participation in interlaboratory trials and of using certified reference materials for this purpose (Rodriguez-Amaya, 1999).

METHOD OF DATA ANALYSIS

Mathematical modeling

Modeling can be defined as the creation of a simplified representation of the world (Wilson and Dainty, 1999). When building models, Occam's Razor should be applied liberally. Occam's Razor states that the simplest explanation of an entity (here: β-carotene metabolism) is preferred (Britannica, 1999). So, the number of pools and variables should be kept to the minimum required to reflect the behavior observed. For effective modeling, decisions should be made about which features of the real world will be included in the model, and to what extent they need to be simplified. These decisions are based on the required function of the model, as well as constraints imposed by our limited understanding of the world (Wilson and Dainty, 1999). For studying carotenoid bioavailability and bioefficacy, three types of mathematical models have been described: compartmental models; area under the curve; and models based on plateau isotopic enrichment reached by multiple dosing (CarRet PIE). Novotny and colleagues (1995) developed one model with five compartments and one model with three compartments to make estimates of the dynamics of β-carotene metabolism, such as the absorption of β-carotene and the conversion of β-carotene to retinol, from data on plasma versus time curves of labeled β-carotene and its metabolite labeled retinol. The compartmental model was based on several assumptions, including an assumption on the outcome parameter. Thus, to describe the absorption of dietary β-carotene an estimate of the absorption of dietary β-carotene was included in the compartmental model. The need for such an assumption decreases the reliability of the final results. In Novotny's compartmental model, the intestinal absorption was constrained to be inside the range of two SD of 15% ± 4.5% based on Bowen and colleagues (1993). In the original publication of this study (Shiau et al, 1994), 2.6 mg (CV, 14%) of a 15 mg dose of β-carotene was absorbed in 11 healthy men with a mean age of 26 y. Burri and Park (1998) used the same compartmental model as Novotny and colleagues to conclude that β-carotene converts to retinol with a high interindividual variability in a study on four females. In the study of Lin and colleagues (2000), fractional absorption of [$^2H_6$]β-carotene was calculated using a number of assumptions such as a plasma β-carotene half-life of 864 h (Novotny et al, 1995); mean sojourn time/1.4) that seem difficult to justify considering the conflicting results of the study on bioavailability (6%) and bioefficacy (41%) of β-carotene (Bowen et al, 1993; Van Vliet et al, 1995).

The other single dose studies (Table 5-2; Parker et al, 1993; You et al, 1996; Tang et al, 1999b; Tang et al, 2000; Lin et al, 2000; Pawlosky et al, 2000; Yao et al, 2000), plot plasma concentrations of labeled compounds versus time. Those studies in which labeled retinol was also administered (Tang et al, 1999b; Tang et al, 2000; Lin et al, 2000) can calculate and compare areas under the curves of plasma concentrations of labeled retinol derived from administered labeled retinol and of labeled retinol derived from administered labeled β-carotene. If the isotopic enrichment of labeled compounds is low, the isotopic enrichment measured at various masses has to be summed, thus increasing the variation of the estimate of isotopic enrichment, thereby decreasing the precision of the estimates of the area under the curves. A limitation of methods based on area under the curve is that they are influenced

by further metabolism of carotenoids such as clearance from serum, metabolism and excretion of carotenoids and retinol (Furr and Clark, 1997), making such methods inappropriate for studying the bioavailability and bioefficacy of carotenoids quantitatively. As already discussed by Van Vliet (1996), single dose studies cannot be used to study the effect of factors on bioavailability and bioefficacy if those factors also affect the clearance of carotenoids or retinol from serum/plasma.

These limitations can be overcome by multiple dosing during which plateau isotopic enrichment is reached as such a regimen diminishes the variation in the estimates of bioefficacy and bioavailability. In our studies in which multiple doses of stable isotopes were used data analysis was conducted by using the CarRet PIE mathematical model based on isotopic enrichment in serum of β-**CAR**otene with [$^{13}C_{10}$]β-carotene and of **RET**inol with [$^{13}C_5$]retinol and with [$^{13}C_{10}$]retinol all at **P**lateau **I**sotopic **E**nrichment (**Chapter 2-4**). Although the appropriate studies have not been carried out, it would be possible to quantify the bioefficacy of β-carotene in fruit and vegetables provided that the intake of unlabeled retinol from food is sufficient to observe a sufficiently large dilution of the isotopic enrichment during the vegetable period compared with the control period. This would enable absolute quantification of the bioavailability and bioefficacy of β-carotene in fruit and vegetables and other categories of food. The narrow confidence interval around the mean bioavailability and bioefficacy of β-carotene in oil, indicates that the CarRet PIE model results in estimates with a high precision. For the quantification of the bioavailability of β-carotene, this model requires an assumption for the stoichiometry of cleavage of β-carotene into retinol. In our estimates of bioavailability we have used the most efficient stoichiometry possible, i.e., 1 mol β-carotene yields 2 mol retinol, thus obtaining estimates of the minimum absorption. The use of a less efficient stoichiometry, e.g., a 1:1 molar equivalent, would result in a higher estimate of the bioavailability. In addition, it was assumed that labeled and unlabeled β-carotene fully mix and both compounds are metabolized in the same way but not necessarily to the same extent.

Statistical analysis of data

In making estimates of bioavailability and bioefficacy, it is necessary to provide a measure of precision related to the biological and analytical variation. Unfortunately, many of the studies carried out up until now have been limited to few subjects and sometimes only one subject rendering such studies difficult to evaluate. As mentioned in the section on defining the aim and hypothesis, the appropriate statistical tests to evaluate the results of the study should be applied.

CONCLUSIONS

A number of isotope techniques have been developed for studying the bioavailability and bioefficacy of dietary carotenoids in humans. From these studies, several conclusions can be derived. Firstly, in studies using isotope techniques it has been shown that β-carotene is converted to retinol in humans (**Chapter 2 and 3**; Parker et al, 1993; Novotny et al, 1995;

You et al, 1996; Burri and Park, 1998; Tang et al, 1999b; Tang et al, 2000; Lin et al, 2000). Secondly, the bioavailability of β-carotene and other carotenoids with or without provitamin A activity - such as 9-*cis*-β-carotene (You et al, 1996) and lutein (Yao et al, 2000), respectively - can be studied using isotope techniques. Thirdly, quantitative data obtained using isotope techniques for studying carotenoid bioavailability and bioefficacy available up until now are limited. In fact, reliable estimates of the bioavailability and bioefficacy of β-carotene in oil have only been derived from our two studies (**Chapter 2 and 3**). From our second study in Indonesia, it can be concluded that the absorption and conversion of β-carotene from pumpkin is 1.7 times more efficiently than that of β-carotene from spinach (**Chapter 3 and 4**). At this point in time, no reliable estimates derived from isotope techniques on the absolute bioavailability and bioefficacy of carotenoids in fruits and vegetables are available. In our second study, the amount of retinol and β-carotene in food provided was low and constant. In future experiments, we plan to provide small amounts of retinol in a highly bioavailable matrix. We expect this to provide a constant and low degree of isotopic enrichment of retinol in food during the run-in and treatment period while the degree of isotopic enrichment of β-carotene in food will be diluted in the treatment period (compared with the run-in period) by the large amount of unlabeled β-carotene in vegetable supplements provided. We expect this dilution in food to result in a measurable dilution in the degree of isotopic enrichment in serum of retinol and β-carotene from which the bioavailability and bioefficacy of β-carotene in food can be estimated. It is envisaged that increased understanding of carotenoid metabolism will enable further development of the design and mathematical models required for obtaining such absolute data.

In conclusion, fortunately many of the technological problems affecting earlier isotope studies have been overcome by the increased sensitivity of GC-MS and LC-MS methods. Of these two methods, the LC-MS methods have the additional advantage that they do not require labor-intensive sample preparation. This will be an essential advantage in the analysis of the large numbers of samples arising from studies with adequate power. LC-MS using APCI has emerged as the most effective and convenient technique for studying the bioavailability, bioconversion and bioefficacy of provitamin A carotenoids.

In our opinion, the use of mathematical models requiring assumptions that are difficult to justify should be reconsidered. In addition, single dose studies have led to analysis of data from measurements approaching the limit of detection. In contrast to studies of mineral metabolism, it will be difficult to overcome problems which will enable intravenous administration of β-carotene and retinol because of their lipophilic nature.

In conclusion, considerable progress has been made in the past decade enabling carotenoid bioavailability and bioefficacy to be studied using isotope techniques. This approach should now be used to quantify the bioavailability and bioefficacy of other provitamin A carotenoids and to quantify the effect of individual SLAMENGHI factors (a mnemonic for factors) which affect the bioavailability and bioefficacy of carotenoids under various conditions (De Pee and West, 1996; Castenmiller and West, 1998). In addition, some of the principles developed can be applied in studies on the bioavailability and bioefficacy of other micronutrients such as folic acid.

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6

Discussion

The aim of the research described in this thesis was to quantify the bioefficacy of β-carotene in foods consumed by school children in Indonesia. This chapter summarizes the main findings, the implications of these findings and provides recommendations for further research. Study of the bioavailability and bioefficacy is important because the contribution of plant foods to vitamin A status can only be substantial when not only the consumption and content of foods but also the bioefficacy of provitamin A carotenoids in such foods is high (**Chapter 1**). Isotopic tracer techniques can meet the need for accurate and precise data on bioavailability, bioconversion and bioefficacy of dietary carotenoids in humans. Isotope techniques have been used by others previously for this purpose but the way in which they have been applied is inappropriate in a number of ways for providing reliable data on the bioavailability and bioefficacy of carotenoids in humans (**Chapter 5**). Therefore, a new stable isotope technique - based on plateau of isotopic enrichment of β-carotene and retinol in serum reached during prolonged intake of multiple low doses of β-carotene and retinol, each specifically labeled with 10 ^{13}C atoms - has been developed (**Chapters 2-4**).

ISOTOPIC TRACER TECHNIQUES FOR STUDYING BIOAVAILABILITY AND BIOEFFICACY OF DIETARY CAROTENOIDS

This new stable isotope technique and those developed up until now by others have been reviewed (**Chapter 5**). Apart from the present technique all others have involved feeding a single dose of labeled β-carotene and/or retinol to a limited number of subjects. Because some methods for the detection of the degree of isotopic enrichment are relatively insensitive, high pharmacological doses of labeled compounds have often been used. After a single physiological dose, the degree of isotopic enrichment in serum approaches baseline values at some time-point. Thus, many measurements are often made when the signal-to-noise ratio is low or, in other words, when the limit of quantitation is approached or even exceeded. This might increase the analytical variation and thus complicate interpretation of the results. For the interpretation of the results from isotopic tracer techniques, mathematical models are needed and those describing the metabolism of a single dose are often based on assumptions that are difficult to justify. Other models have compared areas under the curve of serum concentrations of labeled retinol derived from a single dose of labeled β-carotene and labeled retinol. However, in addition to the need of collecting a sufficient number of blood samples, such methods have serious limitations because various factors affect the clearance from serum, metabolism and excretion of carotenoids and retinol.

The technique described in this thesis is based on reaching a plateau of isotopic enrichment of β-carotene and retinol during prolonged intake of multiple low doses of β-carotene and retinol, each specifically labeled with 10 ^{13}C atoms. The degree of isotopic enrichment of retinol and β-carotene, in either serum or feces, is measured using a highly sensitive method of detection - high performance liquid chromatography coupled with atmospheric pressure chemical ionization liquid chromatography-mass spectrometry (APCI LC-MS). In contrast to other methods the LC-MS method does not require labor-intensive

sample preparation. Therefore, it can be applied when large numbers of samples are generated in studies with adequate power. For the interpretation of data, a mathematical model has been developed based on assumptions that can be readily justified in the light of present knowledge on carotenoid metabolism (**Chapter 5**). The precision of estimates derived from serum seems to be higher than that of estimates derived from feces (**Chapter 4**).

In conclusion, the technique described would appear to be the best now available for use in humans to provide reliable estimates of the bioavailability and bioefficacy of dietary carotenoids (**Chapter 5**).

FINDINGS

Bioavailability and bioefficacy of dietary β-carotene

An amount of 2.6 μg β-carotene in oil has the same vitamin A activity as 1 μg retinol. This corresponds to a bioefficacy of β-carotene of 36%. Compared to retinol, the bioavailability of β-carotene in oil was found to be 86% (**Chapter 2 and 3**). Estimates of the bioavailability and bioefficacy of β-carotene in pumpkin ranged from 1.7 to 1.8 times those of β-carotene in spinach (**Chapter 3 and 4**).

IMPLICATIONS AND RECOMMENDATIONS

Application of stable isotope technique

There appears to be no reason to doubt that the data obtained and the method used in Indonesian children cannot be applied to other population groups in developing countries such as other children, adults (including pregnant and lactating women) and the elderly. Recently, it has been shown that supplementing women not only with β-carotene but also with zinc in the last 2 trimesters of pregnancy substantially increases vitamin A status of both the mothers and their infants until at least 6 mo after consumption of the last supplement (Dijkhuizen et al, 2001). This might indicate a role of zinc in the bioconversion, and thus bioefficacy, of β-carotene. This hypothesis can now be tested by using this stable isotope technique. Because of their proven or possible relationship in provitamin A or vitamin A metabolism, the bioavailability and bioefficacy of β-carotene in oil can be studied similarly in groups with different vitamin A status (Ribaya-Mercado et al, 2000), iron status (Suharno et al, 1993) or exposure to intestinal parasites (Jalal et al, 1998).

In future, it will be necessary to modify the study design in order to assess the absolute bioavailability and bioefficacy of β-carotene and other provitamin A carotenoids in categories of fruit and vegetables and in mixed diets (**Chapter 3**). In addition, instead of providing different foods, in a similar study design, the bioavailability and bioefficacy of different amounts of β-carotene ingested with a meal can be studied. The bioefficacy of high doses of carotenoids will probably be less (Brubacher and Weiser, 1985) but no data are available from humans at the present time.

Moreover, it will be necessary to study the influence of food components such as fat (Roodenburg et al, 2000), fiber (Castenmiller et al, 1999) or pectin (Rock and Swendseid, 1992) - termed 'Effectors' (Castenmiller and West, 1998) - which modulate the absorption and conversion of carotenoids. Up until now, single meals containing or not containing the effector under study have been used in most studies examining the role of such effectors. However, the variation in the results would be less and more reliable if the effectors were built into the whole diet, which should be fed over a period of time.

This plateau isotopic enrichment technique can also be used for studying the bioavailability and bioefficacy of other carotenoids with and without provitamin A activity. For example, when comparing the bioavailability of β-carotene and lutein, the use of isotopically labeled β-carotene and lutein is essential because of the provitamin A role of β-carotene. Earlier studies not based on the use of isotopes (De Pee et al, 1998; Van het Hof et al, 1999) have shown that the bioavailability of lutein, which is more hydrophilic, can be 6 times higher than that of β-carotene. However in their studies, bioavailability refers to the product of arrows 1 and 2 in **Figure 1-4**, which would provide an underestimate of the bioavailability of β-carotene because its bioconversion to retinol (arrow 3) has not been taken into account.

IMPLICATIONS OF DATA ON BIOEFFICACY OF CAROTENOIDS

Most data on the bioefficacy of β-carotene in food have been derived from a two-step process (**Chapter 1**). The first step comprises the bioefficacy of β-carotene in oil compared with that of retinol in oil. Data available including those presented in this thesis are summarized in **Table 3-5**. For the second step - the relative bioavailability of β-carotene in food compared with that of β-carotene in oil - there is still a paucity of data. In controlled dietary trials in Indonesia (De Pee et al, 1998) and Vietnam (Khan et al, 1998) it was found that 26 μg β-carotene in dark green leafy vegetables and 12 μg β-carotene in orange fruits have the same vitamin A activity as 1 μg retinol (bioefficacy of 3.6% and 7.8%, respectively). In a study in China (Tang et al, 1999), 27 μg β-carotene in green and yellow vegetables was found to have the same vitamin A activity as 1 μg retinol. Based on these 3 studies it can be estimated that as much as 21 μg β-carotene in a mixed diet (4:1 vegetables to fruit intake) has the same vitamin A activity as 1 μg retinol (West et al, 2001). This corresponds to a bioefficacy of β-carotene in a mixed diet of 4.5%. This low bioefficacy of β-carotene in dark green leafy vegetables compared with orange fruit has been confirmed by the work described in this thesis (**Chapter 3 and 4**). The data on the first and second step can be used to re-evaluate recommendations on the bioefficacy of carotenoids in food by national and international organizations as part of their guidelines on recommended dietary allowances. The US Institute of Medicine (IOM, 2001) has recently revised their guidelines but it would be interesting to note that if the IOM had used values of bioefficacy of β-carotene in oil available at the time of their deliberations in the range of 24-27% (Booher et al, 1939; Wagner, 1940, Hume and Krebs, 1949; Tang et al, 2000) instead of 47% (Sauberlich et al, 1974), the bioefficacy of β-carotene in a mixed diet would range from 4 to 4.5%.

Two question might arise, firstly, why there is a paucity of reliable data on the bioefficacy of carotenoids in foods ingested by humans and secondly, why is it important to have such data. Firstly, it has been over half a century since the first estimates on the relative efficiency of β-carotene and retinol were published (Booher et al, 1939; Wagner, 1940; Hume and Krebs, 1949). Since then, new knowledge has accumulated about the bioavailability and bioefficacy of dietary carotenoids. In part, this new knowledge derived from a better understanding of the metabolism of β-carotene - such as the discovery of 15,15'-dioxygenase, which is the enzyme responsible for the cleavage of β-carotene into retinol (Lakshmanan et al, 1972) - and because new approaches have been used for studying carotenoid metabolism (Tanumihardjo and Olson, 1988). At the same time, much of the new knowledge derived from the fact that carotenoid metabolism can be studied more readily because new technologies such as high performance liquid chromatography have enabled assessment of concentrations of retinol and carotenoids in serum/plasma (DeRuyter and De Leenheer, 1978; Nierenberg, 1985), food (Micozzi et al, 1990; Epler et al, 1993), breastmilk (Khachik et al, 1997), and tissues (Kaplan et al, 1990; Van Vliet et al, 1995; Haskell et al, 1997). For the plateau isotopic enrichment technique described in this thesis, new liquid chromatography-mass spectrometry methods have been developed (Van Breemen et al, 1998; Wang et al, 2000). In addition, an extraction method was developed for measuring concentration and isotopic enrichment of retinol and carotenoids in feces (**Chapter 4**). These are examples of new technologies which have helped broadening of our knowledge on carotenoid metabolism. It is envisaged, for example that in the future the effect of individual SLAMENGHI factors - as well as the effects of interactions between factors - on the bioavailability and bioefficacy of carotenoids can be quantified separately thus enabling the development of appropriate algorithms.

This leads to the answer to the second question: why is it so important to have reliable data on the relative efficiency of retinol and carotenoids in meeting recommended vitamin A intakes? The following example may clarify the implications of the bioefficacy on the effective supply of vitamin A. In **Chapter 1**, it was shown that if the bioefficacy of β-carotene in food was 8%, an unrealistically high consumption of 12 carrots, 8 portions of spinach or 11 mangos would be needed for a child to meet its daily requirement for vitamin A (400 RE) (FAO/WHO, 1988). If the bioefficacy of β-carotene in food was even lower, 4% - which is a likely prospective as explained above - a child would need to consume 25 carrots, or 17 portions of spinach or 22 mangos to meet a daily requirement for vitamin A. Alternatively, a child could consume 4 eggs or one-seventh of a chicken liver per day. However, most impoverished people cannot afford animal products, thus their consumption of food rich in retinol is low.

IMPLICATIONS FOR THE CONTROL OF VITAMIN A DEFICIENCY

Based on the findings presented and on findings of other studies, it is justified to conclude that approaches other than the promotion of fruit and vegetables are required for eliminating vitamin A deficiency. This does not mean that the consumption of fruit and

vegetables should not be promoted. A combination of approaches (**Chapter 1**) such as immunization, targeted supplementation of vulnerable groups of the population, and food fortification with retinol, seems to be more effective in eliminating vitamin A deficiency. For those with an adequate vitamin A status, social marketing of foods rich in provitamin A carotenoids could help to maintain their vitamin A status. Another approach might be promotion of genetically modified plants such as 'Golden Rice' (Ye et al, 2000). Before the rice is produced and promoted on a large scale, its efficacy (impact under controlled conditions) and effectiveness (impact under field conditions) for controlling vitamin A deficiency should be assessed. A modification of the plateau isotopic enrichment technique described in this thesis could be used for this purpose.

Finally, it should be noted that the discussion on the bioefficacy of β-carotene and on the effectiveness of approaches to eliminating vitamin A deficiency is taking place while the recommended human requirements for vitamin A are at most guesstimates. For proper evaluation of approaches to eliminating vitamin A deficiency, more effort should be directed towards more reliable estimates of vitamin A requirements not only in populations in developed countries but also in those in developing countries.

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Vitamin A deficiency is a serious health problem in developing countries. Improved vitamin A status can reduce morbidity and mortality by 23% in developing countries. Improved vitamin A status can be achieved by reducing the body's demand for vitamin A or by increasing the effective supply of vitamin A or by combining both these approaches. Vitamin A occurs in food as preformed vitamin A (retinol), only present in animal foods and breastmilk, and as provitamin A carotenoids. Foods rich in provitamin A carotenoids are red palm oil, dark-green leafy vegetables, yellow and orange fruits, and red and orange roots and tubers, such as carrots and red sweet potato. Provitamin A carotenoids in plant foods are the major source of vitamin A for a large proportion of the world's population. However, the contribution of plant foods can only be substantial when not only the consumption and provitamin A content of foods but also the bioefficacy of provitamin A carotenoids in such foods is high (**Chapter 1**). With respect to provitamin A carotenoids, bioefficacy is the product of the fraction of the ingested amount which is absorbed (bioavailability) and the fraction of that which is converted to retinol in the body (bioconversion).

In theory, of all carotenoids has the highest vitamin A activity. In their guidelines of 1967 and 1988, the Food and Agricultural Organization (FAO)/World Health Organization (WHO) proposed that the vitamin A activity of 1 μg retinol can be supplied by 3.3 μg β-carotene in oil and 6 μg β-carotene in a mixed diet. Recently, the US Institute of Medicine (IOM) has proposed that the vitamin A activity of 1 μg retinol can be supplied by 2 μg β-carotene in oil and 12 μg β-carotene in a mixed diet. Because a low bioefficacy of β-carotene in plant food has major implications for the effective supply of vitamin A for many impoverished people, accurate and precise data on bioavailability, bioconversion and bioefficacy of dietary carotenoids in humans are required to enable a proper evaluation of food-based approaches for eliminating vitamin A deficiency. Isotopic tracer techniques can supply such data. Isotope techniques have been used by others previously for this purpose but the way in which they have been applied is inappropriate in a number of ways for providing reliable data on the bioavailability and bioefficacy of carotenoids in humans (**Chapter 5**).

Therefore, a new stable isotope technique - based on reaching plateau of isotopic enrichment of β-carotene and retinol in serum during prolonged multiple low doses of β-carotene and retinol, each specifically labeled with 10 ^{13}C atoms - has been developed. The aim of the research described in this thesis was to quantify the bioavailability and bioefficacy of β-carotene in foods typically consumed by school children in Indonesia using this new technique.

ISOTOPIC TRACER TECHNIQUES: A REVIEW OF THE LITERATURE (CHAPTER 5)

Apart from the present technique all others have involved feeding a single dose of labeled β-carotene and/or retinol to a limited number of subjects. Because some methods for the detection of the degree of isotopic enrichment are relatively insensitive, high pharmacological doses of labeled compounds have often been used. After a single physiological dose, the degree of isotopic enrichment in serum approaches baseline values at

some time-point. This might increase the analytical variation and thus complicate interpretation of the results. For the interpretation of the results from isotopic tracer techniques, mathematical models are needed and those describing the metabolism of a single dose are often based on assumptions that are difficult to justify. Other models have compared areas under the curve of serum concentrations of labeled retinol derived from a single dose of labeled β-carotene and labeled retinol. However, in addition to the need of collecting a sufficient number of blood samples, such methods have serious limitations because various factors affect the clearance from serum, metabolism and excretion of carotenoids and retinol. In the next section, the principles of the plateau isotopic enrichment technique and the design of the studies using this technique will be explained.

PLATEAU ISOTOPIC ENRICHMENT TECHNIQUE FOR STUDYING CAROTENOID BIOEFFICACY

The stable isotope technique described in this thesis is based on reaching plateau isotopic enrichment of β-carotene and retinol during prolonged intake of multiple low doses of β-carotene and retinol, each specifically labeled with 10 ^{13}C atoms. To date, this plateau isotopic enrichment technique has been tested in two studies. In the first study (**Chapter 2**), 35 Indonesian children (aged 8-11 y) daily consumed 2 doses of 80 μg [$^{13}C_{10}$]β-carotene and of 80 μg [$^{13}C_{10}$]retinyl palmitate for periods ≤ 10 wk. Over this period, 3 blood samples were drawn per child to obtain serum. Serum, after hexane extraction, was analyzed by APCI LC-MS. Because β-carotene and retinol labeled with stable isotopes weigh more than the unlabeled parent compounds, the degree of isotopic enrichment 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 can be measured by high performance liquid chromatography with atmospheric pressure chemical ionization liquid chromatography-mass spectrometry (APCI LC-MS). From the first study, we concluded that plateau isotopic enrichment of retinol and β-carotene in serum is reached within 21 days, which is important for the design of future studies.

In the second study (**Chapter 3 and 4**) comprising a 3-wk run-in period followed by a 3-wk treatment period, 77 Indonesian school children daily consumed 3 doses of 31 μg [$^{13}C_{10}$]β-carotene and of 21 μg [$^{13}C_{10}$]retinyl palmitate. During the treatment period, half of the subjects daily received 2 portions of 82 g spinach (1.5 mg β-carotene/portion) while the others received 2 portions of 81 g pumpkin (0.7 mg β-carotene/portion). At baseline, and at the end of the run-in and treatment periods, blood samples were drawn and 17 of the 77 children collected stool samples. In blood and stool samples, the degree of isotopic enrichment of retinol and of β-carotene was measured using LC-MS. These LC-MS techniques have emerged as the most effective and convenient for studying the bioavailability, bioconversion and bioefficacy of provitamin A carotenoids. For the interpretation of data, a mathematical model has been developed based on assumptions that can be readily justified in the light of present knowledge on carotenoid metabolism.

FINDINGS ON THE BIOAVAILABILITY AND BIOEFFICACY OF β-CAROTENE

If data from the above-mentioned studies were pooled, an amount of 2.6 μg β-carotene in oil was found to have the same vitamin A activity as 1 μg retinol (**Chapter 2 and 3**). This corresponds to a bioefficacy of 36%. The bioavailability of β-carotene in oil was found to be 86% in these Indonesian children (**Chapter 2 and 3**). The bioavailability and bioefficacy of β-carotene in pumpkin were 1.7 times those of β-carotene in spinach (**Chapter 3**). The precision of estimates derived from serum seems to be higher than that of estimates derived from feces, probably because stool collection was often incomplete (**Chapter 4**). Despite the consumption of large amounts of β-carotene in spinach and pumpkin in the second study, the degree of isotopic enrichment of retinol in serum did not change during the treatment period. Therefore, the absolute bioavailability and bioefficacy of β-carotene in spinach and pumpkin could not be estimated. In future studies, the basal diet should be altered to provide a constant and small intake of retinol in a highly bioavailable matrix.

In conclusion, the technique described would appear to be the best now available for use in humans to provide reliable estimates of the bioavailability and bioefficacy of dietary carotenoids (**Chapter 5**).

FUTURE APPLICATIONS OF THE PLATEAU ISOTOPIC ENRICHMENT TECHNIQUE (CHAPTER 6)

This plateau isotopic enrichment technique can be applied for studying in humans the effect of individual SLAMENGHI factors which affect the bioavailability and bioefficacy of carotenoids (SLAMENGHI is a mnemonic for such factors). For example, the bioefficacy of carotenoids in groups of subjects with different nutritional status with regard to vitamin A, zinc or iron can be studied because these nutrients have been associated with influencing vitamin A and carotenoid metabolism. In future, using a modified design as described above, this technique may be applied for studying the bioefficacy of carotenoids in a mixed diet, and in diets providing different amounts of carotenoids with a meal. Moreover, the effect of 'Effectors' of the absorption and conversion such as fat, fiber or pectin may then be studied. Because many carotenoids, with and without provitamin A activity, can be specifically labeled with ^{13}C atoms, the bioavailability and bioefficacy of these carotenoids and of interaction between such carotenoids can be studied.

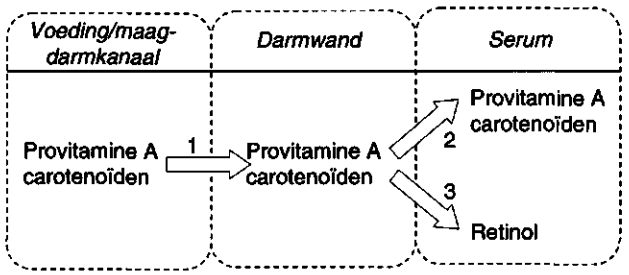
IMPLICATIONS OF THE NEW DATA ON THE BIOEFFICACY OF CAROTENOIDS

The studies described in this thesis have provided the most reliable estimates of the bioefficacy of β-carotene in oil available up until now. There would appear to be no reason to doubt that the data obtained and the method used in Indonesian children cannot be applied to other population groups in developing countries such as other children, adults (including pregnant and lactating women) and the elderly. Based on two recent controlled

dietary trials in Indonesia and Vietnam it has been concluded that as much as 21 μg β-carotene in a mixed diet (4:1 vegetables to fruit intake) has the same vitamin A activity as 1 μg retinol (a bioefficacy of β-carotene in a mixed diet of 4.5%). This low bioefficacy of β-carotene in dark green leafy vegetables compared with orange fruit has been confirmed by the work described in this thesis (**Chapter 3 and 4**). Thus, the current guidelines that the vitamin A activity of 1 μg retinol can be supplied by 6 μg (FAO/WHO) and 12 μg (IOM) of β-carotene in a mixed diet overestimate β-carotene bioefficacy about 2 to 3 times.

The new data on the bioefficacy of β-carotene in oil and in a mixed diet can be used to re-evaluate recommendations on the bioefficacy of carotenoids in food by national and international organizations as part of their guidelines on recommended dietary allowances. In addition, the data enable the formulation and evaluation of appropriate policies and programs for controlling vitamin A deficiency. Because of the low bioefficacy of carotenoids in plant foods approaches other than the promotion of fruit and vegetables are required for eliminating vitamin A deficiency.

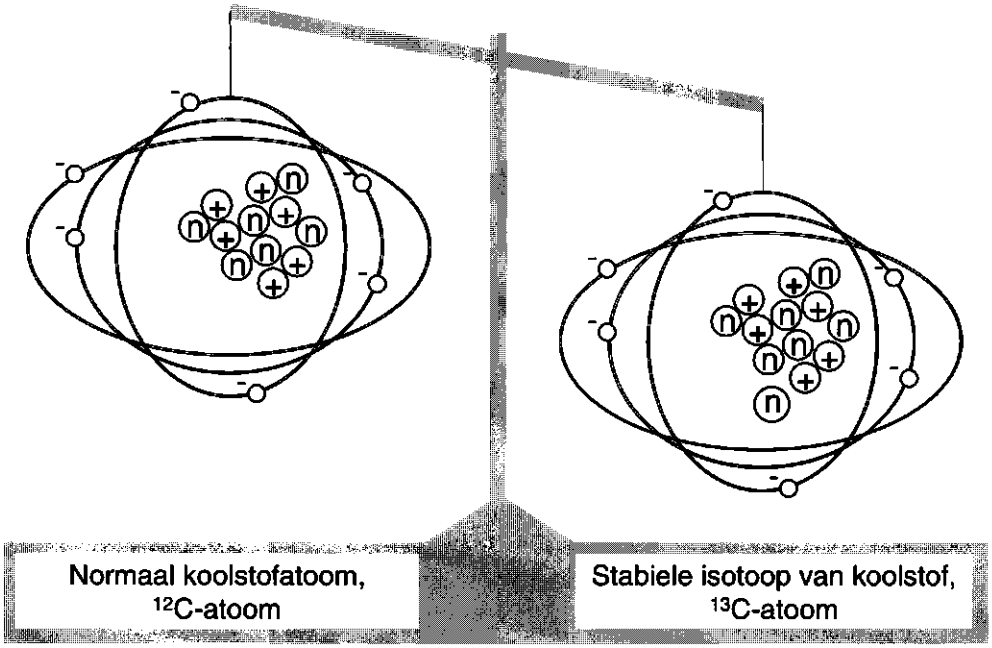
Vitamine A-deficiëntie is in veel ontwikkelingslanden een ernstig gezondheidsprobleem. Een verbeterde vitamine A-status kan ziekte en sterfte verminderen met 23 procent. De vitamine A status kan verbeterd worden door het verminderen van de behoefte van het lichaam aan vitamine A, óf door het vergroten van de werkelijke inname van vitamine A, óf door een combinatie van deze beiden. Vitamine A komt in de voeding voor als voorgevormd vitamine A (ofwel retinol), wat in dierlijke voedingsmiddelen en in borstvoeding zit, én als provitamine A-carotenoïden. Rode palmolie, donkergroene bladgroenten, geel en oranje fruit, en geel en oranje wortels en knollen, zoals zoete rode aardappelen, zijn voedingsmiddelen die rijk zijn aan provitamine A-carotenoïden. Provitamine A-carotenoïden in plantaardige voedingsmiddelen zijn voor een groot deel van de wereldbevolking de belangrijkste bron van vitamine A. Echter, de bijdrage van plantaardige voedingsmiddelen aan de vitamine A status kan alleen substantieel zijn als niet alleen de consumptie én provitamine A-inhoud van die voedingsmiddelen, maar óók de biodoeltreffendheid van provitamine A-carotenoïden in zulke voedingsmiddelen hoog is (**Hoofdstuk 1**). Met betrekking tot provitamine A-carotenoïden is biodoeltreffendheid (product van pijl 1 en 3 in **Figuur S-1**) te definiëren als de fractie van de geconsumeerde hoeveelheid die geabsorbeerd is (biobeschikbaarheid; pijl 1 in Figuur S-1) én de fractie daarvan die in het lichaam omgezet wordt in retinol (bioconversie; pijl 3 in Figuur S-1).



Figuur S-1. De biobeschikbaarheid en biodoeltreffendheid van provitamine A-carotenoïden. Biobeschikbaarheid is aangegeven met pijl 1; bioconversie is aangegeven met pijl 3; en biodoeltreffendheid is het product van pijl 1 en 3.

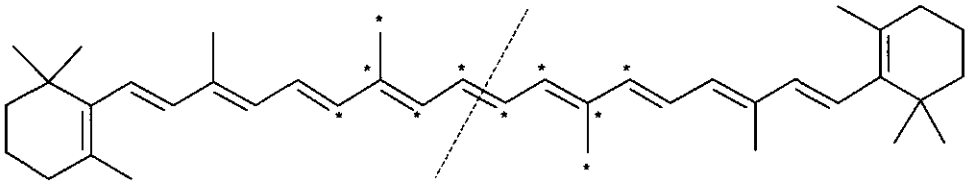
In theorie heeft β-caroteen de hoogste vitamine A activiteit van alle provitamine A-carotenoïden. In hun richtlijnen uit 1967 en 1988, hebben de Voedsel- en Landbouworganisatie van de Verenigde Naties (in het Engels afgekort als FAO) en de Wereld Gezondheidsorganisatie (in het Engels afgekort als WHO) gesteld dat de vitamine A activiteit van 1 microgram (afgekort als μg) retinol tevens geleverd zou kunnen worden door ófwel 3,3 μg β-caroteen in olie ófwel door 6 μg β-caroteen in een gemengde voeding. Onlangs heeft het 'Institute of Medicine' van de Verenigde Staten van Amerika (IOM) gesteld dat de vitamine A activiteit van 1 μg retinol tevens geleverd zou kunnen worden door ófwel 2 μg β-caroteen in olie ófwel door 12 μg β-caroteen in een gemengde voeding. Aangezien een lage biodoeltreffendheid van β-caroteen in plantaardige voedingsmiddelen grote gevolgen heeft voor de daadwerkelijke inname van vitamine A voor veel mensen in ontwikkelingslanden, zijn zeer nauwkeurige getallen over de biobeschikbaarheid, bioconversie en biodoeltreffendheid van carotenoïden in de voeding van mensen nodig. Deze getallen maken een genuanceerde evaluatie mogelijk van de potentie om vitamine A-deficiëntie te elimineren door het stimuleren van de consumptie van bepaalde

voedingsmiddelen. Zulke nauwkeurige getallen kunnen verkregen worden uit studies die gebruik maken van isotooptechnieken, waarbij de isotopen gebruikt worden als markers, om zodoende het caroteenmetabolisme te kunnen volgen (**Figuur S-2**). Voorheen hebben andere onderzoekers isotooptechnieken gebruikt voor het bestuderen van de biobeschikbaarheid en biodoeltreffendheid van β-caroteen in de voeding van de mens. Echter de manier waarop zij die isotooptechnieken hanteerden, had meerdere tekortkomingen, waardoor die studies geen betrouwbare getallen hebben opgeleverd (**Hoofdstuk 5**).

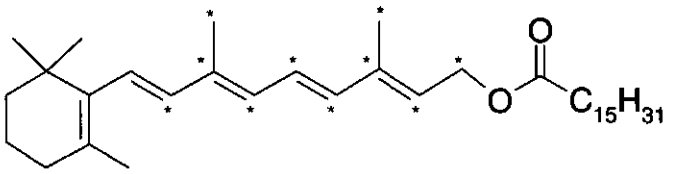


Figuur S-2. Atomen hebben protonen (+) en neutronen (n) in hun kern. Atomen die hetzelfde aantal protonen, maar een verschillend aantal neutronen in hun kern hebben worden isotopen genoemd. Naast radio-actieve isotopen, die radio-actieve straling uitzenden, bestaan er stabiele isotopen. Een normaal koolstofatoom, ofwel een ^{12}C-atoom, heeft 6 protonen en 6 neutronen in de kern. De stabiele isotoop van koolstof, ofwel een ^{13}C-atoom, heeft 6 protonen en 7 neutronen in de kern. Omdat protonen en neutronen even zwaar zijn is een ^{13}C-atoom dus zwaarder dan een ^{12}C-atoom.

Daarom is een nieuwe stabiele isotooptechniek ontwikkeld die gebaseerd is op het bereiken van een plateau van isotoopverrijking van β-caroteen en retinol in serum tijdens langdurige dagelijkse consumptie van meerdere lage doses van β-caroteen en retinol, die beiden op specifieke plaatsen zijn gemarkeerd met 10 ^{13}C atomen (**Figuur S-3**). Het doel van het onderzoek beschreven in dit proefschrift was om de biobeschikbaarheid en biodoeltreffendheid van β-caroteen in voedingsmiddelen in getallen weer te geven met behulp van deze nieuwe isotooptechniek. Voedingsmiddelen die normaal gesproken door Indonesische schoolkinderen geconsumeerd worden zijn hiertoe bestudeerd.



Gemarkeerd [$^{13}C_{10}$]β-caroteen



Gemarkeerd [$^{13}C_{10}$]retinylpalmitaat

Figuur S-3. Een β-caroteenmolekuul (boven) is opgebouwd uit koolstofatomen (weergegeven met de letter C) en waterstofatomen (H). Een retinylpalmitaatmolekuul (onder) is opgebouwd uit koolstof-, waterstof- en zuurstofatomen (O). De sterretjes geven aan op welke plekken in het gemarkeerde molekuul ^{13}C-atomen zijn ingebouwd. β-Caroteenmolekulen die in het lichaam zijn opgenomen en daar omgezet worden in retinol zullen 5 ^{13}C-atomen per retinolmolekuul bevatten (zie stippellijn).

ISOTOOPTECHNIEKEN VOOR HET BESTUDEREN VAN DE BIODOELTREFFENDHEID VAN CAROTENOÏDEN BIJ DE MENS: EEN LITERATUUROVERZICHT (HOOFDSTUK 5)

In de meeste tot nu toe uitgevoerde isotoopstudies heeft een beperkt aantal vrijwilligers slechts een éénmalige dosis met isotopen gemarkeerd β-caroteen en/of retinol geconsumeerd. Omdat sommige detectiemethodes voor het meten van de mate van isotoopverrijking relatief ongevoelig zijn, zijn vaak hoge (farmacologische) doses gemarkeerde stoffen gegeven aan de vrijwilligers. Als een éénmalige lagere (fysiologische) dosis geconsumeerd wordt, benadert de isotoopverrijking van β-caroteen en/of retinol in serum op een gegeven moment de basis-isotoopverrijking in serum (serum is de heldere vloeistof die zich afscheidt bij de stolling van bloed). Het te meten signaal is dan dus nauwelijks te onderscheiden van achtergrondruis, waardoor de analytische nauwkeurigheid verlaagd wordt en de resultaten van de metingen lastig te interpreteren zijn. Voor de interpretatie van de resultaten van studies die gebruik maken van isotooptechnieken zijn wiskundige modellen nodig. Modellen die het metabolisme van een éénmalige dosis beschrijven zijn vaak gebaseerd op niet te bewijzen aannames. Andere modellen hebben de oppervlaktes onder de curve van een grafiek vergeleken. In zulke grafieken worden de concentraties in serum van gemarkeerd retinol - zowel afkomstig van geconsumeerd gemarkeerd β-caroteen (curve 1) als van geconsumeerd gemarkeerd retinol (curve 2) - uitgezet tegen de tijd. Vergelijking van de oppervlaktes onder deze 2 curven geeft een indicatie van de vitamine A activiteit van β-caroteen ten opzichte van die van retinol. Echter,

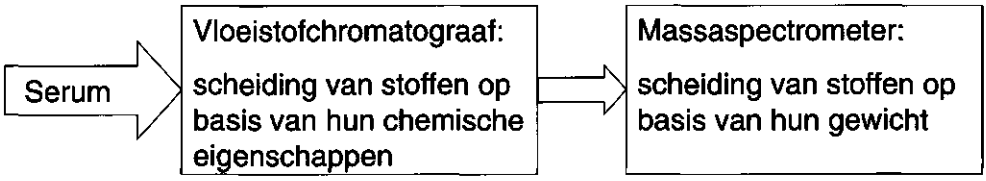
naast de noodzaak van het verzamelen van voldoende bloedmonsters, heeft deze vergelijking ernstige andere beperkingen omdat verschillende factoren de verwijdering van provitamine A-carotenoïden en/of retinol uit serum, het metabolisme van die stoffen en/of de uitscheiding van die stoffen uit het lichaam beïnvloeden. In de volgende paragraaf zullen de principes van de isotooptechniek gebaseerd op 'plateau isotoopverrijking' en de opzet van studies waarin die techniek gebruikt is uitgelegd worden.

PRINCIPE ISOTOOPTECHNIEK GEBASEERD OP 'PLATEAU ISOTOOPVERRIJKING' VOOR HET BESTUDEREN VAN DE BIODOELTREFFENDHEID VAN CAROTENOÏDEN

De stabiele isotooptechniek beschreven in dit proefschrift is gebaseerd op het bereiken van een plateau van isotoopverrijking in serum van β-caroteen en retinol tijdens langdurige dagelijkse consumptie van meerdere lage doses van β-caroteen en retinol, die beiden op specifieke plaatsen zijn gemarkeerd met 10 ^{13}C atomen. Bij voldoende lang consumeren van gemarkeerd β-caroteen en retinol en van gecontroleerde, constante hoeveelheden ongemarkeerd β-caroteen en retinol in de rest van de voeding (de basisvoeding), is na verloop van tijd de hoeveelheid β-caroteen en retinol dat het serum inkomt gelijk aan de hoeveelheid die het serum uitgaat. Op dat moment is een plateau van isotoopverrijking bereikt. Tijdens de eerste studie (**Hoofdstuk 2**) consumeerden 35 Indonesische schoolkinderen (8-11 jaar) gedurende maximaal tien weken dagelijks twee doses van 80 μg [$^{13}C_{10}$]β-caroteen en 80 μg [$^{13}C_{10}$]retinylpalmitaat. Over deze periode zijn drie bloedmonsters per kind genomen. In tegenstelling tot de bewerkelijke detectiemethodes gebruikt in de meeste andere isotooptechnieken, werd het serum na eenvoudige hexaan-extractie geanalyseerd met behulp van een vloeistofchromatograaf gekoppeld aan een massaspectrometer (in het Engels afgekort met LC-MS). Omdat β-caroteen en retinol gemarkeerd met stabiele isotopen zwaarder zijn dan ongemarkeerd β-caroteen en retinol (Figuur S-2) kan de mate van isotoopverrijking van retinol met [$^{13}C_{10}$]retinol (afkomstig van geconsumeerd [$^{13}C_{10}$]retinol) en met [$^{13}C_5$]retinol (afkomstig van geconsumeerd [$^{13}C_{10}$]β-caroteen) en van β-caroteen met [$^{13}C_{10}$]β-caroteen gemeten worden met behulp van LC-MS (**Figuur S-4**). Uit deze eerste studie bleek dat binnen 21 dagen een plateau van isotoopverrijking bereikt werd. Dit is belangrijk voor de opzet van vervolgstudies.

Tijdens de tweede studie (**Hoofdstuk 3 en 4**), bestaande uit een drie weken durende inloopperiode gevolgd door een behandelingsperiode van drie weken, consumeerden 77 Indonesische schoolkinderen drie maal daags 31 μg [$^{13}C_{10}$]β-caroteen en 21 μg [$^{13}C_{10}$]retinylpalmitaat. Tijdens de behandelingsperiode consumeerde de helft van de groep dagelijks ook nog twee porties van 82 gram spinazie (1,5 milligram β-caroteen per portie), terwijl de andere helft van de groep dagelijks twee porties van 81 gram oranje pompoen (0,7 milligram β-caroteen per portie) te eten kreeg. Aan het begin van de studie én aan het eind van de inloop- en de behandelingsperiode zijn bloedmonsters genomen en verzamelden 17 van de 77 kinderen hun ontlasting. De mate van isotoopverrijking van retinol en β-caroteen in bloed en ontlasting werd gemeten met behulp van LC-MS. Van alle detectiemethodes voor het bestuderen van de biobeschikbaarheid, bioconversie en biodoeltreffendheid van

provitamine A-carotenoïden bleek deze LC-MS techniek het meest effectief en praktisch te zijn (**Hoofdstuk 5**). Voor de interpretatie van de gegevens verkregen in deze twee studies is een wiskundig model ontwikkeld dat gebaseerd is op aannames die eenvoudig te verantwoorden zijn in het licht van de huidige kennis over het metabolisme van carotenoïden.



Figuur S-4. Principe van de meting van de mate van isotoopverrijking met behulp van een vloeistofchromatograaf gekoppeld aan een massaspectrometer (in het Engels afgekort met LC-MS). Serum wordt geïnjecteerd in een vloeistofchromatograaf die stoffen scheidt op basis van hun chemische eigenschappen. Zodoende komt op een gegeven moment bijvoorbeeld β-caroteen uit de vloeistofchromatograaf. Aangezien de vloeistofchromatograaf gekoppeld is aan een massaspectrometer kan al dit β-caroteen gescheiden worden op basis van gewicht. β-Caroteen dat afkomstig is van het geconsumeerde gemarkeerde β-caroteen bevat 10 ^{13}C-atomen en is dus zwaarder dan het ongemarkeerde β-caroteen dat van nature aanwezig is. Het percentage gemarkeerd β-caroteen ten opzichte van de totale hoeveelheid β-caroteen is de isotoopverrijking van β-caroteen. Op éénzelfde manier kan de isotoopverrijking van retinol met gemarkeerd retinol bepaald worden.

BEVINDINGEN MET BETREKKING TOT DE BIOBESCHIKBAARHEID EN BIODOELTREFFEND-HEID VAN β-CAROTEEN

De conclusie, na combinatie van de resultaten uit de twee bovenstaande studies, is dat 2,6 μg β-caroteen in olie dezelfde vitamine A activiteit heeft als 1 μg retinol (**Hoofdstuk 2 en 3**). Dit komt overeen met een biodoeltreffendheid van 36 procent. Bij deze Indonesische kinderen was de biobeschikbaarheid van β-caroteen in olie 86 procent (**Hoofdstuk 2 en 3**). Zowel de biobeschikbaarheid als de biodoeltreffendheid van β-caroteen in oranje pompoen was 1,7 keer die van β-caroteen in spinazie (**Hoofdstuk 3**). De precisie van de schattingen gebaseerd op gegevens afkomstig van serum lijken hoger dan die van schattingen gebaseerd op gegevens afkomstig van ontlasting, waarschijnlijk omdat niet altijd alle ontlasting is verzameld (**Hoofdstuk 4**). Ondanks de consumptie van grote hoeveelheden β-caroteen in spinazie en pompoen tijdens de tweede studie, veranderde de mate van isotoopverrijking van retinol in serum niet tijdens de behandelingsperiode in vergelijking met de inloopperiode. Daardoor konden de absolute biobeschikbaarheid en biodoeltreffendheid van β-caroteen in deze groentes niet geschat worden. In toekomstige studies moet de gecontroleerde basisvoeding dusdanig gewijzigd worden opdat die een constante hoeveelheid retinol in een biobeschikbare matrix levert.

Samenvattend lijkt de hier beschreven techniek de beste die tot nu toe beschikbaar is én levert deze techniek betrouwbare en bruikbare schattingen (getallen) van de biobeschikbaarheid en biodoeltreffendheid van carotenoïden in de voeding van de mens (**Hoofdstuk 5**).

TOEKOMSTIGE TOEPASSINGEN VAN DEZE ISOTOOPTECHNIEK (HOOFDSTUK 6)

De stabiele isotooptechniek zoals beschreven in dit proefschrift, kan toegepast worden om bij mensen het effect van individuele SLAMENGHI factoren die de biobeschikbaarheid en biodoeltreffendheid van carotenoïden beïnvloeden te bestuderen (SLAMENGHI is een acroniem voor zulke factoren). Zo kan nu bijvoorbeeld de biodoeltreffendheid van carotenoïden bepaald worden bij groepen vrijwilligers met een uiteenlopende vitamine A-, zink of ijzerstatus (factor N). Dit is van belang omdat gevonden is dat deze stoffen het vitamine A en/of carotenoïden metabolisme beïnvloeden. Met een aangepaste onderzoeksopzet, zoals in de vorige paragraaf beschreven, is deze techniek in de toekomst ook toepasbaar voor het bestuderen van biodoeltreffendheid van carotenoïden in een gemengde voeding (factor M), én voor het bestuderen van de biodoeltreffendheid van carotenoïden bij consumptie van verschillende hoeveelheden carotenoïden (factor A). Het is dan ook mogelijk om het effect van andere voedingsbestanddelen zoals vet, vezel of pectine op de opname en omzetting van carotenoïden te bestuderen (factor E). Veel carotenoïden, met en zonder provitamine A-activiteit, kunnen inmiddels specifiek gemarkeerd worden met ^{13}C atomen. Daardoor is het nu mogelijk de biobeschikbaarheid en biodoeltreffendheid van deze carotenoïden afzonderlijk (factor S) én de interactie tussen deze carotenoïden te bestuderen.

BETEKENIS VAN DEZE NIEUWE BEVINDINGEN OVER DE BIODOELTREFFENDHEID VAN CAROTENOÏDEN

De schattingen (getallen) verkregen uit de studies beschreven in dit proefschrift zijn de meest betrouwbare schattingen van de biodoeltreffendheid van β-caroteen in olie, die tot nu toe beschikbaar zijn. Het lijkt zeer aannemelijk dat de gebruikte methodes en de getallen verkregen bij deze Indonesische schoolkinderen geschikt zijn voor andere groepen in de populatie in ontwikkelingslanden, zoals andere kinderen, volwassenen (inclusief zwangere en borstvoeding gevende vrouwen) en ouderen. Uit twee gecontroleerde voedingsstudies die recent zijn uitgevoerd in Indonesië en Vietnam is geconcludeerd dat een hoeveelheid van 21 μg β-caroteen in een gemengde voeding (inname van groente en fruit in een verhouding van 4 staat tot 1) dezelfde vitamine A activiteit heeft als 1 μg retinol (dit betekent dat de biodoeltreffendheid van β-caroteen in een gemengde voeding 4,5 procent is). De lage biodoeltreffendheid van β-caroteen in donkergroene bladgroenten vergeleken met die van β-caroteen in oranje groente/fruit gevonden in die studies is bevestigd in het werk beschreven in dit proefschrift (**Hoofdstuk 3 en 4**). Dus, de huidige richtlijnen dat de vitamine A activiteit van 1 μg retinol ook geleverd kan worden door 6 μg (FAO/WHO, 1967 en 1988) en 12 μg (IOM, 2000) β-caroteen in een gemengde voeding overschatten de biodoeltreffendheid van β-caroteen met een factor 2 tot 3.

De nieuwe bevindingen van de biodoeltreffendheid van β-caroteen in olie en in een gemengde voeding, zoals beschreven in recente literatuur en in dit proefschrift, kunnen nu gebruikt worden door nationale en internationale organisaties voor het herevalueren van hun

richtlijnen voor de biodoeltreffendheid van β-caroteen en dus de bijdrage van β-caroteen aan de aanbevolen dagelijkse hoeveelheid vitamine A. Daarnaast maken deze nieuwe bevindingen de formulering en evaluatie van adequaat beleid en interventie-programma's voor het elimineren van vitamine A-deficiëntie mogelijk. Gezien de lage biodoeltreffendheid van carotenoïden in plantaardige voedingsmiddelen kan voor het elimineren van vitamine A-deficiëntie niet worden volstaan met de promotie van de groente- en fruitconsumptie.

Defisiensi vitamin A masih merupakan masalah kesehatan yang serius di negara-negara berkembang. Peningkatan status vitamin A dapat menurunkan 23% dari angka kematian dan kesakitan di negara berkembang. Meningkatkan status vitamin A dapat dicapai dengan menurunkan kebutuhan vitamin A atau meningkatkan suplai vitamin A secara efektif atau kombinasi dari keduanya. Vitamin A pada makanan dijumpai dalam bentuk retinol, yang hanya dijumpai pada sumber makanan hewani dan air susu, dan dalam bentuk provitamin A karoten. Makanan yang kaya akan kandungan provitamin A karoten adalah minyak kelapa sawit merah, sayuran berdaun hijau tua, buah-buahan oranye dan kuning, dan akar dan umbi-umbian merah dan oranye seperti wortel dan ketela merah. Provitamin A karoten pada makanan nabati merupakan sumber utama vitamin A bagi mayoritas populasi dunia. Tetapi, manfaat vitamin A dari sumber makanan nabati bukan hanya tergantung dari kandungan provitamin A di dalam makanan itu sendiri, tapi juga tergantung pada bioefikasi provitamin A karoten di dalam makanan tersebut (**Bab 1**). Bioefikasi provitamin A karoten adalah bagian dari makanan yang dicerna dan diabsorpsi (ketersediaan biologis) dan bagian yang dikonversi menjadi retinol di dalam tubuh (biokonversi).

Pedoman FAO/WHO 1967 dan 1988, menyatakan bahwa aktivitas vitamin A sebagai 1 μg retinol didapat dari 3.3 μg β-karoten di dalam minyak dan 6 μg β-karoten pada diet campuran. Menurut data terbaru, US Institute of Medicine (IOM) mengajukan bahwa aktivitas vitamin A yang dinyatakan sebagai 1 μg retinol didapat dari 2 μg β-karoten di dalam minyak dan 12 μg β-karoten pada diet campuran. Rendahnya bioefikasi β-karoten dari sumber makanan nabati merupakan implikasi utama bagi efektivitas suplai vitamin A khususnya pada masyarakat miskin, sehingga data yang akurat dan tepat dalam hal ketersediaan biologis, biokonversi dan bioefikasi asupan karoten bagi manusia dibutuhkan sebagai acuan untuk mengevaluasi pendekatan sumber makanan dalam mengatasi defisiensi vitamin A. Teknik pelacakan dengan isotop dapat memberikan data tersebut. Teknik isotop untuk keperluan tersebut diatas telah dilakukan dalam penelitian terdahulu, tetapi metode yang digunakan kurang dapat menghasilkan data yang dapat dipercaya dalam hal ketersediaan biologis dan bioefikasi dari karoten pada manusia (**Bab 5**).

Oleh karena itu, teknik isotop stabil yang baru dikembangkan dengan berdasarkan pencapaian plateau dari isotop yang diperkaya dengan β-karoten dan retinol dalam serum selama perpanjangan multiplikasi dosis rendah dari β-karoten dan retinol, masing-masing secara spesifik dilabel dengan atom 10 ^{13}C telah dikembangkan. Tujuan dari studi yang diuraikan pada tesis ini adalah untuk meneliti ketersediaan biologis dan bioefikasi dari β-karoten pada jenis makanan yang biasa dikonsumsi oleh anak sekolah di Indonesia dengan menggunakan teknik baru.

TEKNIK PENCARIAN ISOTOP: KAJIAN DAFTAR PUSTAKA (BAB 5)

Selain dari teknik baru ini, penelitian lain telah dilakukan dengan memberikan dosis tunggal dari β-karoten berlabel dan atau retinol berlabel pada beberapa subyek. Karena metodologi untuk mendeteksi tingkatan isotop yang diperkaya relatif tidak sensitif, maka biasa digunakan komponen label yang secara farmakologis berdosis tinggi. Setelah dosis

tunggal fisiologis digunakan, tingkatan isotop pada serum mencapai nilai basal pada beberapa titik waktu. Hal ini dapat meningkatkan variasi analisa dan interpretasi yang rumit dari data yang didapat. Dalam menginterpretasi hasil teknik pelacakan isotop, diperlukan model matematika, dan deskripsi metabolisme dosis tunggal yang biasanya berdasarkan asumsi dimana sulit untuk dicari penjelasannya. Model lain menunjukkan perbandingan area di bawah kurva dari konsentrasi serum label retinol dari turunan dosis tunggal β-karoten berlabel dan retinol berlabel. Tetapi, selain diperlukan jumlah sampel darah yang mencukupi, metode ini juga mempunyai keterbatasan karena berbagai faktor dapat mempengaruhi kejernihan serum, metabolisme dan ekskresi dari karoten dan retinol. Pada bagian berikutnya, akan dijelaskan prinsip dari teknik pengkayaan isotop plateau dan desain studi yang menggunakan teknik ini.

TEKNIK PLATEAU ISOTOP YANG DIPERKAYA UNTUK STUDI BIOEFIKASI KAROTEN

Teknik isotop stabil yang diuraikan dalam tesis ini berdasarkan pencapaian plateau dari isotop yang diperkaya oleh β-karoten dan retinol selama perpanjangan pemberian dosis rendah berulang dari β-karoten dan retinol, yang masing-masing secara spesifik dilabel dengan atom 10 ^{13}C. Untuk keperluan itu, teknik ini sudah diuji dalam dua penelitian. Pada studi pertama (**Bab 2**), 35 anak Indonesia (usia 8-11 tahun) diberi 80 μg [$^{13}C_{10}$] β-karoten dan 80 μg [$^{13}C_{10}$] retinyl palmitat setiap hari selama ≤ 10 minggu. Selama periode penelitian ini, dilakukan pengambilan sampel darah 3 kali dari setiap anak. Setelah diekstraksi dengan hexane, serum dianalisa dengan APCI LC-MS. Oleh karena berat dari β-karoten dan retinol yang dilabel dengan isotop stabil lebih besar dari komponen yang tidak diberi label, tingkatan isotop yang diperkaya oleh retinol dengan [$^{13}C_5$]retinol, yang merupakan turunan dari pemberian [$^{13}C_{10}$]β-karoten dan [$^{13}C_{10}$]retinol, serta β-karoten dan [$^{13}C_{10}$]β-karoten, dapat diukur dengan alat *high performance liquid chromatography* (HPLC) dengan *atmospheric pressure chemical ionization liquid chromatography-mass spectrometry* (APCI LC-MS).

Dari studi yang pertama, kesimpulannya adalah bahwa plateau isotop yang diperkaya dengan retinol dan β-karoten di dalam serum dapat dicapai dalam waktu 21 hari, yang merupakan hal penting untuk mendesain studi berikutnya. Pada studi yang kedua (**Bab 3 dan 4**) percobaan dilakukan selama 3 minggu, diikuti dengan 3 minggu periode intervensi, 77 anak sekolah di Indonesia diberi perlakuan setiap hari dengan 3 dosis 31 μg [$^{13}C_{10}$]β-karoten dan 21 μg [$^{13}C_{10}$] retinyl palmitat. Selama periode intervensi, separuh dari subyek menerima 2 porsi bayam sebanyak 82 g (1.5 mg β-karoten/porsi), dan separuh yang lainnya menerima 2 porsi labu sebanyak 81 g (0.7 mg β-karoten/porsi) setiap hari.

Pada awal dan akhir masa percobaan serta periode intervensi, dilakukan pengumpulan sampel darah dan tinja. Pada sampel darah dan tinja, tingkatan isotop yang diperkaya oleh retinol dan β-karoten diukur dengan menggunakan alat LC-MS. Teknik LC-MS ini merupakan metode gabungan yang dianggap paling efektif dan nyaman untuk studi pengukuran ketersediaan biologis, biokonversi dan bioefikasi dari provitamin A karoten. Untuk menginterpretasi data yang diperoleh, model matematika sudah dikembangkan dengan asumsi yang berdasarkan pengetahuan terbaru mengenai metabolisme karoten.

PENEMUAN HASIL DARI KETERSEDIAAN BIOLOGIS DAN BIOEFIKASI DARI β-KAROTEN

Bila data dari hasil studi yang telah dipaparkan diatas dijadikan satu, maka diperoleh bahwa 2.6 μg β-karoten di dalam minyak setara dengan aktivitas vitamin A yang dinyatakan sebagai 1 μg retinol (**Bab 2 dan 3**). Hal ini berkaitan dengan bioefikasi sebesar 36%.
Pada studi ini, ditemukan bahwa ketersediaan biologis untuk β-karoten di dalam minyak adalah sebesar 86% pada anak di Indonesia (**Bab 2 dan 3**). Ketersediaan biologis dan bioefikasi dari β-karoten pada labu ditemukan 1.7 kali lebih besar dibandingkan dengan β-karoten pada bayam (**Bab 3**). Ketepatan hasil dari pengukuran sampel serum darah tampaknya lebih tinggi daripada hasil pengukuran sampel tinja, hal ini kemungkinan disebabkan oleh pengumpulan tinja yang acap kali tidak lengkap (**Bab 4**).

Walaupun β-karoten pada labu dan bayam dikonsumsi dalam jumlah besar pada studi kedua, tingkatan isotop yang diperkaya oleh retinol pada serum tidak berubah selama periode intervensi. Sehingga, ketersediaan biologis secara absolut dan bioefikasi β-karoten pada bayam dan labu tidak dapat diperkirakan. Untuk studi lanjutan, asupan basal harus diubah untuk mendapatkan asupan retinol yang sedikit dan konstan dalam matriks yang tinggi dalam hal ketersediaan biologisnya.

Kesimpulannya, teknik yang telah diuraikan di atas merupakan cara yang terbaik dan terpercaya untuk digunakan pada manusia dalam memperkirakan ketersediaan biologis dan bioefikasi asupan karoten (**Bab 5**).

APLIKASI TEKNIK PLATEAU ISOTOP YANG DIIPERKAYA UNTUK MAASA YANG AKAN DATANG (BAB 6)

Teknik plateau isotop yang diperkaya ini dapat diaplikasikan untuk melihat efek dari faktor-faktor SLAMENGHI yang dapat mempengaruhi ketersediaan biologis dan bioefikasi karoten pada setiap individu (SLAMENGHI merupakan istilah dari berbagai faktor). Sebagai contoh, bioefikasi dari karoten pada sebuah kelompok yang terdiri dari bebeberapa subyek dengan status gizi yang berbeda dalam hal status vitamin A, seng atau zat besi dapat dipelajari, sebab zat-zat gizi ini berkaitan dengan faktor-faktor yang mempengaruhi metabolisme vitamin A dan karoten.

Untuk masa yang akan datang, dengan memodifikasi desain yang telah diuraikan diatas, teknik tersebut dapat diterapkan untuk studi bioefikasi karoten pada diet campuran, dan pada diet dengan kandungan jumlah karoten yang berbeda pada makanan. Selanjutnya, pengaruh dari 'effectors' pada proses absorpsi dan konversi seperti lemak, serat atau pektin perlu diteliti lebih lanjut. .

Oleh karena berbagai jenis karoten, dengan atau tanpa aktivitas provitamin A secara spesifik dapat dilabel dengan atom ^{13}C, maka ketersediaan biologi dan bioefikasi karoten dan interaksi di antara berbagai jenis karoten dapat dipelajari.

IMPLIKASI DATA BARU DALAM HAL BIOEFIKASI KAROTEN

Studi yang diuraikan dalam tesis ini menunjukkan bahwa pengukuran bioefikasi β-karoten pada minyak yang paling dapat dipercaya sampai saat ini. Sehingga, tidak ada alasan untuk meragukan hasil data yang diperoleh, dan penggunaan metode yang telah dilakukan pada anak-anak di Indonesia ini tidak dapat diterapkan pada kelompok populasi lain di negara-negara berkembang baik pada anak-anak, dewasa (termasuk ibu hamil dan menyusui) maupun pada kelompok usia lanjut. Berdasarkan dari hasil penelitian diet perlakuan-kontrol yang terbaru di Indonesia dan Vietnam, dapat disimpulkan bahwa 21 μg β-karoten pada diet campuran (4:1 perbandingan antara diet sayuran dan buah-buahan), memiliki aktivitas vitamin A yang setara dengan 1 μg retinol (bioefikasi β-karoten pada diet campuran sebesar 4.5%). Perbandingan bioefikasi β-karoten yang rendah pada sayuran berdaun hijau tua dibandingkan dengan buah-buahan oranye telah dibuktikan dari hasil penelitian yang telah diuraikan sebelumnya (**Bab 3 dan 4**).

Dengan demikian, pedoman yang digunakan saat ini dimana aktivitas vitamin A dinyatakan sebagai 1 μg retinol dapat dipenuhi dengan 6 μg (FAO/WHO) dan 12 μg (IOM) β-karoten pada diet campuran menunjukkan estimasi bioefikasi yang berlebihan hingga sekitar 2 –3 kali.

Data terbaru dari bioefikasi β-karoten pada minyak dan perpaduan beberapa data dapat digunakan untuk mengkaji ulang rekomendasi dari bioefikasi karoten di dalam makanan bagi organisasi nasional dan internasional sebagai bagian dari pedoman untuk menentukan angka kecukupan gizi yang dianjurkan.

Selanjutnya, data ini juga dapat digunakan untuk menyusun dan mengevaluasi penentuan kebijaksanaan dan program penanggulangan defisiensi vitamin A. Karena rendahnya bioefikasi karoten dari sumber makanan nabati, maka kegiatan promosi untuk mengkonsumsi makanan selain buah-buahan dan sayur-sayuran dibutuhkan untuk mengatasi kekurangan vitamin A.

La deficiencia de vitamina A es un problema serio de salud pública en países en desarrollo. Al mejorar el estado nutricional de vitamina A se puede reducir la mortalidad en 23%, y la morbilidad. Se puede mejorar el estado de vitamina A ya sea reduciendo la demanda corporal de la vitamina o aumentando la entrega efectiva de la misma o combinando ambas estrategias. La vitamina A se encuentra en los alimentos como vitamina preformada (retinol) solamente en alimentos de origen animal o en la leche materna, o como carotenoides pro-vitamina A. Entre los alimentos ricos en carotenoides pro-vitamina A se encuentran el aceite de palma africana, los vegetales de hojas verde-profundo, las frutas rojas y amarillas, las raíces y tubérculos rojos y amarillos tales como la zanahoria y el camote amarillo. Los carotenoides pro-vitamina A de alimentos de origen vegetal son la fuente más importante de vitamina A para proporción considerable de la población mundial. Sin embargo, la contribución sustancial de tales fuentes depende no solamente del consumo abundante de alimentos con alto contenido de pro-vitamina A sino de que la bioeficacia de los carotenoides pro-vitamina A en tales alimentos sea alta (**Capítulo 1**). Respecto a los carotenoides pro-vitamina A, la bioeficacia es el producto de la fracción de la cantidad ingerida que es absorbida (biodisponibilidad) y la fracción de ésta que es convertida a retinol en el cuerpo (bioconversión).

Teóricamente, de todos los carotenoides pro-vitamina A, el β-caroteno tiene la mayor actividad pro-vitamínica A. En las guías de 1967 y 1988, FAO/OMS propusieron que la actividad de vitamina A de 1 μg de retinol puede ser proporcionada por 3.3 μg de β-caroteno en aceite o 6 μg de β-caroteno en una dieta mixta. Recientemente, el Instituto Estadounidense de Medicina (IOM, siglas en inglés) ha propuesto que la actividad de vitamina A de 1 μg de retinol puede ser proporcionada por 2 μg de β-caroteno en aceite o 12 μg de β-caroteno en una dieta mixta. En vista de que una baja bioeficacia del β-caroteno de alimentos de origen vegetal tiene importantes implicaciones para la provisión efectiva de vitamina A a muchas personas pobres se necesitan datos exactos y precisos sobre la biodisponibilidad, bioconversión y bioeficacia de carotenoides dietéticos para la evaluación apropiada de las estrategias alimentarias para la eliminación de la deficiencia de vitamina A. Las técnicas de rastreo isotópico pueden proporcionar tales datos. Las técnicas isotópicas han sido utilizadas anteriormente para tales propósitos, pero la forma en que han sido aplicadas para obtener datos confiables sobre biodisponibilidad y bioeficacia de carotenoides en humanos es inapropiada desde varios puntos de vista (**Capítulo 5**).

Por lo anterior, se ha desarrollado una nueva técnica con isótopos estables que se basa en alcanzar una meseta de enriquecimiento isotópico de β-caroteno y retinol en el suero mediante múltiples dosis bajas prolongadas de β-caroteno y retinol, cada uno marcado específicamente con 10 átomos de ^{13}C. El objetivo de la investigación descrita en esta tesis fue cuantificar la biodisponibilidad y bioeficacia del β-caroteno de alimentos que son típicamente consumidos por niños escolares en Indonesia, usando esta nueva técnica.

TÉCNICAS DE RASTREO ISOTÓPICO: REVISIÓN DE LA LITERATURA (CAPÍTULO 5)

Aparte de la presente técnica todas las demás han sido efectuadas administrando una dosis única de β-caroteno o retinol marcado a un número limitado de sujetos. Como algunos métodos para la detección del grado de enriquecimiento isotópico son relativamente insensibles, a menudo ha sido necesario el uso de altas dosis farmacológicas de los compuestos marcados. Después de la administración de una dosis fisiológica, el grado de enriquecimiento isotópico en el suero alcanza los valores basales en algún punto del tiempo. Esto puede aumentar la variación analítica y, por tanto, complicar la interpretación de los resultados. Para la interpretación de los resultados de técnicas de rastreo isotópico se necesitan modelos matemáticos, y aquellos que describen el metabolismo de una dosis única a menudo se basan en suposiciones que resultan difíciles de justificar. Otros modelos han comparado las áreas bajo la curva de concentraciones de retinol marcado derivadas de una dosis simple de β-caroteno y/o retinol marcado. Sin embargo, además de la necesidad de recolectar un número suficiente de muestras sanguíneas, tales métodos tienen limitaciones serias porque hay varios factores que afectan el aclaramiento sérico, el metabolismo y la excreción de carotenoides y retinol. En la siguiente sección se explican los principios de la técnica de la meseta de enriquecimiento isotópico y el diseño de estudios que usan dicha técnica.

TÉCNICA DE MESETA DE ENRIQUECIMIENTO ISOTÓPICO PARA EL ESTUDIO DE LA BIO-EFICACIA DE CAROTENOIDES

La técnica de isótopos estables descrita en esta tesis se basa en alcanzar una meseta de enriquecimiento isotópico de β-caroteno y retinol durante ingestión prolongada de dosis bajas múltiples de β-caroteno y retinol, cada uno específicamente marcado con 10 átomos de ^{13}C. A la fecha, esta técnica ha sido probada en dos estudios. En el primer estudio (**Capítulo 2**), 35 niños indonesios (8-11 años de edad) consumieron diariamente dos dosis de 80 μg de [$^{13}C_{10}$]β-caroteno y 80 μg [$^{13}C_{10}$]palmitato de retinilo por períodos ≤ 10 semanas. Durante este período, se obtuvieron tres muestras de sangre por niño para obtener suero. Después de extracción con hexano, el suero fue analizado por espectrometría de masas-cromatografía líquida con ionización química a presión atmosférica (APCI LC-MS, por las siglas en inglés de atmospheric pressure chemical ionization liquid chromatography-mass spectrometry). Como el β-caroteno y el retinol marcado con isótopos estables pesan más que los compuestos no marcados, el grado de enriquecimiento isotópico de retinol con [$^{13}C_5$]retinol - derivado del [$^{13}C_{10}$]β-caroteno administrado - y con [$^{13}C_{10}$]retinol, y de β-caroteno con [$^{13}C_{10}$]β-caroteno puede ser medido por cromatografía líquida de alta resolución con APCI LC-MS. Del primer estudio se concluyó que la meseta de enriquecimiento isotópico de retinol y β-caroteno en suero se alcanza a los 21 días, lo cual es importante para el diseño de futuros estudios. En el segundo estudio (**Capítulos 3 y 4**), que consistió de tres semanas de estabilización seguida de tres semanas de tratamiento, 77 niños escolares indonesios consumieron diariamente tres dosis de 31 μg de [$^{13}C_{10}$]β-caroteno y 21 μg de [$^{13}C_{10}$]palmitato

de retinilo. Durante el período de tratamiento, la mitad de los sujetos recibió diariamente dos porciones de 82 g de espinaca (1.5 mg β-caroteno por porción) mientras que los otros recibieron dos porciones de 81 g de calabaza de pulpa anaranjada (0.7 mg β-caroteno por porción). Se obtuvieron muestras de sangre y (de 17 de los 77 niños) heces al inicio y al final de los períodos de estabilización y tratamiento. Se midió el grado de enriquecimiento isotópico de retinol y β-caroteno en las muestras de sangre y heces usando LC-MS. Estas técnicas de LC-MS han surgido como las más efectivas y convenientes para el estudio de la biodisponibilidad, bioconversión y bioeficacia de los carotenoides pro-vitamina A (**Capítulo 5**). Para la interpretación de los datos, se ha desarrollado un modelo matemático basado en supuestos que pueden ser claramente justificados a la luz del conocimiento actual del metabolismo carotenoide.

HALLAZGOS SOBRE LA BIODISPONIBILIDAD Y BIOEFICACIA DE β-CAROTENO

Si se combinan los datos de los estudios arriba mencionados, se concluye que 2.6 μg de β-caroteno en aceite tienen la misma actividad vitamínica A que 1 μg de retinol (**Capítulos 2 y 3**), lo que corresponde a una bioeficacia de 36%. Se encontró que la biodisponibilidad de β-caroteno en aceite fue de 86% en estos niños indonesios (**Capítulos 2 y 3**). La biodisponibilidad y bioeficacia del β-caroteno de la calabaza anaranjada fue 1.7 veces más que el de la espinaca (**Capítulo 3**). La precisión de los estimaciones derivadas del suero parece ser mayor que la de las estimaciones derivadas de heces, probablemente porque las recolecciones fecales fueron incompletas (**Capítulo 4**). Pese al consumo de grandes cantidades de β-caroteno proveniente de espinaca y calabaza en el segundo estudio, el grado de enriquecimiento isotópico de retinol en el suero no cambió durante el período de tratamiento. Por ello, no se pudo estimar la biodisponibilidad y bioeficacia absolutas del β-caroteno de espinacas y calabazas. En futuros estudios, se debería alterar la dieta basal para proporcionar una ingesta constante y pequeña de retinol en una matriz altamente biodisponible.

En conclusión, la técnica descrita parece ser la mejor disponible para uso en humanos para proveer estimaciones confiables sobre la biodisponibilidad y bioeficacia de carotenoides dietéticos (**Capítulo 5**).

FUTURAS APLICACIONES DE LA TÉCNICA DE MESETA DE ENRIQUECIMIENTO ISOTÓPICO (CAPÍTULO 6)

La técnica de meseta de enriquecimiento isotópico puede ser aplicada en humanos para estudiar los efectos individuales de los factores SLAMENGHI que afectan la biodisponibilidad y bioeficacia de carotenoides (SLAMENGHI es un mnemotécnico para tales factores). Por ejemplo, la bioeficacia de carotenoides en grupos de sujetos con diferentes estados nutricionales respecto a vitamina A, cinc o hierro, ya que estos nutrientes influyen sobre el metabolismo de vitamina A y carotenoides. En el futuro, modificando los

diseños arriba descritos, esta técnica podría aplicarse para el estudio de la bioeficacia de carotenoides en dietas mixtas y en dietas que proveen distintas cantidades de carotenoides en una comida. Más aún, podrían estudiarse también los efectos de 'efectores' de absorción y conversión tales como grasa, fibra y pectina. Como muchos carotenoides, con o sin actividad pro-vitamínica A, pueden ser marcados específicamente con átomos de ^{13}C, entonces se puede estudiar la biodisponibilidad y bioeficacia de estos carotenoides así como la interacción entre ellos.

IMPLICACIONES DE LOS NUEVOS DATOS SOBRE LA BIOEFICACIA DE LOS CAROTENOIDES

Los estudios descritos en esta tesis han proporcionado las estimaciones más confiables de la bioeficacia de β-carotenos en aceite disponibles hasta ahora. No parece haber razón para dudar que los datos obtenidos y el método usado en niños de Indonesia puedan aplicarse a otros grupos de población del mundo en desarrollo tales como niños, adultos (incluyendo embarazadas y madres lactantes) y ancianos. Con base en dos recientes ensayos dietéticos controlados en Indonesia y Vietnam se ha concluido que unos 21 μg de β-caroteno en una dieta mixta (ingesta de verduras:frutas a razón de 4:1) tienen la misma actividad vitamínica A que 1 μg de retinol (que significa una bioeficacia del β-caroteno de una dieta mixta de 4.5%). Esta baja bioeficacia del β-caroteno de verduras de hojas de color verde intenso comparado con frutas anaranjadas ha sido confirmada por el trabajo descrito en esta tesis (**Capítulos 3 y 4**). Por lo tanto, las guías actuales que indican que la actividad vitamínica A de 1 μg de retinol puede ser provista por 6 μg (FAO/OMS) y 12 μg (IOM) de β-caroteno en una dieta mixta sobre-estiman la bioeficacia del β-caroteno en cerca de dos a tres veces.

Los nuevos datos sobre la bioeficacia del β-caroteno en aceite y en una dieta mixta pueden ser usados para re-evaluar las recomendaciones sobre la bioeficacia de carotenoides alimentarios por organizaciones nacionales e internacionales como parte de sus guías sobre recomendaciones dietéticas. Adicionalmente, los datos permiten la formulación y evaluación de políticas y programas apropiados para el control de la deficiencia de vitamina A. Debido a la baja bioeficacia de los carotenoides en alimentos de origen vegetal, se requiere de otras estrategias distintas de la promoción de frutas y vegetales para la eliminación de la deficiencia de vitamina A.

During my PhD, I continuously tried to reduce my 'To do' list. In the meantime, my 'To acknowledge' list grew exponentially. This is the moment to deal with both lists chronologically. I am delighted to finally put in writing, my gratitude to all who contributed to this thesis.

It all started during my Msc (3 January 1994), when Professor Clive West and I visited Professor Lugtenburg and his co-workers in Leiden. Outlining the synthesis pathway on a blackboard, they explained how they would make ^{13}C-labeled retinol and carotenoids. I was impressed. In June 1994 we carried out a pilot study using [$^{13}C_6$]β-carotene and [$^{13}C_6$]retinol. Peter Verdegem and Frans-Jos Jansen synthesized these compounds. Peter and Frans-Jos, I am truly sorry that your work has never been rewarded with a publication, despite the crucial role of your efforts in our decision to continue with 10-fold labeled compounds. In 1997 and 1998, Michiel Verhoeven and Alain Creemers synthesized the labeled compounds used in the research described in this thesis. I have always found it incredibly generous that you have both spent a considerable time of your own PhD projects for this synthesis. Professor Lugtenburg, Johan, I would like to thank you for your continuous and inspiring involvement in this project; it encouraged me to carry out the work and to get the papers written.

At the 11th International Carotenoid Symposium (Leiden, August 1996), I explained to someone that we did not have the results of the pilot study, as we had encountered difficulties in analyzing the samples by liquid chromatography-mass spectrometry (LC-MS). That someone turned out to be Dr Richard van Breemen, and he immediately assured me that he and his co-workers would be able to analyze the samples. Thus, when my PhD started in January 1997, my first duty was a one-month visit to Richard's laboratory in Chicago. Again I was impressed. Accompanied by Clive (the first week only), we quickly learned that the dynamic range of electrospray ionization LC-MS was too low to detect labeled retinol in the presence of large amounts of unlabeled retinol. Clive's conclusion from this visit was: 'We can probably learn how to perform LC-MS analysis, but it would take us at least four years'. Which in my case would be significantly more than that. I am therefore greatly indebted to Yan Wang, Dejan Nikolic, Xiaoying Xu, Yecheng Tan and Yongkai Sun for all sample preparations and analyses they have carried out. Yan Wang, thank you for communicating with me by E-mail and good luck with your own thesis and with your baby. Richard, you have kept your promise, you did analyze the first samples and much more. I have learned a great deal from working with you. I am amazed by the excellent, clear but condensed papers that you write about the development of the method of analysis. I am very grateful for your efforts towards this project in general and for Chapter 5 in particular. I also want to thank you and your wife Denise for your hospitality during my stay in Chicago.

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In July 1999, I returned to Wageningen for the allegedly toughest phase of any PhD, that of analysis data and writing papers. Fortunately, this new phase started with the PhD study tour to South Africa, organized by Margreet, Maud, Alida, Edine, Martijn, Andre and myself. Thank you all for the respectful and energetic teamwork and for keeping the memory of Martijn alive after he suddenly passed away last year.

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correctly, once it works, this isotope technique is like a little egg of Columbus'. In May 2001, while reading the favorable editorial (by Noel Solomons and Robert Russell) corresponding to the publication of our first paper on this isotope technique, I realized that the egg of Columbus had broken. I am sure that in the near future Clive will develop the hatchling into a mature chicken. Clive, I have loved to work with you. I have learned so much from our discussions and your advice in many categories of science and life. I want to thank you for always taking responsibility for what I did and at the same time supporting me to do things my way. Your trust has meant a lot to me. You are one of my role models on many aspects of life, not least of which is the warm atmosphere that you and Helen created for your family.

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Machteld van Lieshout was born on the 11th of July in 1971 in Helmond, the Netherlands. In the summer of 1989, she completed secondary school at the 'Carolus Borromeus College' in Helmond, and enrolled in the study 'Human Nutrition' at the former Wageningen Agricultural University (WAU), the Netherlands. In January 1993, her curiosity for the metabolism of micronutrients brought her to conduct her first research project at the Human and Animal Physiology Group (WAU). Under the supervision of Prof. Daan van der Heide and Dr Petra Versloot, she studied 'Iodide uptake by the thyroid in pregnant rats' using a method to measure continuously the uptake of radioactive iodide (^{125}I) in vivo by the thyroid. In January 1994, she started her second research project at the former Department of Human Nutrition (WAU). Under the supervision of Prof. Clive West, she studied 'The extent of conversion of β-carotene to retinol in man' when she initiated the development of the stable isotope method which became the basis for the work described in this thesis. In January 1995, she conducted her third research project, under the supervision of Dr Karin van het Hof, during which she assisted in organizing and conducting an intervention study with human volunteers at Unilever Research Laboratories at Vlaardingen. In August 1995, she obtained her MSc degree in Human Nutrition. From October 1995 until August 1996, she participated in the 'Professional Exchange Program' of Bureau IELAB of 'Hogeschool Utrecht, the Netherlands'. Within the framework of this program, she was involved in nutrition extension to employees and beneficiaries of a rural development project 'Proyecto Cuchumatanes' in the Western Highlands of Guatemala in Central America. As part of the nutrition extension she wrote a document entitled: '*Manual de capacitación sobre nutrición familiar*' (Manual for training in family nutrition). In October 1996, she attended the 'Third International Graduate Course on Production and Use of Food Composition Data' in Nutrition in Wageningen.

In January 1997, the 'Netherlands Foundation for the Advancement of Tropical Research' (WOTRO-NWO) appointed her as a PhD fellow to resume the development of the above mentioned stable isotope method in a research project entitled: 'Measurement of the bioconversion of β-carotene to retinol in children in Indonesia'. The research in this project was carried out within the framework of collaboration between the Division of Human Nutrition and Epidemiology of Wageningen University, the Nutrition Research and Development Centre in Bogor, Indonesia, the Department of Medicinal Chemistry and Pharmacognosy of the College of Pharmacy of the University of Illinois at Chicago, USA, and the Leiden Institute of Chemistry of Leiden University, in Leiden. She followed the education program of the Graduate School VLAG (advanced courses in Food Technology, Agrobiotechnology, Nutrition and Health Sciences). She participated in the PhD Study Tour to Scandinavia in 1997. In a group of 7 PhD fellows, she organized a two-week study tour for fellow PhD fellows of the Division to South Africa in 1999. In 2001, she was selected to participate in the 7th European Nutrition Leadership Program (ENLP) in Luxembourg. So, now she is a member of the ENLP Alumni Association.

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