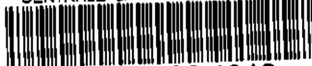
# Spinach as a source of carotenoids, folate and antioxidant activity

**Jacqueline J.M. Castenmiller**



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# Spinach as a source of carotenoids, folate and antioxidant activity

**Jacqueline Jozefine Maria Castenmiller**

**Proefschrift**

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# Stellingen

1. De relatieve biobeschikbaarheid van β-caroteen in spinazie is laag, maar kan worden verbeterd door het verder bewerken van heel-blad spinazie.
   Dit proefschrift

2. Over de antioxidative rol van de hoeveelheid β-caroteen in het Nederlandse voedingspatroon kan nog geen duidelijke uitspraak worden gedaan.
   Dit proefschrift

3. No supplements should be recommended without solid evidence of safety and efficacy.
   Gebaseerd op: Mares-Perlman JA. Too soon for lutein supplements. Am J Clin Nutr 1999;70:431-2

4. In retrospect, it was naieve to think that one food component, β-carotene, would be a universal protector against epidermal cancers and/or cardiovascular disease.
   Russell RM. Physiological and clinical significance of carotenoids. Internat J Vit Nutr Res 1998;68:349-53

5. Vele kruiden en specerijen verfijnen de smaak en bevorderen de oxidatieve stabiliteit van voedingsmiddelen.
   FAIR project CT95-0158 'Natural antioxidants in foods'

6. Personen, die verslaafd zijn aan wortels, zijn allen rokers.
   Kaplan R. Carrot addiction. Aust NZ J Psychiatry 1996;30:698-700; Cerny L, Cerny K. Can carrots be addictive? An extraordinary form of drug dependence. Br J Addict 1992;87:1195-7

7. Er bestaat een duidelijk verschil tussen onderzoek uitgevoerd op een zaterdagmiddag en onderzoek dat vele zaterdagen heeft gekost.
   Naar aanleiding van een opmerking van professor JGAJ Hautvast in 'De toekomst van Karel: De toekomst van eten', uitgezonden door de AVRO op 23 oktober 1999

8. The reason why we have two ears and only one mouth is that we may listen the more and talk the less.
   Zeno of Citium, ca 300 BC

9. Opvoeden is de kunst zichzelf in principe overbodig te maken.

10. Het waren niet de mannen die voor de grote sprong in de evolutie van ons brein ongeveer twee miljoen jaar geleden hebben gezorgd. Het geheim zit in het koken door de vrouwen: plantaardige voeding werd daardoor in een klap tot een gemakkelijk verteerbare en goedkope energiebron.

    Wrangham RW, e.a. The raw and the stolen. Cooking and the ecology of human origin. Current Anthropology 1999;40:567-94

11. Het terugdringen van chemische bestrijdingsmiddelen zoals aanbevolen in het meerjarenplan 'Gewasbescherming' is onvoldoende tot zijn recht gekomen door de grote vrijblijvendheid van het gewasbeschermingsbeleid.

12. Issues affecting women are not soft or marginal, but are central to decisions involving all nations.

    Hillary Rodham Clinton, 1996

Stellingen behorend bij het proefschrift:
'Spinach as a source of carotenoids, folate and antioxidant activity'

Jacqueline J.M. Castenmiller
Wageningen, 5 januari 2000

# ABSTRACT


**Spinach as a source of carotenoids, folate and antioxidant activity**

*PhD thesis by Jacqueline J.M. Castenmiller, Division of Human Nutrition and Epidemiology, Wageningen University, The Netherlands, 5 January 2000*

Fruits and vegetables are generally considered important contributors to a healthy diet and an increased intake of fruits and vegetables is related to a decreased risk of cancers, cardiovascular disease, and other diseases. In this thesis two aspects of spinach, a dark-green, leafy vegetable, are examined. The first aspect is the bioavailability of the carotenoids and folate present in spinach. The second aspect is the antioxidant activity of spinach consumption in humans and the antioxidant capacity of spinach products. The literature on carotenoid bioavailability and bioconversion was reviewed for each of the SLAMENGHI factors. These factors include: Species of carotenoid; molecular Linkage; Amount of carotenoid consumed in a meal; Matrix in which the carotenoid is incorporated; Effectors of absorption; Nutrient status of the host; Genetic factors; Host related factors; and Interactions.

A dietary intervention study with 70 healthy human subjects divided over six treatment groups was conducted to examine the effect of the food matrix on the bioavailability of carotenoids and folate and to evaluate the effect of spinach intake on biomarkers of antioxidant activity. Four groups received a basic diet plus a spinach product (whole-leaf, minced, enzymatically liquefied, and liquefied spinach plus added dietary fibre), one group received the basic diet plus a carotenoid supplement of β-carotene, lutein and a small amount of zeaxanthin dissolved in oil, and one group received the basic diet only. Consumption of spinach (20 g/MJ), containing the carotenoids β-carotene and lutein, increased serum concentrations of β-carotene, lutein, α-carotene and retinol and decreased the serum concentration of lycopene compared with the control group. Compared with the synthetic carotenoid supplement, the relative bioavailability of β-carotene was low, 5.1-9.5%, but much higher for lutein, 45-55%. Serum β-carotene responses differed significantly between the whole-leaf and liquefied spinach groups and between the minced and liquefied spinach groups. The plasma folate response was significantly greater in the spinach groups compared with the control group. Intake of minced and liquefied spinach resulted in greater plasma folate responses than consumption of whole-leaf spinach. Thus, disruption of the food matrix (cell wall structure) and loss of cellular structure had an effect on the

bioavailability of β-carotene and folate, whereas the bioavailability of lutein was not affected. Addition of dietary fibre to the liquefied spinach to compensate for the fibre that was broken down during liquefaction had no effect on serum carotenoid or plasma folate responses.

Consumption of spinach or the carotenoid supplement resulted in an increased erythrocyte glutathione reductase activity, and decreased erythrocyte catalase activity and serum α-tocopherol concentration. These changes were related to serum lutein concentrations. The antioxidant capacity of the differently processed spinach products was determined in different oxidation systems. Corrected for the total phenolics content of the spinach, the whole-leaf spinach extract showed the strongest inhibition of hydroperoxide formation, followed by extracts from liquefied and minced spinach. The calculated amount of lutein in the spinach samples used (amounts were adjusted for the total phenolics content of spinach) was correlated with the inhibition of hydroperoxide formation. In a meatball model at 100 and 200 g spinach/kg meat, whole-leaf and minced spinach were prooxidative (increased the formation of thiobarbituric acid-reactive substances); the liquefied spinach became antioxidative at 200 g/kg. Minced and liquefied spinach were antioxidatively active with respect to the mean inhibition of hexanal formation, whereas whole-leaf spinach showed prooxidant activity.

In conclusion, the results of the studies described in this thesis demonstrated an effect of the food matrix on β-carotene and folate, but not on lutein, bioavailability and support the finding that β-carotene is not a good antioxidant in man or in foods, but indicate that lutein may play a role as antioxidant.

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# 1

# General introduction

## HEALTH EFFECTS OF FRUITS AND VEGETABLES

Cancer and cardiovascular disease are major causes of chronic disease deaths and are thus major public health problems. Apart from genetic and environmental factors, life-style is considered to influence the aetiology of chronic diseases. Life-style factors include diet, smoking, drinking and physical activity. Fruits and vegetables are generally considered important contributors to a healthy diet and, for some years now, health professionals have been advising the general public to increase their intake of fruits and vegetables to reduce the risk of specific cancers, cardiovascular disease, neural tube defects, and cataracts. Although the mechanisms are not fully understood, carotenoids, folic acid and dietary fibre appear to play important roles in the prevention of these diseases (Kushi et al. 1995).

Estimates have been made of the proportion of cancer cases and cardiovascular deaths that could be prevented by increasing fruit and vegetable consumption. For cancer incidence, the best estimate is 19% (7-28%); CHD mortality risk could be reduced by 20-40%, whereas stroke mortality could be reduced by 0-25% with high intake of fruits and vegetables (Klerk et al. 1998). Law & Morris (1998) have tried to quantify the relationship between fruits and vegetables consumption and the incidence of ischaemic heart disease. They conclude that the risk of ischaemic heart disease is about 15% lower at the 90th than at the 10th centile of fruit and vegetable consumption.

A diet high in fruits and vegetables provides a variety of food components, which promote health. Table 1 lists a number of nutrients and nonnutrients present in fruits and vegetables. However, fruits and vegetables further contain natural substances that may have adverse effects on human health: nitrate, aflatoxin and other mycotoxins, and goitrogens. There may also be some adverse

**Table 1. Nutrients and nonnutrients in fruits and vegetables**

| Nutrients | Nonnutrients |
|---|---|
| Carotenoids | Dietary fibre |
| Ascorbic acid | Glucosinolates |
| Fat-soluble vitamins such as tocopherols | Phenols, including flavonoids |
| B-vitamins such as folic acid | Organosulfides/organo sulfur compounds |
| Trace elements such as selenium | Phytoestrogens/isoflavones |
| Potassium | Protease inhibitors |
| | Phytosterols |

effects of fruit and vegetable consumption, due to contamination of food with heavy metals and pesticides.

## CAROTENOIDS AND HEALTH

Carotenoids are widespread in nature. They are a group of fat-soluble pigments found in fruits and vegetables, insects, birds, and other plant-eating animal species, including humans. Carotenoids have light absorbing properties. Light absorption occurs in the low-energy, visible range of the spectrum, giving fruits and plants their brilliant yellow, orange, red and green colours. Carotenoids are also found in all green plant tissues, where they occur in the photosynthetic pigment-protein complexes of the chloroplasts (in leaves). In these tissues, the colour of carotenoids is masked by the green of the chlorophyll, but is revealed during the degradation of chlorophyll. This phenomenon can be seen for example during the ripening of fruits and in leaves with the onset of autumn. Also, many yellow or orange flowers owe their colour to carotenoids, which are located in chromoplasts (Britton et al. 1995b). Most carotenoids can be described by the general formula $C_{40}H_{56}O_n$ where n is 0-6. Only few carotenoids can be detected in human blood, including α-carotene, β-carotene, lutein, zeaxanthin, β-cryptoxanthin, lycopene and canthaxanthin (see Figure 1). Carotenoids such as α-carotene, β-carotene and β-cryptoxanthin are precursors of retinol (vitamin A).

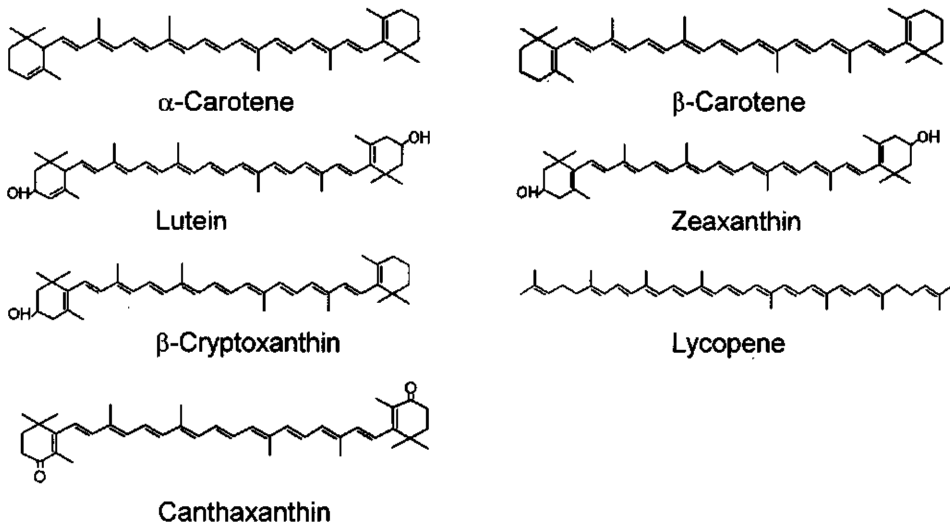



**Figure 1.** Structure of major carotenoids found in human blood

Those carotenoids containing oxygen are also referred to as oxycarotenoids or xanthophylls.

Based on intake or biomarkers of intake, carotenoids have been postulated to play a protective role in angina pectoris (Riemersma et al. 1991), heart disease (Kardinaal et al. 1993, van Poppel 1994) and cancer (Stahelin et al. 1991), particularly cancer of epithelial tissues (van Poppel 1996), lung (Dartigues et al. 1990, Knekt et al. 1990, Smith & Waller 1991) and stomach cancer (Chen et al. 1992, Cipriani et al. 1991). The hypothesis that β-carotene plays a role in cancer prevention is based on studies suggesting that carotenoids function as antioxidants, as enhancers of immune function, as precursors of retinoids and/or by stimulation of cell-cell communication. Epidemiological studies reveal associations but not cause and effect. In order to determine whether β-carotene is the active phytochemical in cancer prevention, four major intervention trials in humans have been completed, all of which included β-carotene supplementation with or without other nutrients. Two studies, provided evidence suggesting that β-carotene increased the risk of lung cancer in high-risk groups of male Finnish smokers (ATBC Cancer Prevention Study Group 1994) and of smokers or workers who had been occupationally exposed to asbestos (CARET study; Omenn et al. 1996). Another study showed neither negative nor positive effects on the incidence of cancer in a healthy population supplemented for 12 years with β-carotene (The Physicians Health Study; Hennekens et al. 1996). One study, which involved combined supplementation of β-carotene with tocopherol (vitamin E) and selenium to marginally malnourished people, showed lower mortality from gastric and total cancers in the supplemented group (Blot et al. 1993). Thus, with respect to the chemopreventive effect of fruits and vegetables, these intervention trials indicated that β-carotene alone is probably not the active phytochemical in the prevention of cancer.

With respect to cardiovascular disease, the clinical trials in which β-carotene was given without other antioxidants found no evidence that supplementation had a protective effect. The trials included the ATBC study (Rapola et al. 1997); the Physicians Health Study (Hennekens et al. 1996); the trial involving those exposed to asbestos or heavy smoking (Omenn et al. 1996, Rimm et al. 1993); and a study involving patients at high risk for recurrent nonmelanoma skin cancer (Greenberg et al. 1996). Three of the four studies found increases in cardiovascular disease mortality in the supplemented groups ranging from 12 to 26%. The Physicians Health Study found no difference (Kritchevsky 1999, Russell 1998).

There are a number of associations reported between carotenoid intake and

disease. The inverse associations between lycopene intake and prostate cancer and between lutein intake and age-related macular degeneration (AMD) are each supported by one main study that has examined specifically these carotenoids but are not corroborated by other similar studies (Cooper et al. 1999). A number of possible confounding factors may explain the inconsistencies between the trials and observational epidemiological evidence. The intake and serum concentrations of other carotenoids, which are correlated with serum β-carotene concentrations, might be important factors, as might other phytochemicals or nutrients (Kritchevsky 1999). Dietary intakes of the carotenoids lutein and zeaxanthin, which are primarily obtained from dark-green, leafy vegetables, have been found to be most strongly associated with reduced risk for age-related macular degeneration (Seddon et al. 1994).

With regard to the health effect of fruits and vegetables, with emphasis of the possible role of β-carotene and other carotenoids, smoking and alcohol intake may play a role and should be taken into account in future studies. Both the ATBC and CARET populations included persons who were in the initial stage of developing lung cancer prior to beginning β-carotene supplementation. The gas phase of cigarette smoke is a rich source of free radicals and is highly oxidative. Under conditions of high oxidative stress and phagocyte-mediated inflammatory responses, β-carotene and other carotenoids may be oxidised to epoxides and other reactive derivatives, which then act as pro-oxidants and as chemical messengers that stimulate cellular proliferation, especially in carcinogen-altered epithelial cells (Boosalis et al. 1996, Irribarren et al. 1997). In such a situation, β-carotene or its metabolites could have promoted the latter stages of cancer. Thus, it could be hypothesised that β-carotene or its metabolites protect against cancer initiation (van Poppel et al. 1992) but act as promoters in the later stages of cancer development.

Studies in humans have shown that for a given β-carotene intake, alcohol consumption and plasma β-carotene concentrations are correlated (Ahmed et al. 1994). Whereas alcoholics generally have low plasma β-carotene concentrations, presumably reflecting a low intake, alcohol intake per se might in fact increase serum concentrations of β-carotene in humans even with as few as two drinks per day (Forman et al. 1995). The combination of an increase in serum β-carotene concentrations and a relative lack of a corresponding rise in retinol concentrations suggest a block in the conversion of β-carotene to retinol by ethanol. Because alcohol increases β-carotene concentrations, and because cardiovascular complications are apparently associated with elevated β-carotene concentrations,

Leo & Lieber (1999) suggest that it is possible that β-carotene is cardiotoxic, a possibility that is still largely unexplored. Furthermore, the hepatotoxicity of the combination of alcohol and β-carotene was found to be exacerbated when the latter is given as beadlets (Leo et al. 1997). Beadlets resulted in a proliferation of the smooth endoplasmic reticulum and in leakage of mitochondrial glutamate dehydrogenase into plasma, reflecting mitochondrial injury (Leo et al. 1997). The reason for this toxicity is not clear. The composition of the beadlets is proprietary; of the known ingredients, none have been identified as toxic. Indeed subsequently, the data of the ATBC study and CARET showed that the increased incidence of pulmonary cancer was related to the amount of alcohol consumed by the participants (Albanes et al. 1996, Albanes et al. 1997, Omenn et al. 1996).

## FOLIC ACID AND HEALTH

The term folate refers to a group of compounds that exhibit vitamin activity similar to that of pteroyl-L-glutamic acid (folic acid). Pteroic acid and hydrogenated and methylated derivatives can be conjugated to one glutamyl residue (monoglutamyl folates) or to more glutamyl residues (polyglutamyl folates). Folate is essential for haematopoiesis but is now receiving attention in other areas. Folates are abundant in many of the same fruits and vegetables that are rich in carotenoids. Folic acid is a leading candidate for being primarily responsible for reducing plasma homocysteine concentrations. A low intake of folate is associated with increased plasma homocysteine levels (Selhub et al. 1993), cardiovascular disease (Rimm et al. 1998) and colon cancer (Giovannucci et al. 1995). An elevated plasma homocysteine concentration is a risk factor for neural tube defects (Steegers-Theunissen et al. 1991) and cardiovascular disease (Graham et al. 1997, Kang et al. 1992, Nygård et al. 1995, Pancharuniti et al. 1994, Verhoef et al. 1996). Folic acid, vitamin $B_{12}$, methionine, and choline are intimately interrelated in methyl group metabolism. Inadequate folate intake first leads to a decrease in plasma folate concentration, then to a decrease in erythrocyte folate concentration, a rise in homocysteine concentration, and ultimately to megaloblastic changes in the bone marrow and other rapidly dividing cells. Folate deficiency causes extensive incorporation of uracil instead of thymine into human DNA, leading to chromosomal breaks. This mechanism is the likely cause of the increased cancer risk, and perhaps the cognitive defects associated with low folate intake (Ames 1998).

## BIOAVAILABILITY OF FOOD CONSTITUENTS

Accurate information on the bioavailability of nutrients and nonnutrients is needed in order to devise food-based strategies for long-term alleviation of deficiency and/or to devise food or supplement-based interventions to reduce risk for chronic diseases, which appear linked to these food constituents. Thus, it is important to be able to estimate the uptake of the food constituents from the gastrointestinal tract; the extent of their metabolism in the intestinal wall and in other tissues of the body, including conversion to the active form of a nutrient; the concentrations reached and maintained in various organs and tissues of the body; and the rate and mode of excretion from the body. Vitamin toxicity is a growing problem and here, too, bioavailability and bioconversion is important. For example, the self-limiting conversion of carotenoids to retinol apparently prevents retinol toxicity (Bates 1989), although very high intakes of some carotenoids can lead to hyperpigmentation of tissues (Dimitrov et al. 1988, Diplock 1995).

The term bioavailability is used by a variety of investigators in various fields of research. The following series of definitions is used to clarify the situation, especially since the well-established toxicological/pharmacological definition of bioavailability is not compatible with the nutritional use of the term. Bioavailability can be defined as the proportion of a nutrient ingested which becomes available to the body for metabolic processes (Macrae et al. 1993) or the proportion of a nutrient being capable of being absorbed and available for use or storage or more briefly the proportion of a nutrient that can be used (Bender 1989). In order for a nutrient to be capable of being used - or available - it must either be present in the diet in a form that can be transported across the mucosa, or the ingested forms must be capable of being transformed into transportable forms and the nutrient must be absorbed in a form that can be utilised in normal metabolism. More recently bioavailability has been defined as the fraction of an ingested nutrient that is available for utilisation in normal physiological functions or for storage (Jackson 1997). The term bioconversion is used to refer to the conversion to the active form of a nutrient. The overall process from ingestion to the formation of an active form of a nutrient is termed bioefficacy although this is often referred to as bioconversion.

### Factors affecting bioavailability and bioconversion

The factors that affect nutrient, and nonnutrient, bioavailability, and in some cases bioconversion, include: Species of carotenoid; molecular Linkage; Amount of carotenoid consumed in a meal; Matrix in which the nutrient is incorporated;

Effectors of absorption and bioconversion; Nutrient status of the host; Genetic factors; Host related factors; and Interactions. By using the capitalised letter, the mnemonic SLAMENGHI has been introduced to order these factors (Castenmiller & West 1998, de Pee & West 1996, West & Castenmiller 1998). The bioavailability of iron has been examined thoroughly (Benito & Miller 1998, Hurrell 1997), whereas little information is available on the bioavailability and bioconversion of other food components, especially nonnutrients. Current knowledge of each of the SLAMENGHI factors for carotenoids is discussed in Chapter 2. Some of the factors can be manipulated, for example, the amount consumed, the food matrix or the amount of fat or dietary fibre in a meal.

## BIOAVAILABILITY OF CAROTENOIDS

### Absorption and transport of carotenoids

Carotenoids are absorbed in a similar manner to other fat-soluble compounds. They are incorporated into mixed micelles, which are formed from triacylglycerols, phospholipids, other fat-soluble compounds and bile salts. The carotenoids are then absorbed intact in the small intestine by passive diffusion into the enterocytes or cleaved in the intestinal mucosa, forming retinyl esters, and subsequently incorporated in chylomicrons and transported in chylomicrons to the liver. Carotenoids, and tocopherols, can be resecreted from the liver with lipoproteins for transport by plasma to other tissues. In man, about 75-80% of the carotenoid hydrocarbons (β-carotene and lycopene) is transported in large, less dense low-density lipoproteins (LDL), 12% in very low-density lipoproteins (VLDL) and 8% in high-density lipoproteins (HDL) (Krinsky et al. 1958, Lowe et al. 1999, Mathews-Roth & Gulbrandsen 1974), whereas lutein and zeaxanthin are more evenly distributed between the smaller, more dense LDL particles and high density lipoproteins (Cornwell et al. 1962, Lowe et al. 1999). Among all lipoproteins, transfer of β-carotene may occur (Johnson & Russell 1992).

Carotenoids may interact with each other during intestinal absorption, metabolism and serum clearance (Gaziano et al. 1995, Kostic et al. 1995). The concentration of various carotenoids in serum and other tissues depends not only on their absorption but also on differential uptake and subsequent metabolism, including the conversion of provitamin A carotenoids to retinol, by various tissues. For example, after ingestion of β-carotene, both β-carotene and retinol are found in serum, liver and adipose tissue (Krinsky et al. 1990). Lutein has been shown to accumulate in the eye (Nussbaum et al. 1981), while α-carotene, β-carotene,

lycopene, zeaxanthin, and cryptoxanthin accumulate in a variety of tissues (Kaplan et al. 1990).

Provitamin A carotenoids are converted to retinol by the action of 15-15'-carotenoid dioxygenase. This process occurs primarily in the enterocytes although enzyme activity is found in other tissues such as the liver. It is not yet established beyond doubt whether just one enzyme exists and whether the cleavage of β-carotene is central or not (Wolf 1995). The formation of β-apo-13-carotenone and beta-apo-14'-carotenal provides direct evidence for an enzymatic eccentric cleavage mechanism (Tang et al. 1991). Parker et al. (1993) used $^{13}C$-labelled β-carotene in humans and recovered most of the label in retinyl esters. This finding would suggest that β-carotene cleavage is central; central oxidation of β-carotene theoretically yields two molecules of retinol. Other provitamin A carotenoids apparently yield 1 mol retinol per mol carotenoid undergoing metabolism.

**Methods to determine bioavailability of carotenoids**

Various methods have been used up until now to measure carotenoid bioavailability and bioconversion. The balance method compares the amount of carotenoid recovered in the faeces with that fed. Studies from India indicated that between one quarter and a half of β-carotene in amaranth was recovered in faeces suggesting that the remainder was absorbed and converted to retinol (Lala & Reddy 1970). Three to five year old children absorbed 75% of a single dose of 1200 μg β-carotene from an alga (Annapurna et al. 1991) and 45% of a supplement of 14 mg carotene with oil was absorbed in a placebo controlled experiment with 9-16 year old boys (Roels et al. 1958). Shiau et al. (1994) assessed the retention time of β-carotene during transit through the intestine by using the total gut washout method. For subjects receiving no meal, 83% of ingested β-carotene was recovered in rectal effluent collected within 24 hours post-β-carotene administration, whereas the quantity of β-carotene in faeces of individuals receiving meals was 49-71%. Goodman and colleagues (1966) administered $^{14}C$-labelled to subjects and recovered 9 and 17% of the radioactivity of β-carotene in lymph over a 20-h period. This result suggests that balance studies overestimate absorption. Probably, carotenes are broken down in the large intestine. Nowadays medical ethical committees are reluctant to approve studies using radioactive isotope-labelled compounds, such as $^{14}C$-labelled β-carotene and retinol, in human subjects. More recently, uniformly labelled $^{13}C$-β-carotene (Parker et al. 1993) and $^{2}H$-labelled $d_8$-β-carotene (Dueker et al. 1994,

Novotny et al. 1995) have been used to measure relative β-carotene bioavailability and conversion to retinol. However, without the administration of labelled retinol it is not possible to quantify the contribution, which β-carotene makes to retinol synthesis. At the Division of Human Nutrition and Epidemiology (Wageningen University), a method has been developed using $^{13}C_{10}$-β-carotene and $^{13}C_{10}$-retinyl palmitate to quantify the bioavailability and bioconversion of β-carotene (van Lieshout et al. 1999).

Most studies on bioavailability and bioconversion have measured β-carotene and retinol concentrations in serum or plasma. With the methods used, it was not possible to study the metabolism of β-carotene. Relative bioavailability can be measured by comparing the serum response of a carotenoid-rich food relative to a synthetic supplement of the carotenoid dissolved in oil (Brown et al. 1989, Jensen et al. 1986, Masaki et al. 1993, Micozzi et al. 1992). Another modification of the serum response is to measure the concentration of β-carotene and retinyl esters and other carotenoids in various lipoprotein fractions (van Vliet et al. 1995, van den Berg 1998). Results from several studies comparing weighed intakes of carotenoids and mean plasma concentrations suggest that plasma carotenoid concentrations are indicative of dietary intake, but the large intra-individual variation in plasma concentrations indicates that any assessment of longer-term status from data at any one time-point should be treated with caution (Scott et al. 1996).

## BIOAVAILABILITY OF FOLATE

Based on a review of the literature, Brouwer et al. (1999) concluded that the food matrix and the amount of folic acid consumed are the main factors influencing folate bioavailability.

### Absorption and transport of folate

Natural folates are present primarily in the polyglutamate form. Before absorption, glutamate moieties distant to the proximal glutamate residue must be removed by the enzyme conjugase pteroylpolyglutamate hydrolase. After hydrolysis to monoglutamates, two different transport systems are present. Folates can be bound to membrane-associated folate binding proteins and transported across the brush border membrane by a carrier-mediated mechanism. Active mucosal transport is accelerated by glucose and galactose and impaired by unidentified factors present in many foods. At high intraluminal concentrations of folate (>10

μM) a nonsaturable diffusion-mediated transport system plays a major role in folate absorption (Mason et al. 1990). Folic acid absorbed from the intestine at physiological concentrations is largely converted to reduced forms and then methylated or formylated, whereas at higher concentrations, it is transported through the enterocytes without such modifications (Herbert & Das 1994). Folate is delivered to bone marrow cells, reticulocytes, liver, cerebrospinal fluid, and renal tubular cells. Prior to tissue storage or use as a coenzyme, folate monoglutamate is converted to the polyglutamate form.

### Methods to determine bioavailability of folate

Folate bioavailability can be determined by measuring urinary excretion of labelled folate (Gregory et al. 1991, Gregory et al. 1992) and area-under-the-curve (AUC) of serum folate (Bailey et al. 1988, Pfeiffer et al. 1997, Tamura & Stokstad 1973). The primary indicator of folate adequacy selected is red cell folate, which reflects tissue folate stores and is an indicator of long-term status. Plasma homocysteine concentration increases when inadequate quantities of folate are available to donate the methyl group that is required to convert homocysteine to methionine. Serum or plasma folate concentration is considered a sensitive indicator of dietary folate intake (Jacques et al. 1993). High intakes of 500 μg of folic acid or more have no additional effect on improving the functional parameter of folate status, plasma total homocysteine concentration. It is not clear whether high doses of folic acid pose health risks (Brouwer et al. 1999).

## ROLE AND MECHANISM OF ACTION OF ANTIOXIDANTS

It is difficult to interpret whether the apparent benefits of fruit and vegetable intake are due to antioxidant vitamins, other nutrients, nonnutrients, dietary habits or other lifestyle characteristics. Foods and the human body are under constant oxidative stress from free radicals, reactive oxygen and nitrogen species and other prooxidants. Antioxidants are needed to prevent the formation and oppose the actions of reactive oxygen and nitrogen species, which are generated in vivo and cause damage to DNA, lipids, proteins, and other biomolecules. It is however, important to emphasise that not all reactions in living systems that involve free radicals are damaging; thus, several normal biological processes in vivo depend on free radicals (Diplock et al. 1994). Cells have intact prooxidant/antioxidant systems that function continuously to generate and to detoxify oxidants during normal aerobic metabolism. Oxidative stress is an

imbalance between prooxidants and antioxidants in favour of the prooxidants and has been demonstrated to play a role in many diseases, including cancer, cardiovascular and pulmonary diseases (Bast et al. 1998). Radicals of oxygen (superoxide anion and hydroxyl, alkyoxyl, and peroxyl radicals), reactive nonradical oxygen species (hydrogen peroxide and singlet oxygen) and radicals of carbon, nitrogen, and sulfur constitute the variety of reactive molecules that cause oxidative stress to cells. Radicals are defined as any atom or molecule that contain one or more orbital electrons with unpaired spin states; the radical may be a very small molecule such as oxygen or it may be a part of a large biomolecule such as a protein, carbohydrate, lipid, or nucleic acid (Diplock 1994, Thomas 1994). They are in many instances highly reactive because the unpaired state is thermodynamically unstable. The effectiveness of an antioxidant against oxidative stress depends on its ability to counteract the specific molecules causing the stress and the cellular or extracellular location of the source of these molecules.

In response to oxidative stress, biological tissues have developed multicomponent, multiphasic antioxidants (Decker 1998). Antioxidants might act through donation of electrons or hydrogen atoms (Bast et al. 1998). However, the first line of defence, which is largely enzymatic, is to prevent the generation of radical species derived from molecular oxygen. The enzymes superoxide dismutase, catalase and glutathione peroxidase are ubiquitously distributed in all aerobic organisms (Madhavi et al. 1996). Superoxide dismutase prevents the accumulation of superoxide anion ($O_2^{\bullet-}$), which is not membrane-permeable, by converting it to hydrogen peroxide ($H_2O_2$), which is metabolised by catalase or glutathione peroxidase to water. The second line of defence is to reverse damage or prevent further damage to cellular constituents such as lipids and nucleic acids. To support both the first and second line of defence, a pool of antioxidants is required. These include endogenous constituents, such as serum proteins and their thiol groups, bilirubin, urate and glutathione and dietary constituents, such as ascorbic acid, tocopherols, carotenoids and flavonoids. The second line of oxidative defence can most clearly be seen in the case of lipid peroxidation. The polyunsaturated fatty acids (PUFA:H) of cell membranes are susceptible to free radical attack. A free radical ($OH^{\bullet}$) can extract a hydrogen atom from PUFA:H, forming a PUFA radical ($PUFA^{\bullet}$). In combination with oxygen, a peroxyl radical is produced ($PUFA{:}OO^{\bullet}$) which reacts with PUFA:H to form a hydroperoxide (PUFA:OOH) and another $PUFA^{\bullet}$. Moreover in the presence of iron or copper PUFA:OOH can undergo one electron reduction to form more free radicals ($PUFA{:}O^{\bullet}$ and $OH^{\bullet}$) (Duthie 1993). Primary antioxidants can react with peroxyl radicals before they react further with unsaturated lipid molecules and convert

them to more stable products. The best-known primary antioxidants are phenolics and tocopherols. Secondary antioxidants are compounds that retard the rate of chain initiation by various mechanisms other than the pathway followed by the primary antioxidants. The secondary antioxidants reduce the rate of autoxidation of lipids by such processes as binding metal ions, scavenging oxygen, decomposing hydroperoxides to nonradical products, absorbing UV radiation, and deactivating singlet oxygen (Madhavi et al. 1996). Some of the major antioxidant defence mechanisms within the cell are depicted in Figure 2.

There is no doubt that antioxidants are of great importance in the aetiology of a number of different human diseases. It is critical to provide evidence that a diet with a high antioxidant capacity and activity from fruits and vegetables can either increase the overall antioxidant capacity or change the relative balance between individual antioxidant components in the human body; otherwise, any antioxidant hypotheses related to the protection of fruits and vegetables against diseases would not be sustained. Antioxidants may act synergistically. Palozza & Krinsky (1992) showed that β-carotene can act synergistically with α-tocopherol in rat liver microsomes: β-carotene regenerated α-tocopherol from the α-tocopheroxy radical. Tocopherol functions synergistically with ascorbic acid, which can react with tocopherol radicals to regenerate tocopherol. Glutathione reduces the ascorbyl free radical and ascorbate can regenerate α-tocopherol from the α-tocopherol free radical (Haenen & Bast 1983) or repair the β-carotene radical (Bohm et al. 1998). Tocopherol, especially together with ascorbic acid, destroys nitrite, an essential component in the food chain associated with cancer of the stomach, oesophagus and liver (Weisburger 1991).

**Carotenoids as antioxidants**

Carotenoids have been shown to possess antioxidant activity in vitro. Nevertheless, there is no convincing evidence that carotenoids possess antioxidant activity in humans. Carotenoids such as α-carotene, β-carotene, lycopene, and some oxycarotenoids, such as zeaxanthin and lutein, exert antioxidant functions in lipid phases by physical or chemical quenching of singlet oxygen ($^1O_2$) or by reacting with a variety of free radicals (Burton & Ingold 1984, Sies & Stahl 1995). The antioxidant action is limited to low oxygen partial pressures, less than 150 torr, and at higher oxygen pressures β-carotene may become prooxidant (Krinsky 1993).

In plant chloroplasts, carotenoids are important scavengers of singlet oxygen generated by an excited state of chlorophyll. The ability to quench singlet oxygen is related to the number of double bonds in the molecule. Carotenoids with 9 to 11

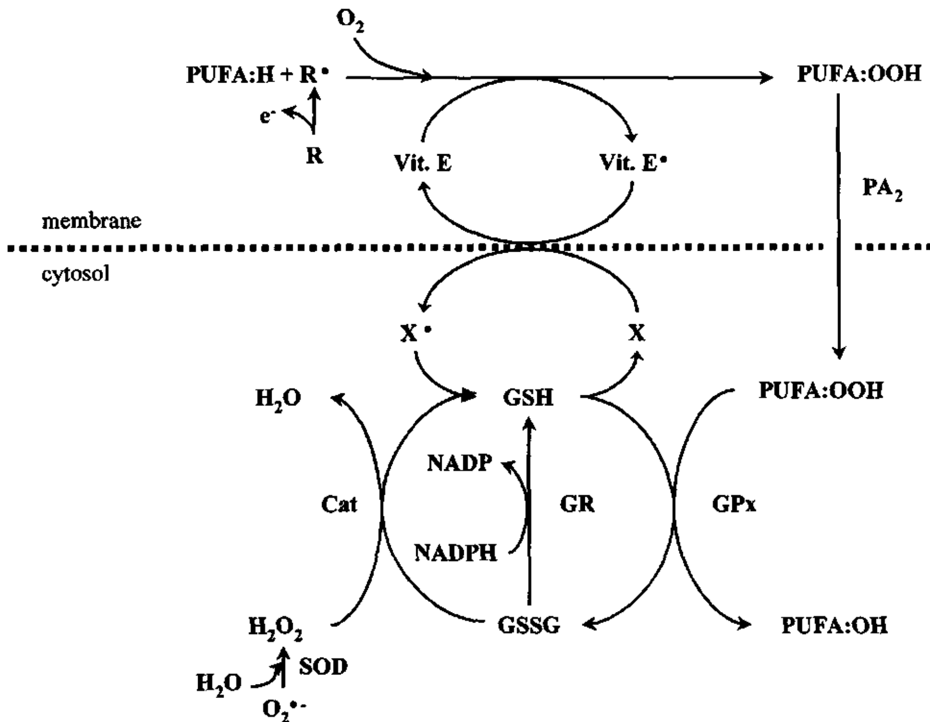
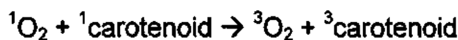



**Figure 2**. Major antioxidant defence mechanisms within the cell. R•, free radical; PUFA:H, polyunsaturated fatty acid; PUFA:OOH, fatty acid hydroperoxide; $PA_2$, phospholipase $A_2$; X, can be either ascorbic acid or a carotenoid while X• is the corresponding radical; GSH, reduced glutathione; GSSG, oxidised glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; Cat, catalase; SOD, superoxide dismutase

conjugated double bonds, which includes all the carotenoids in Figure 1, are electron-rich and can absorb the energy from the singlet oxygen and distribute it over all the single and double bonds in the molecule. These carotenoids are reported to be better quenchers than those with eight or fewer conjugated double bonds are (Hirayama et al. 1994). Thus, carotenoids are able to take over and absorb the energy from molecules in high-energy states. In this way carotenoids can quench the higher-energy form of oxygen, singlet oxygen ($^1O_2$). After this reaction, the oxygen returns to its basic triplet state ($^3O_2$):



$$^1O_2 + {}^1\text{carotenoid} \rightarrow {}^3O_2 + {}^3\text{carotenoid}$$

The 'energised' carotenoid ($^3$carotenoid) then releases the absorbed energy in the form of heat, thereby restoring it to its normal energy level.

$$^{3}\text{carotenoid} \rightarrow \text{carotenoid} + \text{heat}$$

Carotenoids are therefore not destroyed during the process and can repeat the process with additional singlet oxygen molecules (Bast et al. 1998, Britton 1995a, Krinsky 1994, Madhavi et al. 1996).

The proposed antioxidant mechanisms of carotenoids against free radicals, as in lipid oxidation, includes electron transfer, which results in the formation of carotenoid radical cations, addition reactions forming carotenoid-adduct radicals and further radical reactions, in which carotenoids act as hydrogen atom donors forming neutral carotenoid radicals (Bast et al. 1998, Haila 1999). Electron transfer results in the formation of a carotenoid radical cation, as follows:

$$\text{carotenoid} \rightarrow \text{carotenoid}^{\bullet +} + e^{-}$$

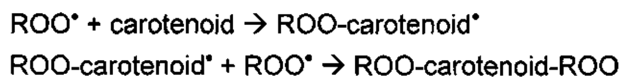
The resulting electron might be used to neutralise free radicals ($R^{\bullet}$):

$$R^{\bullet} + e^{-} \rightarrow R^{-}$$

The carotenoid radical cation might also undergo a dismutation reaction, whereby a radical termination occurs:

$$2\ \text{carotenoid}^{\bullet +} \rightarrow \text{carotenoid} + \text{carotenoid}^{2+}$$

Different carotenoid structures will differ in their ability to participate in electron transfer reactions. Addition reactions form carotenoid-adduct radicals, which can react further to form a non-radical product:



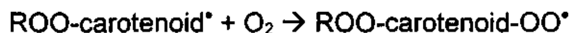
$$ROO^{\bullet} + \text{carotenoid} \rightarrow ROO\text{-carotenoid}^{\bullet}$$
$$ROO\text{-carotenoid}^{\bullet} + ROO^{\bullet} \rightarrow ROO\text{-carotenoid-}ROO$$

Hydrogen abstraction leads to formation of the neutral carotenoid radical:

$$R^{\bullet} + \text{carotenoid} \rightarrow RH + \text{carotenoid}^{\bullet}$$

At higher oxygen pressure, carotenoid-adduct radicals can react with oxygen to

generate a carotenoid-adduct peroxyl radical:



$$ROO\text{-carotenoid}^{\bullet} + O_2 \rightarrow ROO\text{-carotenoid-}OO^{\bullet}$$

This latter compound might have a prooxidant activity.

From this short description it can be concluded that the possible antioxidative role of carotenoids in nutrition is complex.

## OUTLINE OF THE THESIS

The objective of this thesis is to study the bioavailability of carotenoids and folate, with special emphasis on the effect of the food matrix on carotenoid bioavailability and to obtain insight in the antioxidant or prooxidant activity of various spinach products in man and in spinach products.

In Chapter 2, an overview of the literature is given on the state of our knowledge with regard to factors affecting the bioavailability and bioconversion of carotenoids. As indicated by the mnemonic (SLAMENGHI) the bioavailability of carotenoids is influenced by extrinsic and intrinsic factors while dietary and nutrient interactions may also play a role. The effect of the food matrix and dietary fibre on the bioavailability of carotenoids and folate was examined in a controlled dietary intervention study described in Chapters 3 and 4, respectively. Whether the bioavailability or intake of carotenoids (β-carotene and lutein) has an effect on markers of enzymatic and non-enzymatic antioxidant activities in human blood is examined in Chapter 5. And in Chapter 6 the antioxidant activity of differently processed spinach products was tested in different models.

Spinach was selected as the experimental vegetable because in Europe, spinach is the most popular frozen vegetable. The mean intake of carotenoids in the Netherlands is estimated to be for α-carotene, β-carotene and lutein plus zeaxanthin, 0.7, 3.0, and 2.5 mg/d, respectively. The most important foods contributing to the intake of β-carotene were carrots, while spinach, endive and kale contributed to the intake of β-carotene and lutein plus zeaxanthin (Goldbohm et al. 1998). The results are discussed and the conclusions are presented in Chapter 7.

## REFERENCES


Ahmed S, Leo MA, Lieber CS. Interaction between alcohol and beta-carotene in patients with alcoholic liver disease. Am J Clin Nutr 1994;60:430-6.

Albanes D, Heinonen OP, Taylor PR, Virtamo J, Edwards BK, Rautalahti M, Hartman AM, Palmgren J, Freedman LS, Haapakoski J, Barrett MJ, Pietinen P, Malila N, Tala E, Liippo K, Salomaa ER, Tangrea JA, Teppo L, Askin FB, Taskinen E, Erozan Y, Greenwald P, Huttunen JK. Alpha-Tocopherol and beta-carotene supplements and lung cancer incidence in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study: effects of base-line characteristics and study compliance. J Natl Cancer Inst 1996;88:1560-70.

Albanes D, Virtamo J, Taylor PR, Rautalahti M, Pietinen P, Heinonen OP. 1997. Effects of supplemental ⍺-carotene, cigarette smoking, and alcohol consumption on serum carotenoids in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. Am J Clin Nutr 1997;66:366-72.

Ames BN. Micronutrients prevent cancer and delay aging. Toxicol Lett 1998;102-103:5-18.

Annapurna V, Shah N, Bhaskaram P, Bamji MS, Reddy V. Bioavailability of Spirulina carotenes in preschool children. J Clin Biochem Nutr 1991;10:145-52.

Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group (ATBC). The effect of vitamin E and beta-carotene on the incidence of lung cancer and other cancers in male smokers. N Engl J Med 1994;330:1029-35.

Bailey LB, Barton LE, Hillier SE, Cerda JJ. Bioavailability of mono and polyglutamyl folate in human subjects. Nutr Rep Int 1988;38:509-18.

Bast A, Haenen GRMM, van den Berg R, van den Berg H. Antioxidant effects of carotenoids. Internat J Vit Nutr Res 1998;68;399-403.

Bates C. Vitamins workshop: report. In: Southgate DAT, Johnson IT, Fenwick GR (Eds). Nutrient availability: chemical and biological aspects. Cambridge, UK: The Royal Society of Chemistry, 1989:390-1.

Bender AE. Nutritional significance of bioavailability. In: Southgate DAT, Johnson IT, Fenwick GR (Eds). Nutrient availability: chemical and biological aspects. Cambridge, UK: The Royal Society of Chemistry, 1989:3-9.

Benito P, Miller D. Iron absorption and bioavailability: An updated review. Nutr Res 1998;18:581-603.

Berg H van den, van Vliet T. Effect of simultaneous, single oral doses of β-carotene with lutein or lycopene on the β-carotene and retinyl ester responses in the triacylglycerol-rich lipoprotein fraction of men. Am J Clin Nutr 1998;68:82-9.

Blot WJ, Li JY, Taylor PR, Guo W, Dawsey S, Wang GQ, Yang CS, Zheng SF, Gail M, Li GY, Liu BQ, Tangrea J, Sun YH, Fusheng L, Fraumeni Jr, Zhang YH, Bing L. Nutrition intervention trials in Linxian, China: Supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. J Natl Cancer Inst 1993;85:1483-92.

Bohm F, Edge R, McGarvey DJ, Truscott TG. Beta-carotene with vitamins E and C offers synergistic cell protection against NOx. FEBS Lett 1998;436:387-9.

Boosalis MG, Snowdon DA, Tully CL, Gross MD. Acute phase response and plasma carotenoid concentrations in older women: findings from the Nun Study. Nutrition 1996;12:475-8.

Bowen HT, Omaye ST. Oxidative changes associated with β-carotene and α-tocopherol enrichment of human low-density lipoproteins. J Am Coll Nutr 1998;17:171-9.

Britton G. Structure and properties of carotenoids in relation to function. FASEB J 1995a;9:1551-8.

Britton G, Liaaen-Jensen S, Pfander H. Carotenoids today and challenges for the future. In: Britton G, Liaaen-Jensen S, Pfander H (Eds). Carotenoids. Volume 1A: isolation and analysis. Chapter 2, pp. 13-26. Basel: Birkhäuser Verlag, 1995b. [ISBN 3-7643-2908-4/3-7643-2936-X].

Brouwer IA, van Dusseldorp M, West CE, Steegers-Theunissen RPM. Bioavailability of folate and folic acid: Review of the literature. In: Brouwer IA. Folic acid, folate and homocysteine: Human intervention studies. PhD thesis. Wageningen, The Netherlands: Wageningen Agricultural University, 1999.

Brown ED, Micozzi MS, Craft NE, Bieri JG, Beecher G, Edwards BK, Rose A, Taylor PR, Smith JC. Plasma carotenoids in normal men after a single ingestion of vegetables or purified β-carotene. Am J Clin Nutr 1989;49:1258-65.

Burton GW, Ingold KU. β-Carotene: an unusual type of lipid antioxidant. Science 1984;224:569-73.

Castenmiller JJM, West CE. Bioavailability and bioconversion of carotenoids. Annu Rev Nutr 1998;18:19-38.

Chen J, Geissler C, Parpia B, Li J, Campbell TC. Antioxidant status and cancer mortality in China. Int J Epidemiol 1992;21:625-35.

Cipriani F, Buiatti E, Palli D. Gastric cancer in Italy. Italian J Gastroenterology 1991;23:429-35.

Cooper DA, Eldridge AL, Peters JC. Dietary carotenoids and certain cancers, heart disease, and age-related macular degeneration: A review of recent research. Nutr Rev 1999;57:201-14.

Cornwell DG, Kruger FA, Robinson HB. Studies on the absorption of beta-carotene and the distribution of total carotenoid in human serum lipoproteins after oral administration. J Lipid Res 1962;3:65-70.

Dartigues JF, Dabis F, Gros N, Moise A, Bois G, Salamon R, Dilhuydy JM, Courty G. Dietary vitamin A, beta carotene and risk of epidermoid lung cancer in south-western France. Eur J Epidemiol 1990;6:261-5.

Decker EA. Strategies for manipulating the prooxidative/antioxidative balance of foods to maximize oxidative stability. Trends Food Sci Technol 1998;9:241-8.

Dimitrov NV, Meyer C, Ullrey DE, Chenoweth W, Michelakis A, Malone W, Boone C, Fink G. Bioavailability of β-carotene in humans. Am J Clin Nutr 1988;48:298-304.

Diplock AT. Antioxidants and disease prevention. Molec Aspects Med 1994;15:293-376.

Diplock AT. Safety of antioxidant vitamins and beta-carotene. Am J Clin Nutr 1995;62(suppl.):1510S-16S.

Dueker SR, Jones AD, Smith GM, Clifford AJ. Stable isotope methods for the study of β-carotene-$d_8$ metabolism in humans utilizing tandem mass spectrometry and high performance liquid chromatography. Anal Chem 1994;66:4177-85.

Duthie G. Vitamin E (tocopherols). In: Garrow JS, James WPT (Eds). Human nutrition and dietetics, 9th ed. Edinburgh: Longman Group UK Limited (Churchil Livingstone), 1993.

Forman MR, Beecher GR, Lanza E, Reichman ME, Graubard BI, Campbell WS, Marr T, Yong LC, Judd JT, Taylor PR. Effect of alcohol consumption on plasma carotenoid concentrations in premenopausal women: a controlled dietary study. Am J Clin Nutr 1995;62:131-5.

Gaziano JM, Johnson EJ, Russell RM, Manson JE, Stampfer MJ, Ridker PM, Frei B, Hennekens CH, Krinsky NI. Discrimination in absorption or transport of β-carotene isomers after oral supplementation with either all-*trans*- or 9-*cis*-β-carotene. Am J Clin Nutr 1995;61:1248-52.

Giovannucci E, Ascherio A, Rimm EB, Stampfer MJ, Colditz GA, Willett WC. Intake of carotenoids and retinol in relation to risk of prostate cancer. J Natl Cancer Inst 1995;87:1767-76.

Goodman DS, Blomstrand R, Werner B, Huang HS, Shiratori T. The intestinal absorption and metabolism of vitamin A and β-carotene in man. J Clin Invest 1966;45:1615-23.

Goldbohm RA, Brants HAM, Hulshof KFAM, van den Brandt PA. The contribution of various foods to intake of vitamin A and carotenoids in the Netherlands. Internat J Vit Nutr Res 1998;68:378-83.

Graham IM, Daly LE, Refsum HM, et al. Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. J Am Med Assoc 1997;277:1775-81.

Greenberg ER, Baron JA, Karagas MR, Stukel TA, Nierenberg DW, Stevens MM, Mandel JS, Haile RW. Mortality associated with low plasma concentration of beta carotene and the effect of oral supplementation. J Am Med Assoc 1996;275:699-703.

Gregory JF 3d, Bhandari SD, Bailey LB, Toth JP, Baumgartner TG, Cerda JJ. Relative bioavailability of deuterium-labeled monoglutamyl tetrahydrofolates and folic acid in human subjects. Am J Clin Nutr 1992;55:1147-53.

Gregory JF 3d, Bhandari SD, Bailey LB, Toth JP, Baumgartner TG, Cerda JJ. Relative bioavailability of deuterium-labeled monoglutamyl and hexaglutamyl folates in human subjects. Am J Clin Nutr 1991;53:736-40.

Haenen GR, Bast A. Protection against lipid peroxidation by a microsomal glutathione-dependent labile factor. FEBS Lett 1983;159:24-8.

Haila K. Effects of carotenoids and carotenoid-tocopherol interaction on lipid oxidation *in vitro*. 1) Scavenging of free radicals. 2) Formation and decomposition of hydroperoxides. PhD thesis, EKT-series 1165. Helsinki, Finland: University of Helsinki, Department of Applied Chemistry and Microbiology, 1999.

Hennekens CH, Buring J, Manson JE, Stampfer M, Rosner B, Cook NR, Belanger C, La Motte F, Gaziano JM, Ridber PM, Willett W, Peto R. Lack of effect of long-term supplementation with β-carotene on the incidence of malignant neoplasms and cardiovascular disease. New Engl J Med 1996;334:1145-9.

Herbert V, Das KC. Folic acid and vitamin $B_{12}$. In: Shils ME, Olson JA, Shike M (Eds). Modern nutrition in health and disease, 8th ed., Chapter 25. Philadelphia, USA: Lea & Febiger, 1994:402-25.

Hirayama O, Nakamura K, Hamada S, Kobayashi Y. Singlet oxygen quenching ability of naturally occurring carotenoids. Lipids 1994;29:149-50.

Hurrell RF. Bioavailability of iron. Eur J Clin Nutr 1997;51(suppl.1):S4-S8.

Irribarren C, Folsom AR, Jacobs DR Jr, Gross MD, Belcher JD, Eckfeldt JH, Kritchevsky SB. Patterns of covariation of serum β-carotene and α-tocopherol in middle-aged adults: The Atherosclerosis Risk in Communities (ARIC) Study. Nutr Metab Cardiovasc Dis 1997;7:445-58.

Jackson MJ. The assessment of bioavailability of micronutrients: introduction. Eur J Clin Nutr 1997;51(suppl.1):S1-S2.

Jacques PF, Sulsky SI, Sadowski JA, Philips JC, Rush D, Willett WC. Comparison of micronutrient intake measured by a dietary questionnaire and biochemical indicators of micronutrient status. Am J Clin Nutr 1993;57:182-9.

Jensen SD, Spiller GA, Pattison, Whittam JH, Scala J. Acute effects of dietary carotenes on serum alpha and beta carotene in humans. Nutr Rep Int 1986;33:117-22.

Johnson EJ, Russell RM. Distribution of orally administered β-carotene among lipoproteins in healthy men. Am J Clin Nutr 1992;56:128-35.

Kang S, Wong PW, Malinow MR. Hyperhomocysteinemia as a risk factor for occlusive vascular disease. Annu Rev Nutr 1992;12:279-98.

Kaplan LA, Lau JM, Stein EA. Carotenoid composition, concentrations and relationships in various human organs. Clin Physiol Biochem 1990;8:1-10.

Kardinaal AFM, Kok FJ, Ringstad J, Gomez-Aracena J, Mazaev VP, Kohlmeier L, Martin BC, Aro A, Kark JD, Delgado-Rodriguez M, Riemersma RA, van 't Veer P, Huttunen JK, Martin-Moreno JM. Antioxidants in adipose tissue and risk of myocardial infarction: the EURAMIC study. Lancet 1993;342:1379-84.

Klerk M, Jansen MCJF, van 't Veer P, Kok FJ. Fruits and vegetables in chronic disease prevention. Wageningen, the Netherlands: Division of Human Nutrition & Epidemiology, Wageningen Agricultural University, 1998.

Knekt P, Aromaa A, Maatela J, Aaran RK, Nikkari T, Hakama M, Hakulinen T, Peto R, Teppo L. Serum vitamin A and subsequent risk of cancer: Cancer incidence follow-up of the Finnish Mobile Clinic

Health Examination Survey. Am J Epidemiol 1990;132:857-70.

Kostic D, White WS, Olson JA. Intestinal absorption, serum clearance, and interactions between lutein and β-carotene when administered to human adults in separate or combined oral doses. Am J Clin Nutr 1995;62:604-10.

Krinsky NI, Mathews-Roth MM, Welankiwar S, Sehgal PK, Lausen NC, Russett M. The metabolism of [14C]beta-carotene and the presence of other carotenoids in rats and monkeys. J Nutr 1990;120:81-7.

Krinsky NI. The biological properties of carotenoids. Pure Appl Chem 1994;66:1003-10.

Krinsky NI, Cornwell, DG, Oncley JL. The transport of vitamin A and carotenoids in human plasma. Ach Biochem Biophys 1958;73:233-46.

Krinsky NI. Actions of carotenoids in biological systems. Annu Rev Nutr 1993;13:561-87.

Kritchevsky SB. β-Carotene, carotenoids and the prevention of coronary heart disease. J Nutr 1999;129:5-8.

Kushi LH, Lenart EB, Willett WC. Health implications of Mediterranean diets in light of contemporary knowledge. 1. Plant foods and dairy products. Am J Clin Nutr 1995;61(suppl.):1407S-15S.

Lala VR, Reddy V. Absorption of β-carotene from green leafy vegetables in undernourished children. Am J Clin Nutr 1970;23:110-3.

Law MR, Morris JK. By how much does fruit and vegetable consumption reduce the risk of ischaemic heart disease? Eur J Clin Nutr 1998;52:549-56.

Leo MA, Lieber CS. Alcohol, vitamin A, and β-carotene: adverse interactions, including hepatotoxicity and carcinogenicity. Am J Clin Nutr 1999;69:1071-85.

Leo MA, Aleynik S, Aleynik M, Lieber CS. β-carotene beadlets potentiate hepatotoxicity of alcohol. Am J Clin Nutr 1997;66:1461-9.

Lieshout M van, West CE, Lugtenburg J, van Breemen RB, Muhilal. Quantification of bioavailability and bioconversion of β-carotene using [$^{13}C_{10}$]-β-carotene and [$^{13}C_{10}$]-retinyl palmitate. XIX IVACG Meeting Report. Washington, DCV: IVACG, 1999:87 (Abstr).

Lowe GM, Bilton RF, Davies IG, Ford TC, Billington D, Young AJ. Carotenoid composition and antioxidant potential in subfractions of human low-density lipoprotein. Ann Clin Biochem 1999;36:323-32.

Macrae R, Robinson RK, Sadler MJ. Encyclopedia of food science, food technology and nutrition. London: Academic Press, 1993.

Madhavi DL, Despande SS, Salunkhe DK (eds.). Food antioxidants: technological, toxicological, and health perspectives. New York: Marcel Dekker, Inc., 1996.

Masaki K, Doi T, Yuge K, Yoshioka Y, Nagata Y. Plasma β-carotene response to intake of β-carotene drink in normal men. Ann NY Acad Sci 1993;691:290-2.

Mason JB, Shoda R, Haskell M, Selhub J, Rosenberg IH. Carrier affinity as a mechanism for the pH-dependence of folate transport in the small intestine. Biochim Biophys Acta 1990;1024:331-5.

Mathews-Roth M, Gulbrandsen CL. Transport of beta-carotene in serum of individuals with carotenemia. Clin Chem 1974;20:1578-9.

Micozzi MS, Brown ED, Edwards BK, Bieri JG, Taylor PR, Khachik F, Beecher GR, Smith JC Jr. Plasma carotenoid response to chronic intake of selected foods and β-carotene supplements in men. Am J Clin Nutr 1992;55:1120-5.

Novotny JA, Dueker SR, Zech LA, Clifford AJ. Compartmental analysis of the dynamics of β-carotene metabolism in an adult volunteer. J Lipid Res 1995;36:1825-38.

Nussbaum JJ, Pruett RC, Delori FC. Historic perspectives. Macular yellow pigment. The first 200 years. Retina 1981;1:296-310.

Nygård O, Vollset SE, Refsum H, Stensvold I, Tverdal A, Nordrehaug JE, Ueland PM, Kvale G. Total

plasma homocysteine and cardiovascular risk profile. The Hordaland Homocysteine Study. J Am Med Assoc 1995;274:1526-33.

Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S, Hammar S. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. N Engl J Med 1996;334:1150-5.

Palozza P, Krinsky NI. *β-Carotene and α-tocopherol are synergistic antioxidants.* Arch Biochem Biophys 1992;297:184-7.

Pancharuniti N, Lewis CA, Sauberlich HE, Perkins LL, Go RC, Alvarez JO, Macaluso M, Acton RT, Copeland RB, Cousins AL, et al. Plasma homocysteine, folate and vitamin B12 concentrations and risk for early-onset coronary artery disease. Am J Clin Nutr 1994;59:940-8 and Am J Clin Nutr 1996;63:609 (erratum).

Parker RS, Swanson JE, Marmor B, Goodman KJ, Spielman AB, Brenna JT, Viereck SM, Canfield WK. Study of β-carotene metabolism in humans using $^{13}C$-β-carotene and high precision isotope ratio mass spectrometry. Ann NY Acad Sci 1993; 691:86-95.

Pee S de, West CE. Dietary carotenoids and their role in combating vitamin A deficiency: a review of the literature. Eur J Clin Nutr 1996;50(suppl.3):S38-S53.

Pfeiffer CM, Rogers LM, Bailey LB, Gregory JF 3rd. Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual-label stable-isotope protocol. Am J Clin Nutr 1997;66:1388-97.

Poppel G van. Epidemiological evidence for β-carotene in prevention of cancer and cardiovascular disease. Eur J Clin Nutr 1996;50(suppl.3):S57-S61.

Poppel G van, Kardinaal A, Princen H, Kok FJ. Antioxidants and coronary heart disease. Ann Med 1994;26:429-34.

Poppel G van, Kok FJ, Hermus RJJ. Beta-carotene supplementation in smokers reduces the frequency of micronuclei in sputum. Br J Cancer 1992;66:1164-8.

Rapola JM, Virtamo J, Ripatti S, Huttunen JK, Albanes D, Taylor PR, Heinonen OP. Randomised trial of alpha-tocopherol and beta-carotene supplements on incidence of major coronary events in men with previous myocardial infarction. Lancet 1997;349:1715-20.

Riemersma RA, Wood D, Macintyre C, Elton R, Gay K, Oliver M. Risk of angina pectoris and plasma concentrations of vitamins A, C, and E and carotene. Lancet 1991;337:1-5.

Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin E consumption and the risk of coronary heart disease in men N Engl J Med 1993;328:1450-6.

Rimm EB, Willett WC, Hu FB, Sampson L, Colditz GA, Manson JE, Hennekens C, Stampfer MJ. Folate and vitamin B6 from diet and supplements in relation to risk of coronary heart disease among women. J Am Med Assoc 1998;279:359-64.

Roels OA, Trout M, Dujacquier R. Carotene balances on boys in Ruanda where vitamin A deficiency is prevalent. J Nutr 1958;65:115-27.

Russell RM. Physiological and clinical significance of carotenoids. Internat J Vit Nutr Res 1998;68:349-53.

Scott KJ, Thurnham DI, Hart DJ, Bingham SA, Day K. The correlation between the intake of lutein, lycopene and beta-carotene from vegetables and fruits, and blood plasma concentrations in a group of women aged 50-65 years in the UK. Br J Nutr 1996;75:409-18.

Seddon JM, Ajani UA, Sperduto RD, Hiller R, Blair N, Burton TC, Farber MD, Gragoudas ES, Haller J, Miller DT, et al. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. J Am Med Assoc 1994;272:1413-20 and J Am Med Assoc 1995;273:622.

Selhub J, Jacques PF, Wilson PWF, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. J Am Med Assoc 1993;270:2693-8.

*Shiau A, Mobarhan S, Stacewicz-Sapuntzakis M, Benya R, Liao Y, Ford C, Bowen P, Friedman H,* Frommel TO. Assessment of the intestinal retention of β-carotene in humans. J Am Coll Nutr

1994;13:369-75.

Sies H, Stahl W. Vitamins E and C, β-carotene, and other carotenoids as antioxidants. Am J Clin Nutr 1995;62:1315S-21S.

Smith AH, Waller KD. Serum beta-carotene in persons with cancer and their immediate families. Am J Epidemiol 1991;133:661-71.

Stahelin HB, Gey KF, Eichholzer M, Ludin E, Bernasconi F, Thurneysen J, Brubacher G. Plasma antioxidant vitamins and subsequent cancer mortality in the 12-year follow-up of the prospective Basel (Switzerland) study. Am J Epidemiol 1991;133:766-75.

Steegers-Theunissen RPM, Boers GHJ, Trijbels JMF, Eskens TKAB. Neural tube defects and derangement of homocysteine metabolism (letter). N Engl J Med 1991;324:199-200.

Tamura T, Stokstad EL. The availability of food folate in man. Br J Haematol 1973;25:513-32.

Tang G, Wang WD, Russell RM, Krinsky NI. Characterization of beta-apo-13-carotenone and beta-apo-14'-carotenal as enzymatic products of the excentric cleavage of beta-carotene. Biochem 1991;30:9829-34.

Thomas JA. Oxidative stress, oxidative defense, and dietary constituents. In: Shils ME, Olson JA, Shike M (Eds). Modern nutrition in health and disease, 8th ed., Chapter 33. Philadelphia, USA: Lea & Febiger, 1994:501-12.

Vliet T van, Schreurs WHP, van den Berg H. Intestinal β-carotene absorption and cleavage in man: response of β-carotene and retinyl esters in the triglyceride-rich lipoprotein fraction after a single oral dose of β-carotene. Am J Clin Nutr 1995;62:110-6.

Verhoef P, Hennekens CH, Malinow MR, Kok FJ, Willett WC, Stampfer MJ. Homocyst(e)ine metabolism and risk of myocardial infarction: Relationship with vitamin B6, vitamin B12 and folate. Am J Epidemiol 1996;143:845-60.

Weisburger JH. Nutritional approach to cancer prevention with emphasis on vitamins, antioxidants, and carotenoids. Am J Clin Nutr 1991;53:226S-37S.

West CE, Castenmiller JJM. Quantification of the "SLAMENGHI" factors for carotenoid bioavailability and bioconversion. Internat J Vit Nutr Res 1998;68:371-7.

Wolf G. The enzymatic cleavage of beta-carotene: still controversial. Nutr Rev 1995;53:134-7.

# 2

# Bioavailability and bioconversion of carotenoids

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## ABSTRACT

Factors that influence the bioavailability of carotenoids and their bioconversion to retinol are species of carotenoids, molecular linkage, amount of carotenoids consumed in a meal, matrix in which the carotenoid is incorporated, effectors of absorption and bioconversion, nutrient status of the host, genetic factors, host-related factors, and mathematical interactions. In this paper, current knowledge of these factors is examined. Although data are not sufficiently comparable to allow an extensive systematic comparison of results, a number of conclusions can be drawn from the information now available.

## INTRODUCTION

Carotenoids are important to man and other animals as precursors of vitamin A and retinoids. In addition, they act as antioxidants, immunoenhancers, inhibitors of mutagenesis and transformation, inhibitors of premalignant lesions, screening pigments in primate fovea, and nonphotochemical fluorescence quenchers. Increased dietary intake of carotenoids is associated with decreased risk of (*a*) macular degeneration and cataracts, (*b*) some cancers, and (*c*) some cardiovascular events (Krinsky 1993). Carotenoids also have a widespread function or use in coloration.

### Naming

Most carotenoids can be described by the general formula $C_{40}H_{56}O_n$ where *n* is 0-6. Hydrocarbons (*n* = 0) are termed carotenes and oxygenated carotenoids are called xanthophylls. According to the International Union of Pure and Applied Chemistry (IUPAC 1959) recommendation for semi-systematic names, the trivial names for carotenes, such as α-carotene and β-carotene, should be replaced by β,ε-carotene and β,β-carotene respectively. However, the trivial names are well-established and more convenient to use, so the trivial names rather than the IUPAC semi-systematic names are used in this paper.

### Bioavailability

Bioavailability is the fraction of an ingested nutrient that is available for utilisation in normal physiological functions or for storage (Jackson 1997). Published information on carotenoid bioavailability is based mainly on measurement of carotenoids in serum or plasma after ingestion. It should be noted that at steady state, plasma

carotenoids amount to approximately one percent of the total body content of carotenoids, whereas the highest concentration can be found in the liver (Schmitz et al. 1991).

There are various methods for determining bioavailability of carotenoids: the balance method (Lala & Reddy 1970, Roels et al. 1958, Shiau et al. 1994), measurement of recovery of radioactive β-carotene in lymph (Blomstrand & Werner 1967, Goodman et al. 1966), use of uniformly labelled β-carotene (Dueker et al. 1994, Novotny et al. 1995), or comparison of the response in serum or lipoprotein fractions of carotenoids with standard doses of carotenoids. Most research has concentrated until now on determining serum or plasma concentrations of provitamin A carotenoids, especially β-carotene. Comparatively little is known about the occurrence, function, and bioavailability of non-provitamin A carotenoids. The concentration in serum may not be the best indicator of carotenoid status (Fotouhi et al. 1996, Kardinaal et al. 1995) because these concentrations are a result of absorption and removal from serum for storage, bioconversion, or excretion. Furthermore, fasting serum carotenoid concentrations can be affected, sometimes quite rapidly, by a number of factors, such as smoking.

**Bioconversion**

Carotene bioconversion, strictly speaking, is the proportion of bioavailable carotene converted to retinol. Often however, the term covers both the bioavailability and bioconversion process. Provitamin A carotenoids are converted to retinol by the action of 15-15'-carotenoid dioxygenase. This process occurs primarily in the enterocytes although enzyme activity is found in other tissues, such as the liver. There is still controversy about whether the cleavage is central or eccentric. Per molecule of β-carotene, central cleavage would result in the formation of two molecules of retinal, which could be reduced to retinol and then esterified, whereas eccentric cleavage would produce one molecule of a β-apo-carotenal that could be converted not only to retinal but also to β-apo-carotenoic acids and subsequently to retinoic acid. Van Vliet and co-workers (1995) suggested that the ratio of the response of retinyl esters to β-carotene may be a good indicator of intestinal β-carotene conversion. Of absorbed β-carotene, 60-70% was converted, mainly into retinyl esters, but several details with respect to the cleavage reaction remain to be elucidated (van Vliet et al. 1996a).

There is no information about the conversion of carotenoids that escape bioconversion in intestinal enterocytes. In vivo carotenoid conversion always refers to bioavailability plus bioconversion of carotenoids. In the future, it would be worthwhile to obtain information about bioavailability, bioconversion, and the

composite of both.

## FACTORS AFFECTING THE CAROTENOID BIOAVAILABILITY AND BIOCONVERSION

De Pee & West (1996) reviewed the evidence that carotene-rich fruits and vegetables can overcome vitamin A deficiency and concluded that the positive effects that get reported often come from studies with poor experimental designs. They noted that a number of factors influence the bioavailability of carotenoids, which they grouped in the mnemonic SLAMENGHI (Castenmiller & West 1997, de Pee & West 1996): Species of carotenoids, molecular Linkage, Amount of carotenoids consumed in a meal, Matrix in which the carotenoid is incorporated, Effectors of absorption and bioconversion, Nutrient status of the host, Genetic factors, Host-related factors, and mathematical Interactions. The purpose of this paper is to discuss current knowledge of the factors affecting bioavailability and bioconversion of carotenoids. Prediction of carotenoid bioavailability from different foods and under specified circumstances would be possible if each of these factors could be quantified and if observed additive effects can be split into their SLAMENGHI components. This review is restricted mainly to studies of humans but will refer to animal studies where relevant.

## SPECIES OF CAROTENOIDS

### Bioavailability

The naturally occurring configuration of carotenoids in plant foods is usually the all-*trans* isomer. In general, *cis* isomers are more polar, less prone to crystallisation, and more soluble in oils and hydrocarbon solvents. The *cis* isomeric composition increases with food processing, particularly heating, and the quantity formed appears related to the severity and extent of heat treatment. Rushin and co-workers (1990) demonstrated that *cis* isomers (mainly 13-*cis*) had not formed after blood was drawn and that *cis* isomers of β-carotene were present at significant concentrations in the human circulation. The all-*trans* form is absorbed more readily in humans than the 9-*cis* form. When multiple doses of mixtures of the two were ingested, the proportion of the 9-*cis* form found in serum was less than one sixth of the amount found in the carotenoid mixture (Ben-Amotz & Levy 1996, Gaziano et al. 1995, Jensen et al. 1987, Tamai et al. 1995). Also, after a single dose, the increase

of all-*trans*-β-carotene in serum or in the chylomicron fraction of serum was much higher than for the 9-*cis* isomer (Stahl et al. 1993, Stahl et al. 1995), which indicates a strong preferential absorption or transport of the all-*trans* isomer in comparison with the 9-*cis* isomer. After a single oral dose, more than 95% of plasma $^{13}C$-labeled *trans*-β-carotene and labelled retinol was derived from labelled 9-*cis*-β-carotene. This indicates that a significant portion of the labelled 9-*cis*-β-carotene dose was isomerised to labelled *trans*-β-carotene before entering the blood stream (You et al. 1996). When lycopene uptake from processed and unprocessed tomato juice was studied in humans, the *cis* isomer was somewhat better absorbed than the all-*trans* form (Stahl & Sies 1992). This would be related to the better solubility of the *cis* form.

Larger amounts of β-carotene were incorporated into the micelles when the carotene mixtures contained 86% rather than 5% 9-*cis*-β-carotene, and an increase in the level of total carotene in the solution was accompanied by a constant or even enhanced carotene incorporation (Levin & Mokady 1995).

In all studies on isomeric forms (see Table 1), the effect of algae supplements (containing similar amounts of 9-*cis*- and all-*trans*-β-carotene) was smaller than the response to similar concentrations of synthetic β-carotene (mainly all-*trans*). Based on studies with multiple doses, a daily intake of *cis*-β-carotene from algae or synthetic β-carotene would results in a 0.018- and 0.003- μmol β-carotene increase per mg, respectively, in serum. For all-*trans*-β-carotene, these values are 0.027 μmol for algae and 0.032 μmol for synthetic β-carotene.

### Bioconversion

All carotenoids containing at least one unsubstituted β-ionone ring and a polyene side-chain attached containing at least 11 carbon atoms are potential precursors of vitamin A, with β-carotene showing the highest vitamin A activity on a molar basis. Data on the relative bioconversion of various carotenoids to retinol is presented in Table 2. These data are based on the storage of retinol in the liver when its carotenoids are fed and therefore comprise not only bioconversion but also bioavailability. The FAO/WHO Expert Consultation on vitamin A requirements has assumed that the vitamin A activity of other provitamin A carotenoids, including *cis* isomers of β-carotene, is 50% that of β-carotene (FAO/WHO Joint Expert Consultation 1988). Studies using intestinal preparations have generally supported these data: α-carotene, 29% (van Vliet et al. 1996c); β-cryptoxanthin, 55% (van Vliet et al. 1996c); and *cis*-β-carotene, 7% (Nagao & Olson 1994). Intestinal perfusion studies with ferrets have shown that absorption, clearance and metabolism of all-

## Table 1. SLAMENGHI factor S (Species): *cis-trans* isomers

| Study | Details[a] | SLAMENGHI factor: Bioavailability[b] | SLAMENGHI factor: Bioconversion[c] |
|---|---|---|---|
| Ben-Amotz & Levy (1996) | 2 x 15 M, 20-30 y; 40 mg β-carotene daily for 14 days from alga *Dunaliella bardawil* (42% all-*trans*, 43% 9-*cis*) or synthetic β-carotene (97% all-*trans*), or placebo | All-*trans*-β-carotene: 0.037, 0.522[d]; *cis*-β-carotene: 0.006, 0.016[d] | 0.037, 1.60[d] |
| Gaziano et al. (1995) | 7 M and 17 F; 100 mg β-carotene daily for 6 days from *Dunaliella salina* (50% all-*trans*) or synthetic all-*trans*-β-carotene | All-*trans*-β-carotene: 0.808, 1.523[d]; *cis*-β-carotene: 0.059, 0.095[d] | |
| Jensen et al. (1987) | 6 M and 10 F, 18-60 y; 24 mg β-carotene daily for 7 days from *Dunaliella salina* (all-*trans*:9-*cis* = 40:60) or carrots (98% all-*trans*) or placebo | All-*trans*-β-carotene: 0.687, 0.492[e]; *cis*-β-carotene: 0.047, 0.017[e] | |
| Stahl et al. (1993) | 3 M and 2 F, 23-37 y; single dose of 5.6 µmol β-carotene/kg body weight and 0.17 µmol/kg body weight of α-carotene from *Dunaliella salina* (54% all-*trans*, 37% 9-*cis*, 9% other *cis* isomers, 1% α-carotene) | Serum increase of α-carotene was 5.6% of increase of all-*trans*-β-carotene, reflecting ration in Betatene; no 9-*cis* was found in serum | |
| Stahl & Sies (1992) | 5 M and 1 F, 22-36 y; single dose of 2.5 µmol lycopene per kg body weight as heated tomato juice (20-30% *cis* isomers) | *cis*-Lycopene better absorbed than all-*trans* form | |
| Tamai et al. (1995) | 30 M, 20-25 y; 60 mg β-carotene daily for 44 weeks from *Dunaliella bardawil* (all-*trans*:9-*cis* = 50:50) or synthetic all-*trans*-β-carotene or placebo | All-*trans*-β-carotene: 0.559, 1.21[f]; *cis*-β-carotene: 0.084, 0.149[f] | No change |
| Tang et al. (1996) | 7 M and 5 F, 23-68 y; single dose of 120 mg β-carotene | *trans*-β-Carotene: 0.780; *cis*-β-carotene: 0.020 | |
| You et al. (1996) | 2 M and 1 F, 23-38 y; single dose of 1 mg 99.4% 9-*cis*-β-carotene | Labelling all-*trans*: >95% of plasma *trans*-β-carotene and retinol was derived from 9-*cis*-β-carotene | No 9-*cis* retinol detected |

[a] M, male; F, female; y, years.
[b] Unless otherwise stated, increase in serum or plasma carotenoid concentration shown as micromoles per liter.
[c] Unless otherwise stated, increase in serum or plasma retinol concentration shown in micromoles per liter.
[d] Results from alga and synthetic β-carotene, respectively.
[e] Results from alga and carrot, respectively.
[f] Results estimated from alga and synthetic β-carotene, respectively.

**Table 2. SLAMENGHI factor S(pecies): relative bioconversion of carotenoids[a]**

| Carotenoid | Relative bioconversion (% on a weight basis) |
|---|---|
| β-Carotene | 100[b] |
| 9-*cis*-β-Carotene | 38[c] |
| 13-*cis*-β-Carotene | 53[c] |
| α-Carotene | 50-54[b]; 29[d] |
| γ-Carotene | 42-50[b] |
| β-Zeacarotene | 20-40[b] |
| β-Cryptoxanthin | 50-60[b]; 55[d] |
| 3,4-Dehydro-β-carotene | 75[b] |
| 2,2'-Dimethyl-β-carotene | 50[b] |
| β-Carotene-5',6'-monoepoxide | 21[b] |
| α-Carotene-5,6-monoepoxide | 25[b] |
| 4-Keto-β-carotene | 44-50[b] |
| 3-Keto-β-carotene | 52[b] |
| 4-Hydroxy-β-carotene | 48[b] |
| β-apo-8'-Carotenal | 72[b] |
| β-apo-12'-Carotenal | 120[b] |
| Non-provitamin A carotenoids[e] | 0[b] |

[a] Unless otherwise stated, the carotenoids are in *trans* form.
[b] Bauernfeind (1972).
[c] Zechmeister (1949).
[d] van Vliet et al. (1996c).
[e] Non-provitamin A carotenoids such as lutein, lycopene, zeaxanthin, and canthaxanthin do not have at least one unsubstituted β-ionone ring and a polyene side-chain attached containing at least 11 carbon atoms.

*trans*- and 9-*cis*-β-carotene produced similar amounts of retinoic acid, with 9-*cis*-β-carotene giving rise to equal amounts of all-*trans*- and 9-*cis*-retinoic acid (Hébuterne et al. 1995).

## MOLECULAR LINKAGE

There are few data on the effect of carotenoid esters, which are common in fruits and vegetables, on bioavailability. Herbst et al. (1997) found that lutein diesters showed a trend toward greater bioavailability than free lutein suggesting that the human gut is very efficient in cleaving esters of lutein, and therefore, esterified lutein in food may be equally or better bioavailable than free lutein. No β-cryptoxanthin or esters from other carotenoids were detected in chylomicrons or serum after consumption of tangerine juice (Wingerath et al. 1995). Since esters are not absorbed by the enterocyte, we would not expect molecular linkage to have an effect on bioconversion.

## AMOUNT OF CAROTENOIDS CONSUMED IN A MEAL

### Bioavailability of β-carotene

Serum β-carotene concentrations vary depending on the amount of β-carotene in a meal. Shiau et al. (1994) reported that with a meal, only 35-71% of β-carotene was recovered in rectal effluent as compared with 83% without a meal. The kinetics of serum response to orally ingested β-carotene seems independent of dose (Canfield et al. 1997, Henderson et al. 1989), but other factors play a role because the responses observed in different studies vary considerably.

A large number of studies have measured serum or plasma response to supplementation with carotenoids, mainly β-carotene. Other studies have examined the effect of supplementing a meal with a mixture of carotenoids has on such responses. Sometimes these studies have used large, pharmacological doses of β-carotene, and often they have not been well controlled. The between-individuals response to repeated doses of β-carotene, which lead to plateau concentrations (Costantino et al. 1988), appears to be generally less variable than the response to single doses. An overview of studies is given in the Annex (and posted on a website: Supplementary Materials section at http://www.AnnualReviews.org). Prince & Frisoli (1993) found that administering β-carotene daily in three divided doses with meals increased the serum β-carotene concentration three times more than when the same total dose was administered once daily. The studies listed in the Annex are not strictly comparable, often because the matrix in which the carotene was supplied is different (see below), and, for example, the β-carotene preparations used were different. Some supplements contained carotenoids in water-soluble beadlets whereas others contained crystalline carotenoids dissolved or

resuspended in oil. Calves receiving the commercial beadlet sources had higher serum carotenoid levels than calves receiving carotenoids in oil. When fed water-soluble β-carotene beadlets, calves had peak β-carotene levels 28 times higher than when fed crystalline β-carotene (Bierer et al. 1995).

Data from single-dose studies indicate that in normal subjects the efficiency of carotenoid absorption varies widely. Serum β-carotene concentrations rise quickly after a single oral dose and usually peak at 5 h and remain elevated for more than 24 h. Omission of a meal delays the time to reach peak concentrations. A delayed and less-efficient absorption was reported with higher doses (Nierenberg et al. 1991). Some subjects do not show a plasma response after supplementation (Gärtner et al. 1997, Johnson & Russell 1992). After a meal, there may be large intra-individual variability in triglyceride response. Thus, it may be advisable to adjust carotenoid values in serum for triglyceride response after a single dose of β-carotene (van Vliet et al. 1995). Most studies on carotenoid bioavailability do not report triglyceride responses.

To study the linear and dose-response relationship between β-carotene supplementation and increases in serum β-carotene concentrations, a meta-analysis was carried out that included all studies with a daily β-carotene supplement of <50 mg that lasted <1 year. The analysis included 31 studies, and the heterogeneity between the study results was apparent. The 95% between-study interval in serum response per mg of supplemented β-carotene ranged from 0.0317 µmol to 0.0388 µmol/liter. The duration of β-carotene supplementation was a significant predictor of β-carotene response.

**Bioavailability of other carotenoids**

Carotenoids may interact with each other during intestinal absorption, metabolism, and serum clearance. Data suggest that the concentration of α-carotene in plasma (Carughi & Hooper 1994) and chylomicrons (Gärtner et al. 1996) and the concentration of lycopene in plasma (Carughi & Hooper 1994) reflect the content of these carotenoids in the meal or supplement.

Some studies showed no effect of β-carotene on serum levels of α-carotene, cryptoxanthin, lycopene, and lutein (Fotouhi et al. 1996, Henderson et al. 1989), whereas others found significantly different carotenoid concentrations after supplementation with β-carotene. The plasma lutein concentration was significantly reduced after multiple (Micozzi et al. 1992) and single doses (Kostic et al. 1995) of β-carotene. In the presence of high amounts of β-carotene, the uptake from the intestinal lumen into chylomicrons of lutein and zeaxanthin as compared to all-*trans*-

β-carotene was preferred (Gärtner et al. 1996). β-Carotene supplementation reduced the lycopene concentrations in low-density lipoproteins (Gaziano et al. 1995) and serum significantly (Prince & Frisoli 1993). In contrast, Wahlqvist et al. (1994) found an increase in lycopene concentration after β-carotene supplementation, but only in men. α-Carotene concentrations were increased after β-carotene supplementation (de Pee et al. 1995, Micozzi et al. 1992, Wahlqvist et al. 1994). The ingestion of concurrent doses of β-carotene and canthaxanthin reduced the peak serum canthaxanthin concentration, but canthaxanthin did not inhibit the appearance of β-carotene in serum (White et al. 1994).

In summary, β-carotene supplementation increases concentrations of α-carotene in a dose-dependent but non-linear way and would appear to decrease serum concentrations of lutein, lycopene, and canthaxanthin. Related to dietary intake in milligrams per day, the increases in serum carotenoid concentration for other carotenoids are 0.06-0.15 μmol of lutein/liter, 0-0.09 μmol of lycopene/liter, and 0.11-0.27 μmol of α-carotene/liter.

**Bioconversion**

In 1967, the FAO/WHO (1967) concluded that when consumed in a mixed meal, 6 μg of β-carotene - or 12 μg of other provitamin A carotenoids - is absorbed and converted to 1 μg of retinol. However, the FAO/WHO Joint Expert Consultation recognised in 1988 that retinol equivalence is related to dose level. When the β-carotene intake per meal is $<1000$ or $>4000$ μg, the amount of β-carotene equivalent to 1 μg of retinol was considered to be 4 and 10 μg, respectively (FAO/WHO Joint Expert Consultation 1988).

In rats, low intake of vitamin A increases cleavage activity as measured in vitro (van Vliet et al. 1996b, Villard & Bates 1986), and a high intake of β-carotene increases cleavage activity in the liver of rats (van Vliet et al. 1996b). Based on the difference in liver vitamin A contents between β-carotene-supplemented and unsupplemented rats, β-carotene conversion factors were estimated at 9:1 for rats fed high amounts of vitamin A and 4:1 for rats fed normal and low amounts of vitamin A (van Vliet et al. 1996b). Intestinal β-carotene cleavage activity was higher in vitamin A-deficient rats than in rats with a high intake of vitamin A or β-carotene. The addition of lutein to an incubation of β-carotene reduced retinal formation, whereas lycopene had no effect (van Vliet et al. 1996c).

## MATRIX IN WHICH THE CAROTENOID IS INCORPORATED

### Bioavailability

β-Carotene dissolved in oil is absorbed far more readily than β-carotene from foods. It is possible to calculate relative bioavailability by comparing the effect a food has on serum β-carotene values with the effect pure β-carotene has. It should be noted that "pure" β-carotene can be water-soluble β-carotene beadlets or β-carotene in a suspension with oil. Bioavailability of β-carotene from these preparations may be different. Compared with the serum response from β-carotene supplements, the bioavailability of β-carotene from various foods is as follows: stir-fried vegetables, 7% (de Pee et al. 1995); carrots, 18-26% (Brown et al. 1989, Micozzi et al. 1992, Törrönen et al. 1996); and spinach, 7% (JJM Castenmiller, CE West et al. unpublished information). A subsequent study in Indonesia (de Pee et al. 1998) found the serum response of β-carotene from fruits to be four times that from vegetables. The low bioavailability of carotenoids from dark-green leafy vegetables may be attributed to their entrapment and complexing to proteins in chloroplasts and within cell structures. Such entrapment may not only be physical (matrix effect) but also molecular (effector effect, see below). The α- and β-carotene in carrots exist as crystals up to 1000 μm in length. Although soluble in the intestinal contents, the transit time is probably insufficient for extensive solubilisation to take place during this passage through the intestinal tract. Similarly, lycopene also exists in tomatoes in the crystalline form. In orange and yellow fruits (mango, papaya, etc) and in pumpkin and sweet potato, carotenoids are dissolved in oil droplets in chromoplasts and can be readily extracted during digestion. Cooking increases the bioavailability of carotenoids, possibly because of the softening or disruption of plant cell walls and the disruption of carotenoid-protein complexes (Erdman et al. 1988). Stahl & Sies (1992) reported that lycopene concentrations in human serum increased from processed but not unprocessed tomato juice. Bioavailability of lycopene was greater from tomato paste than from fresh tomatoes (Gärtner et al. 1997).

### Bioconversion

The FAO/WHO Joint Expert Consultation (1988) concluded that 6 μg of β-carotene from a mixed meal provided 1 μg of retinol. For β-carotene dissolved in oil, the International Union of Pure and Applied Chemistry concluded from two studies (Hume & Krebs 1949, Wagner 1940) that 3.33 μg provided 1 μg of retinol (IUPAC 1959). The effect of the matrix, however, is almost certainly limited to phenomena that take place in the lumen of the small intestine. Thus, reported effects of the

matrix on the overall yield of retinol from carotenoids can be attributed to matrix effects on bioavailability. The earlier studies on the yield of retinol from carotenoids have been reviewed by de Pee & West (1996). From a carefully carried study of schoolchildren in Indonesia, it has been calculated that 1 μg of retinol was provided by 26 μg (95% confidence interval, 13-76) of β-carotene from dark-green leafy vegetables and carrots and by 12 μg (95% confidence interval, 6-29) from yellow and orange fruits. Almost identical data have been found in a study of lactating women in Vietnam (NG Khan, CE West, S De Pee, D Bosch, HH Khôi, JGAJ Hautvast, unpublished data).

## EFFECTORS OF ABSORPTION AND BIOCONVERSION

Absorption of carotenoids is similar to that of other lipids. A variety of nutrients consumed together with carotenoids may affect carotenoid absorption, metabolism, and/or bioconversion. The presence of protein in the small intestine helps stabilise fat emulsions and enhances micelle formation and carotenoid uptake. Lecithin may enhance triglyceride absorption through facilitating micelle formation, and long-chain fatty acids increase cholesterol absorption and, thus, the absorption of fat-soluble vitamins. A decrease in carotenoid bioavailability may be caused by interaction in the gastrointestinal tract with drugs or constituents of foods, such as sulfides and acids, in the gastrointestinal tract (Peiser & Yang 1979, Wedzicha & Lamikanra 1983). Gastric pH also plays a role: a single 120-mg dose of β-carotene increased plasma concentrations of β-carotene at normal gastric pH to a level twice as high as that at a gastric pH of 6.4 (Tang et al. 1996).

### Intake of dietary fat

The absorption and bioconversion of β-carotene is markedly reduced when the intake of fat is very low (Jialal et al. 1991, Prince & Frisoli 1993). Addition of a small quantity of fat to the diet greatly improves the absorption of vegetable carotenoids (Roels et al. 1958), with optimal absorption requiring an intake of at least 5 g of fat per day (Jayarajan et al. 1980). We conclude that a minimum amount of fat is necessary for uptake of carotenoids. There is no dose-response relationship above the threshold value, but there is some increase in the serum β-carotene response to a high-fat diet (Dimitrov et al. 1988, Nierenberg et al. 1991, Shiau et al. 1994). Some data suggest that polyunsaturated fatty acid-rich dietary fat increases the serum response to β-carotene more than does mono-unsaturated fatty acid-rich dietary fat (Kasper & Ernst 1969). The serum response to dietary β-carotene is related to the

serum response to dietary triglyceride (Henderson et al. 1989). The solubility of β-carotene (apolar) and zeaxanthin (polar) decreases with increased chain length in triglyceride fatty acids (Borel et al. 1996), which may explain why, after a single 120-mg dose of β-carotene, the β-carotene response in chylomicrons was lower after ingestion of a meal comprised of triglycerides from $C_8$ and $C_{10}$ rather than $C_{16}$ and $C_{18}$ fatty acids. Also, the concentration of retinyl palmitate in chylomicrons was lower, although the conversion to retinol was not significantly different between the two meals (Borel et al. 1997). Sucrose polyester, a nonabsorbable fat analogue, reduces plasma carotenoid concentrations markedly (Weststrate & van het Hof 1995).

**Intake of dietary fibre**

In a study to examine the effect of dietary fibre on serum carotene values in humans, the plasma β-carotene was reduced by 42% when pectin was given (Rock & Swendseid 1992). Results from studies with chickens suggest that the extent of methyl esterification of the pectin is important in determining the extent of inhibition of absorption (Erdman et al. 1986). One of the effects of dietary fibre on lipid metabolism centres on its interaction with bile acids, resulting in increased faecal excretion of bile acids and, thus, decreased absorption of fats and fat-soluble substances, such as carotenoids and cholesterol.

**Intake of alcohol**

Several investigators (Albanes et al. 1997, Lecomte et al. 1994, Stryker et al. 1988) have reported that drinkers, compared with subjects with low or no alcohol intake, had lower β-carotene levels, whereas plasma retinol levels were similar (Lecomte et al. 1994) or higher (Albanes et al. 1997, Simonetti et al. 1995). The response was not related to the level of alcohol consumption (Albanes et al. 1997). Conversely, after withdrawal, plasma carotenoid levels increased, whereas retinol concentration diminished (Lecomte et al. 1994). In a 6-month controlled dietary study involving women who were non-smokers, plasma α- and β-carotene concentrations were significantly higher, 19% and 13%, respectively, and the lutein/zeaxanthin concentration was significantly lower during the alcohol-intake phase of the study (Forman et al. 1995). The combination of an increase in plasma and liver β-carotene after ingesting ethanol and a relative lack of a corresponding rise in retinol suggests that alcohol interferes with the conversion of β-carotene to vitamin A (Leo et al. 1992). When ethanol was used in intestinal rat and hamster preparations as a solvent for β-carotene, retinal formation was reduced to 55% (van Vliet et al. 1996c).

### Intake of other food constituents

Supplementation with vitamin E plus ascorbic acid did not alter serum β-carotene levels (Nierenberg et al. 1994).

## NUTRIENT STATUS OF THE HOST

### Bioavailability

The absorption of carotenoids is likely to be dependent on vitamin A status. Mattson & Deuel (1943) showed that high vitamin A intake reduces carotenoid pigmentation in chickens, an effect confirmed by others (Thompson 1975). Supplementation with vitamin A decreases the absorption of β-carotene but of canthaxanthin as well (Sklan et al. 1989). It has been common practice to feed chickens high levels of vitamin A to produce pale poultry meat. It could be argued, although it is unlikely, that part of this effect is due not to an increased vitamin A status but to the concurrent ingestion of retinol with other carotenoids.

### Bioconversion

Feeding β-carotene-rich foods to humans leads to an increase in serum retinol levels only when these are initially low (Charoenkiatkul et al. 1985, Lala & Reddy 1970). Treatment with β-carotene does not significantly modify the vitamin A levels in vitamin A-replete subjects. It is assumed that a plasma level of β-carotene equal or higher than 1.1 μmol/liter reflects a nutritional intake of provitamins sufficient to support homeostasis of retinol. Novotny et al. (1995) estimated from a study of one vitamin A-replete person that 22% of a single 40-mg dose of β-carotene was absorbed and that 18.5 μg of dietary β-carotene is equivalent to 1 μg of retinol.

Since ingestion of β-carotene does not lead to vitamin A toxicity, it is tempting to suggest that not only current β-carotene intake but also circulating β-carotene levels (carotene status) inhibits carotenoid bioconversion. At lower levels of β-carotene intake, retinol levels in serum and liver are increased by ingestion of β-carotene, whereas ingestion of canthaxanthin reduces retinol levels in serum and liver (Sklan et al. 1989). This could be interpreted as inhibition of bioconversion but could be readily explained by the lack of provitamin A precursor. No studies on the effect of vitamin A status on dioxygenase activity have been carried out with humans, though studies of rats (van Vliet et al. 1996b, Villard & Bates 1986) have shown that diets low in vitamin A do increase dioxygenase activity (Brubacher & Weiser 1985). Retinol deficiency can arise despite a plentiful supply of retinol or carotene. Low-

protein diets reduce dioxygenase activity in rats (Gronowska-Senger & Wolf 1970) or limit the production of retinol-binding protein. Zinc also affects the synthesis of retinol-binding protein and may also influence the conversion of β-carotene to vitamin A through retinal reductase, which is zinc dependent. Zinc-deficient rats had serum retinol levels not significantly different from controls. However, their retinol liver reserves were much lower. The results suggest that zinc deficiency impairs the efficiency of β-carotene utilisation in the rat (Takruri & Thurnham 1981).

## GENETIC FACTORS

Data from the Cardia study (Slatterly et al. 1995) showed that, in the United States, Caucasian men and women take supplements containing vitamins A and β-carotene twice as frequently as do African American men and women. They also have lower mean plasma lutein concentrations and higher mean plasma retinol concentrations than their African American counterparts have. When adjusted for influencing factors, the plasma β-carotene concentration was also higher in African Americans than in white haemodialysis patients in the United States (Rock et al. 1997). In Nigerian women (Adams-Campbell et al. 1992), β-carotene levels were 1.2-13 times higher than was found in the US population. However, many of these effects could be attributed to diet. Failure to split β-carotene in humans is rare but can lead (*a*) to metabolic carotenemia with normal or even low intakes of carotenoids (Monk 1982) or (*b*) to vitamin A deficiency if retinol intake is low (McLaren & Zekian 1971).

## HOST-RELATED FACTORS

Host-related factors may explain many of the differences observed in the serum response to ingestion of dietary carotenoids. One of the best predictors of the response to an oral dose of β-carotene is the initial serum β-carotene concentration (Albanes et al. 1992, Nierenberg et al. 1991). Such initial serum carotenoid levels could be attributed to factors already discussed, such as long-term intake (status) and genetic factors. In addition, postabsorption metabolism and not bioavailability may be responsible for the differences observed. This could explain many of the effects of body weight (Nierenberg et al. 1991), use of alcohol, and smoking. Thus, in the discussion below, studies that indicate differences in bioavailability and not just serum concentrations of carotenoids are highlighted.

**Effect of sex and age**

The serum response to β-carotene is higher in women than in men (Nierenberg et al. 1991); however, part of this effect could be attributed to differences in body weight and body composition. There is no evidence for a reduced serum response with ageing, which could be explained by the passive nature of the absorption process. In one study, the serum response was higher in older people (Maiani et al. 1989), whereas in other studies the opposite was found (Sugarman et al. 1991, Yeum et al. 1996).

**Effect of illness and protein-energy malnutrition**

Absorption of fat-soluble substances including carotenoids is impaired in any disease in which there is fat maldigestion and malabsorption. Fat maldigestion arises when there is impaired production of lipase and bile acids and impaired neutralisation of chyme in the duodenum (Das et al. 1996, Lala & Reddy 1970).

## MATHEMATICAL INTERACTIONS

Mathematical interactions refers to the difference in effect observed when two factors play a role together compared with the product of the effects observed separately. However, data are not yet available to allow any estimate of mathematical interactions.

## CONCLUSIONS

Carotenoid bioavailability and bioconversion are influenced by a number of factors, the SLAMENGHI factors. Each carotenoid seems to show an individual pattern of absorption, plasma transport, and metabolism. We investigated the state of knowledge with regard to SLAMANGHI factors.

We conclude that, at this stage, data are not sufficiently comparable to allow systematic comparison of results and often the effects studied with regard to bioavailability cannot be attributed to individual factors. However, a number of conclusions can be drawn.

The bioavailability and provitamin A activity of the various carotenoids and geometrical isomers of carotenoids differ. The absorption of all-*trans*-β-carotene is higher than that of 9-*cis*-β-carotene. Also, the bioconversion of β-carotene is higher for the all-*trans* isomer than for the *cis* isomers. The vitamin A activity of other

provitamin A carotenoids is lower than that of β-carotene. Carotenoid esters in foods are readily split in the gut, and thus, molecular linkage does not play a role in carotenoid bioavailability and bioconversion. A positive relationship between intake and response of β-carotene suggests that absorption is a nonsaturable passive process. β-Carotene supplementation was shown in a number of studies to increase serum concentrations of β-carotene and decrease serum concentrations of lutein, lycopene, and canthaxanthin. Recent studies of rats (van Vliet et al. 1996b) confirm the dose-related factors for the conversion of β-carotene from a mixed meal, as suggested by FAO/WHO (1988). In vitro studies would suggest that lutein interferes with the conversion of β-carotene to retinol (van Vliet et al. 1996c), and this may explain, in part, the low conversion to retinol of β-carotene from dark-green leafy vegetables (de Pee et al. 1995). Relatively few properly designed studies have been carried out to examine the effect of food matrix on carotenoid bioavailability (de Pee & West 1996). From these studies it can be concluded that bioavailability of β-carotene from foods is low, particularly from dark-green leafy vegetables (7%). Due to the low bioavailability, it has been calculated that 1 μg of retinol is provided by 26 μg of β-carotene from dark-green leafy vegetables and carrots and by 12 μg from yellow and orange fruits (de Pee et al. 1998). Intake of dietary fat has a positive effect on β-carotene bioavailability and dietary fibre has a negative effect, and alcohol intake seems to interfere with the bioconversion of β-carotene to retinol. Nutrient status and genetic factors related to the host may explain some of the differences observed. Effects of season, sex, age, and smoking are largely explained by differences in long- and short-term intakes of carotenoids. Few controlled supplementation studies have been carried out to study the effect of these variables on carotenoid response in serum.

There is a need for more carefully designed studies to define the role of individual SLAMENGHI factors. The improvement in high-performance liquid chromatography methods for the quantification of carotenoids and the current development of isotopic methods should lead to the generation of much data in the coming years.

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## REFERENCES


Adams-Campbell LL, Nwankwo MU, Ukoli FA, Omene JA, Kuller LH. Serum retinol, carotenoids, vitamin E, and cholesterol in Nigerian women. J Nutr Biochem 1992;3:58-61.

Albanes D, Virtamo J, Rautalahti M, Haukka J, Palmgren J, et al. β-Carotene before and after β-carotene supplementation. Eur J Clin Nutr 1992;46:15-24.

Albanes D, Virtamo J, Taylor PR, Rautalahti M, Pietinen P, Heinonen OP. Effects of supplemental β-carotene, cigarette smoking, and alcohol consumption on serum carotenoids in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. Am J Clin Nutr 1997;66:366-72.

Bauernfeind JC. Carotenoid vitamin A precursors and analogs in foods and feeds. J Agric Food Chem 1972;20:456-73.

Ben-Amotz A, Levy Y. Bioavailability of a natural isomer mixture compared with synthetic all-*trans* β-carotene in human serum. Am J Clin Nutr 1996;63:729-34.

Bierer TL, Merchen NR, Erdman JW Jr. Comparative absorption and transport of five common carotenoids in preruminant calves. J Nutr 1995;125:1569-77.

Blomstrand R, Werner B. Studies on the intestinal absorption of radioactive β-carotene and vitamin A in man. Scand J Clin Lab Invest 1967;19:339-45.

Borel P, Grolier P, Armand M, Partier A, Lafont H, et al. Carotenoids in biological emulsions: solubility, surface-to-core distribution, and release from lipid droplets. J Lipid Res 1996;37:250-61.

Borel P, Grolier P, Mekki N, Simonet L, Rochette C, et al. Medium-chain triglycerides reduce β-carotene bioavailability in healthy humans as compared to long-chain triglycerides. In: Int. Congr. Nutr., 16th, Ottawa, pp 123. Ottawa, Canada: IUNS Secretariat, 1997.

Brown ED, Micozzi MS, Craft NE, Bieri JG, Beecher G, et al. Plasma carotenoids in normal men after a single ingestion of vegetables or purified β-carotene. Am J Clin Nutr 1989;49:1258-65.

Brubacher G, Weiser H. The vitamin A activity of β-carotene. Internat J Vit Nutr Res 1985;55:5-15.

Canfield LM, Giuliano AR, Neilson EM, Yap HH, Graver EJ, Cui HA, Blashill BM. β-Carotene in breast milk and serum is increased after a single β-carotene dose. Am J Clin Nutr 1997;66:52-61.

Carughi A, Hooper FG. Plasma carotenoid concentrations before and after supplementation with a carotenoid mixture. Am J Clin Nutr 1994;59:896-9.

Castenmiller JJM, West CE. Bioavailability of carotenoids. Pure Appl Chem 1997;69:2145-50.

Charoenkiatkul S, Valyasevi A, Tontisirin K. Dietary approaches to the prevention of vitamin A deficiency. Food Nutr Bull 1985;7:72-5.

Costantino JP, Kuller LH, Begg L, Redmond CK, Bates MW. Serum level changes after administration of a pharmacologic dose of β-carotene. Am J Clin Nutr 1988;48:1277-83.

Das BS, Thurnham DI, Das DB. Plasma α-tocopherol, retinol, and carotenoids in children with falciparum malaria. Am J Clin Nutr 1996;64:94-100.

Dimitrov NV, Meyer C, Ullrey DE, Chenoweth W, Michelakis A, et al. Bioavailability of β-carotene in humans. Am J Clin Nutr 1988;48:298-304.

Dueker SR, Jones AD, Smith GM, Clifford AJ. Stable isotope methods for the study of β-carotene-$d_8$ metabolism in humans utilizing tandem mass spectrometry and high-performance liquid chromatography. Anal Chem 1994;66:4177-85.

Erdman JW, Fahey GJ Jr, White CB. Effects of purified dietary fiber sources on β-carotene utilization in the chick. J Nutr 1986;116:2415-23.

Erdman JW, Poor CL, Dietz JM. Factors affecting the bioavailability of vitamin A, carotenoids and vitamin E. J Agric Food Chem 1988;42:214-21.

FAO/WHO Joint Expert Consultation. Requirements of Vitamin A, Iron, Folate and Vitamin $B_{12}$. FAO Food Nutr Ser No 23. Rome: Food Agric Org, 1988, 107 pp.

FAO/WHO. Requirements of Vitamin A, Thiamine, Riboflavin and Niacin. FAO Food Nutr Ser No 8. Rome: Food Agric Org WHO Technical Report Series No 362. Geneva: WHO, 1967.

Forman MR, Beecher GR, Lanza E, Reichman ME, Graubard BI, et al. Effect of alcohol consumption on plasma carotenoid concentrations in premenopausal women: a controlled dietary study. Am J Clin Nutr 1995;62:131-5.

Fotouhi N, Meydani M, Santos MS, Meydani SN, Hennekens CH, Gaziano JM. Carotenoid and tocopherol concentrations in plasma, peripheral blood mononuclear cells, and red blood cells after long-term β-carotene supplementation in men. Am J Clin Nutr 1996;63:553-8.

Gärtner C, Stahl W, Sies H. Preferential increase in chylomicron levels of the xanthophylls lutein and zeaxanthin compared to β-carotene in the human. Internat J Vit Nutr Res 1996;66:119-25.

Gärtner C, Stahl W, Sies H. Lycopene is more bioavailable from tomato paste than from fresh tomatoes. Am J Clin Nutr 1997;66:116-22.

Gaziano JM, Johnson EJ, Russell RM, Manson JE, Stampfer MJ, et al. Discrimination in absorption or transport of β-carotene isomers after oral supplementation with either all-*trans*- or 9-*cis*-β-carotene. Am J Clin Nutr 1995;61:1248-52.

Goodman DS, Blomstrand R, Werner B, Huang HS, Shiratori T. The intestinal absorption and metabolism of vitamin A and beta-carotene in man. J Clin Invest 1966;45:1615-23.

Gronowska-Senger A, Wolf G. Effect of dietary proteins on the enzyme from rat and human intestine which converts β-carotene to retinal. J Nutr 1970;100:300-8.

Hébuterne X, Wang XD, Johnson EJ, Krinsky NI, Russell RM. Intestinal absorption and metabolism of 9-*cis*-β-carotene in vivo: biosynthesis of 9-*cis*-retinoic acid. J Lipid Res 1995;36:1264-73.

Henderson CT, Mobarhan S, Bowen P, Stacewicz-Sapuntzakis M, Langenberg P, et al. Normal serum response to oral β-carotene in humans. J Am Coll Nutr 1989;8:625-35.

Herbst S, Bowen P, Hussain E, Stacewicz-Sapuntzakis M, Damayanti B, Burns J. Evaluation of the bioavailability of lutein (L) and lutein diesters (LD) in humans. FASEB J 1997;11:A447 (Abstr).

Hume EM, Krebs HA. Vitamin A requirements of human adults. Med Res Counc Spec Rep No 264. London: HM Stationery Office, 1949, 145 pp.

Int Union of Pure Appl Chem (IUPAC). The vitamin A Potency of Beta-Carotene, pp 1-11. London: Butterworth Sci, 1959.

Jackson MJ. The assessment of bioavailability of micronutrients: introduction. Eur J Clin Nutr 1997;51(suppl.1):S1-S2.

Jayarajan P, Reddy V, Mohanram M. Effect of dietary fat on absorption of β-carotene from green leafy vegetables. J Med Ind Res 1980;71:53-6.

Jensen CD, Howes TW, Spiller GA, Pattison TS, Whittam JH, Scala J. Observations on the effects of ingesting *cis* and *trans*-beta-carotene isomers on human serum concentrations. Nutr Rep Int 1987;35:413-22.

Jialal I, Norkus EP, Cristol L, Grundy SM. β-Carotene inhibits the oxidative modification of low-density lipoprotein. Biochim Biophys Acta 1991;1086:134-8.

Johnson EJ, Russell RM. Distribution of orally administered β-carotene among lipoproteins in healthy men. Am J Clin Nutr 1992;56:128-35.

Kardinaal AFM, van 't Veer P, Brants HAM, van den Berg H, van Schoonhoven J, Hermus RJJ. Relations between antioxidant vitamins in adipose tissue, plasma, and diet. Am J Epidemiol 1995;141:440-50.

Kasper H, Ernst B. Der Einfluss des Nahrungsfettes auf die Vitamine-A- und Carotineresorption. Int Z Vit Forsch 1969;39:23-9.

Kostic D, White WS, Olson JA. Intestinal absorption, serum clearance, and interactions between lutein and β-carotene when administered to human adults in separate or combined oral doses. Am J Clin

Nutr 1995;62:604-10.

Krinsky NI. Actions of carotenoids in biological systems. Annu Rev Nutr 1993;13:561-87.

Lala VR, Reddy V. Absorption of β-carotene from green leafy vegetables in undernourished children. Am J Clin Nutr 1970;23:110-3.

Lecomte E, Herberth B, Pirollet P, Chancerelle Y, Arnaud J, et al. Effect of alcohol consumption on blood antioxidant nutrients and oxidative stress indicators. Am J Clin Nutr 1994;60:255-61.

Leo MA, Kim CI, Lowe N, Lieber CS. Interaction of ethanol with β-carotene: delayed blood clearance and enhanced hepatotoxicity. Hepatology 1992;15:883-91.

Levin G, Mokady S. Incorporation of all-*trans*- or 9-*cis*-β-carotene into mixed micelles in vitro. Lipids 1995;30:177-9.

Maiani G, Mobarhan S, Ceccanti M, Ranaldi L, Gettner S, et al. Beta-carotene serum response in young and elderly females. Eur J Clin Nutr 1989;43:749-61.

Mattson FH, Deuel HJ Jr. Studies on carotenoid metabolism III. The effect of a high vitamin A diet on the carotenoid metabolism of chickens. J Nutr 1943;25:103-12.

McLaren DS, Zekian B. Failure of enzymatic cleavage of β-carotene. Am J Dis Child 1971;121:278-80.

Micozzi MS, Brown ED, Edwards BK, Bieri JG, Taylor PR, et al. Plasma carotenoid response to chronic intake of selected foods and β-carotene supplements in men. Am J Clin Nutr 1992;55:1120-5.

Monk BE. Metabolic carotenaemia. Br J Dermatol 1982;106:485-8.

Nagao A, Olson JA. Enzymatic formation of 9-*cis*, 13-*cis* and all-*trans* retinals from isomers of beta-carotene. FASEB J 1994;8:968-73.

Nierenberg DW, Stukel TA, Baron JA, Dain BJ, Greenberg ER, The Skin Cancer Prevention Study Group. Determinants of increase in plasma concentration of β-carotene after chronic oral supplementation. Am J Clin Nutr 1991;53:1443-9.

Nierenberg DW, Stukel TA, Mott LA, Greenberg ER. Steady-state serum concentration of alpha-tocopherol not altered by supplementation with oral β-carotene. J Nat Cancer Inst 1994;86:117-20.

Novotny JA, Dueker SR, Zech LA, Clifford AJ. Compartmental analysis of the dynamics of β-carotene metabolism in an adult volunteer. J Lipid Res 1995;36:1825-38.

Pee S de, West CE. Dietary carotenoids and their role in combating vitamin A deficiency: a review of the literature. Eur J Clin Nutr 1996;50(suppl.3):S38-S53.

Pee S de, West CE, Muhilal, Karyadi D, Hautvast JGAJ. Lack of improvement in vitamin A status with increased consumption of dark-green leafy vegetables. Lancet 1995;346:75-81.

Pee S de, West CE, Permaesih D, Martuti S, Muhilal, Hautvast JGAJ. Orange fruit is more effective than are dark-green, leafy vegetables in increasing serum concentrations of retinol and β-carotene in schoolchildren in Indonesia. Am J Clin Nutr 1998;68:1058-67.

Peiser GD, Yang SF. Sulphite-mediated destruction of β-carotene. J Agric Food Chem 1979;27:446-9.

Prince MR, Frisoli JK. Beta-carotene accumulation in serum and skin. Am J Clin Nutr 1993;57:175-81.

Rock CL, Jahnke MG, Gorenflo DW, Swartz RD, Messana JM. Racial group differences in plasma concentrations of antioxidant vitamins and carotenoids in hemodialysis patients. Am J Clin Nutr 1997;65:844-50.

Rock CL, Swendseid ME. Plasma β-carotene response in humans after meals supplemented with dietary pectin. Am J Clin Nutr 1992;55:96-9.

Roels OA, Trout M, Dujacquier R. Carotene balances on boys in Ruanda where vitamin A deficiency is prevalent. J Nutr 1958;65:115-27.

Rushin WG, Catignani GL, Schwartz SJ. Determination of β-carotene and its *cis* isomers in serum. Clin Chem 1990;36:1986-9.

Schmitz HH, Poor CL, Wellman RB, Erdman JW Jr. Concentrations of selected carotenoids and vitamin A in human liver, kidney and lung tissue. J Nutr 1991;121:1613-21.

Shiau A, Mobarhan S, Stacewicz-Sapuntzakis M, Benya R, Liao Y, et al. Assessment of the intestinal retention of β-carotene in humans. J Am Coll Nutr 1994;13:369-75.

Simonetti P, Brusamolino A, Pellegrini N, Viani P, Clemente G, et al. Evaluation of the effect of alcohol consumption in erythrocyte lipids and vitamins in a healthy population. Alcohol Clin Exp Res 1995;19:517-22.

Sklan D, Yosefov T, Friedman A. The effects of vitamin A, beta-carotene and canthaxanthin on vitamin A metabolism and immune responses in the chick. Internat J Vit Nutr Res 1989;59:245-50.

Slatterly ML, Jacobs DR Jr, Dyer A, Benson J, Hilner JE, Caan BJ. Dietary antioxidants and plasma lipids: The Cardia Study. J Am Coll Nutr 1995;14:635-42.

Stahl W, Schwarz W, Sies H. Human serum concentrations of all-*trans* β- and α-carotene but not 9-*cis* β-carotene increase upon ingestion of a natural isomer mixture obtained from Dunaliella salina (Betatene). J Nutr 1993;123:847-51.

Stahl W, Schwarz W, von Laar J, Sies H. All-*trans* β-carotene preferentially accumulates in human chylomicrons and very low density lipoproteins compared with the 9-*cis* geometrical isomer. J Nutr 1995;125:2128-33.

Stahl W, Sies H. Uptake of lycopene and its geometrical isomers is greater from heat-processed than from unprocessed tomato juice in humans. J Nutr 1992;122:2161-6.

Stryker WS, Kaplan LA, Stein EA, Stampfer MJ, Sober A, Willett WC. The relation of diet, cigarette smoking, and alcohol consumption to plasma beta-carotene and alpha-tocopherol levels. Am J Epidemiol 1988;127:283-96.

Sugarman SB, Mobarhan S, Bowen PE, Stacewicz-Sapuntzakis M, Langenberg P, et al. Serum time curve characteristics of a fixed dose of β-carotene in young and old men. J Am Coll Nutr 1991;10:297-307.

Takruri HRH, Thurnham DI. Effect of zinc deficiency on the conversion of β-carotene to retinol as indicated by liver stores. Proc Nutr Soc 1981;41:53A.

Tamai H, Morinobu T, Murata T, Manago M, Mino M. 9-*cis* β-carotene in human plasma and blood cells after ingestion of β-carotene. Lipids 1995;30:493-8.

Tang G, Serfaty-Lacrosniere C, Camilo ME, Russell RM. Gastric acidity influences the blood response to a β-carotene dose in humans. Am J Clin Nutr 1996;64:622-6.

Thompson SY. Role of carotene and vitamin A in animal feeding. World Rev Nutr Diet 1975;21:224-80.

Törrönen R, Lehmusaho M, Häkkinen S, Hänninen O, Mykkänen H. Serum β-carotene response to supplementation with raw carrots, carrot juice or purified β-carotene in healthy non-smoking women. Nutr Res 1996;16:565-75.

Villard L, Bates CJ. Carotene dioxygenase (EC 1.13.11.21) activity in rat intestine; effects of vitamin A deficiency and of pregnancy. Br J Nutr 1986;56:115-22.

Vliet T van. Absorption of β-carotene and other carotenoids in humans and animal models. Eur J Clin Nutr 1996a;50(suppl.3):S32-S37.

Vliet T van, Fentener van Vlissingen M, van Schaik F, van den Berg H. β-Carotene absorption and cleavage in rats is affected by the vitamin A concentration of the diet. J Nutr 1996b;126:499-508.

Vliet T van, van Schaik F, Schreurs WHP, van den Berg H. In vitro measurement of β-carotene cleavage activity: methodological considerations and the effect of other carotenoids on β-carotene cleavage. Internat J Vit Nutr Res 1996c;66:77-85.

Vliet T van, Schreurs WHP, van den Berg H. Intestinal β-carotene absorption and cleavage in men: response of β-carotene and retinyl esters in the triglyceride-rich lipoprotein fraction after a single oral dose of β-carotene. Am J Clin Nutr 1995;62:110-6.

Wagner KH. Die experimentelle Avitamineose A beim Menschen. Hoppe-Seyler's Zeitsch Physiol Chem 1940;246:153-89 (Correction 1940;264:59).

Wahlqvist ML, Wattanapenpaiboon N, Macrae FA, Lambert JR, MacLennan R, Hsu-Hage BHH, Australian Polyp Prevention Project Investigators. Changes in serum carotenoids in subjects with colorectal adenomas after 24 mo of β-carotene supplementation. Am J Clin Nutr 1994;60:936-43.

Wedzicha BL, Lamikanra O. Sulphite mediated destruction of β-carotene: the partial characterisation of reaction products. Food Chem 1983;10:275-83.

Weststrate JA, van het Hof KH. Sucrose polyester and plasma carotenoid concentrations in healthy subjects. Am J Clin Nutr 1995;62:591-7.

White WS, Stacewicz-Sapuntzakis M, Erdman JW Jr, Bowen PE. Pharmacokinetics of β-carotene and canthaxanthin after ingestion of individual and combined doses by human subjects. J Am Coll Nutr 1994;13:665-71.

Wingerath T, Stahl W, Sies H. β-Cryptoxanthin selectively increases in human chylomicrons upon ingestion of tangerine concentrate rich in β-cryptoxanthin esters. Arch Biochem Biophys 1995;324:385-90.

Yeum KJ, Booth SL, Sadowski JA, Liu C, Tang G, et al. Human plasma carotenoid response to the ingestion of controlled diets high in fruits and vegetables. Am J Clin Nutr 1996;64:594-602.

You CS, Parker RS, Goodman KJ, Swanson JE, Corso TN. Evidence of *cis-trans* isomerization of 9-*cis*-β-carotene during absorption in humans. Am J Clin Nutr 1996;64:177-83.

Zechmeister L. Stereoisomeric provitamins A. Vitam Horm 1949;7:57-81.

## ANNEX. STUDIES ON BIOAVAILABILITY AND BIOCONVERSION OF CAROTENOIDS

*Supplemental Materials section of the Annual Reviews' Web site (http://www.AnnualReviews.org)*

Many studies have measured serum or plasma response to supplementation with carotenoids, mainly β-carotene. Other studies have examined the effect supplementing a meal with a mixture of carotenoids has on such responses. Sometimes these studies have used large, pharmacological doses of β-carotene, and often they have not been well controlled. The between-individuals response to repeated doses of β-carotene, which lead to plateau concentrations (Costantino et al. 1988), appears to be generally less variable than the response to single doses. An overview of such studies is given here.

| Study | | | | SLAMENGHI |
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| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | factor[d] |
| Abbey et al. 1995 | 15 M, 41 y, smokers; 145 mg vitamin C from orange juice and 16 mg β-carotene from carrot juice or placebo daily for 3 wk | β-Carotene: 0.52-0.20 | Retinol: 2.23-2.20 | A, M |
| Albanes et al. 1992 | 222 M, 30-69 y; 20 mg β-carotene daily for 2 mo | β-Carotene: 4.99-0.53 | | A |
| Albanes et al. 1997 | 491 M ($n$ = 237 supplement, $n$ = 254 placebo), 58-76 y, smokers; 20 mg β-carotene daily for 6.7 y | (Quit smoking, smoking < 20 cigarettes daily, smoking ≥ 20 cigarettes daily)<br>β-carotene: 5.74-0.43, 6.61-0.39, 5.11-0.32;<br>β-cryptoxanthin: 0.26-0.17, 0.20-0.13, 0.15-0.20;<br>α-carotene: 1.66-0.67, 1.40-0.75, 1.40-0.52;<br>lutein: n.s., 0.15-0.18, n.s.<br>β-Carotene smokers: 5.81-0.37<br>(Low alcohol ≤ 12.9 g/d, high alcohol consumption > 12.9 g/d)<br>β-carotene: 6.54-0.46, 5.71-0.29;<br>β-cryptoxanthin: 0.23-0.13, 0.18-0.11;<br>α-carotene: 0.16-0.08, 0.14-0.05 | Retinol: 2.05-1.94 (n.s.) | A, E, H |

| Study | | | | SLAMENGHI |
|---|---|---|---|---|
| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | factor[d] |
| Allard et al. 1994 | 38 M non-smokers (treatment: $n$ = 18; placebo: $n$ = 20) and 25 M smokers (treatment $n$ = 14; placebo: $n$ = 11), 20-75 y; 20 mg β-carotene daily for 4 wk or placebo | (Non-smokers, smokers) β-carotene: 3.50-0.41-(0.38-0.43), 3.38-0.29-(0.27-0.27); α-carotene: n.s. | | A, H |
| Baker et al. 1996 | 91 M in 7 groups, 20-55 y; 30 mg β-carotene daily for 14 d | Carotene: 5.85-3.35 | n.s. | A |
| Ben-Amotz & Levy 1996 | 30 M, 20-30 y; 40 mg all-*trans*-β-carotene daily for 14 d or placebo | All-*trans*-β-carotene: 0.764-0.279-(0.224-0.261); *Cis*-β-carotene: 0.016 | Retinol: 1.60 | A |
| Borel et al. 1997 | 8 M, young, healthy; single dose of 120 mg all-*trans*-β-carotene plus 40 g LCT (99% $C_{16}$+$C_{18}$ fatty acids) or 40 g MCT (95% $C_8$+$C_{10}$ fatty acids) | Chylomicron TG and β-carotene response lower after intake MCT-meal than after LCT-meal; β-carotene: 72/274 nmol/L.h | Chylomicron retinyl palmitate: MCT < LCT; conversion to vitamin A (RP response/β-carotene response) n.s. | E |
| Bos et al. 1992 | 123 M + F ($n$ = 62 β-carotene treatment, $n$ = 61 placebo), 39 y, smokers (21 cigarettes per day, smoking for at least 20 y); 20 mg β-carotene daily for 14 wk or placebo | (β-Carotene group, placebo) β-carotene: 4.57-0.35; 0.28-0.30 | | A |
| Brown et al. 1989 | 30 M + 8 M (placebo), 20-45 y, nonsmokers; single dose of 12 or 30 mg pure β-carotene ($n$ = 2 x 15), broccoli (6 mg β-carotene and 5 mg lutein, $n$ = 7), tomato juice (12 mg lycopene, $n$ = 8), carrots (29 mg β-carotene + 9 mg α-carotene, $n$ = 7) or placebo, cross-over design (measurements over 11 d) | (12 mg, 30 mg, carrot) (baseline: 0.341 and 0.330) β-carotene: 0.330+0.032 = 0.362, 0.538+0.032 = 0.570, 0.114+0.032 = 0.146<br>Intake of broccoli or tomato juice did not change any plasma carotenoids | | A, M |

| Study | | | | SLAMENGHI |
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| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | factor[d] |
| Bulux et al. 1994 | 16-17 M + F/group, 7-12 y, vitamin A depleted (*n* = 4: vitamin A deficient); 6 mg β-carotene daily for 20 d from synthetic β-carotene or carrots with 10 g fat or 1000 RE retinyl palmitate | (Synthetic, carrots)<br>β-carotene: 0.92-0.27 = 0.65, 0 | n.s. Baseline plasma retinol: 1.23, subjects had significant intestinal infestation | A, M |
| Canfield et al. 1997 | 12 F, lactating, 23-36 y; single dose of 60 or 210 mg β-carotene with breakfast | (60 mg, 210 mg)<br>β-Carotene: 1.85-0.75 = 1.1, 1.2<br>no effect on the major other carotenoids | n.s. | A |
| Carughi & Hooper 1994 | 4 M and 7 F, 22-52 y, non-smoking; after 2 wk run-in, 6 capsules with concentrate (8.5 mg β-carotene, 3.5 mg α-carotene, 0.5 mg lycopene per day) made into paste with olive oil daily for 4 wk | (Significant differences)<br>β-carotene: 1.450-0.501;<br>α-carotene: 0.665-0.186;<br>lycopene: 0.569-0.525; | | A |
| Cooney et al. 1991 | 14 M + F; 30 mg β-carotene daily for 1 wk | Sixfold increase in β-carotene serum levels, lycopene concentration remained constant | | A |
| Costantino et al. 1988 | 300 M, 50-75 y; 15 mg β-carotene daily for 1 y or placebo | β-Carotene after 4 mo: 3.3-0.3 = 3.0 | | A |
| Das et al. 1996 | 28 M and 22 F in each of three groups: with severe malaria, mild malaria, controls; age and sex matched; 2-11 y; India | (Control, mild and severe malaria groups, significant differences with control and between severe and mild malaria)<br>lutein: 0.423, 0.267, 0.258 (n.s. with mild);<br>β-cryptoxanthin: 0.119, 0.064, 0.042;<br>lycopene: 0.068, 0.038, 0.014;<br>α-carotene: 0.035, 0.019, 0.007;<br>β-carotene: 0.310, 0.150, 0.076 | (Control, mild, severe)<br>retinol: 1.10, 0.70, 0.43 | H |

| Study | | | | SLAMENGHI |
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| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | factor[d] |
| Dimitrov et al. 1988 | 15 M + F, 21-63 y, 45 mg β-carotene daily for 30 d; 11 M + F, 15 mg β-carotene daily for 8 wk; 9 M + F, 45 mg β-carotene daily with high fat (> 63 g fat) diet for 5 d followed by 16 d normal fat diet | 45 mg: range placebo: 0.15-0.45; range β-carotene: 0.9-4.8. 15 mg: range placebo: 0.15-0.45; range β-carotene group: 0.4-3.1<br>(High fat diet, low fat diet)<br>45 mg β-carotene: 3.2-0.15, 1.2-0.15 | n.s. | A, E |
| Drewnowski et al. 1997 | 361 M and 476 F, 18-94 y; cross-sectional study in France | Women had significant higher serum β-carotene concentrations than men (0.97 vs. 0.70 μmol/L); no effect of age; for men BMI negatively associated β-carotene concentration; negative correlations with energy and fat intakes and with alcohol use, serum cholesterol levels and tobacco use | | H |
| Forman et al. 1995 | 18 F, 21-39 y, non-smoking; 30 g alcohol daily for 3 menstrual cycles, 3 alcohol-free cycles; cross-over design (paired differences); intake of 6 mg total carotenoids per day during these 6 mo | (Alcohol, no alcohol)<br>α-carotene: 0.0174, 0.0142;<br>β-carotene: 0.7626, 0.7079;<br>lutein/zeaxanthin: 0.07744, 0.8624 | n.s. | E |
| Forman et al. 1996 | 12 F, 20-34 y; approximately 10 mg carotenoids daily during 2 cycles (2.5 mg β-carotene, 2.5 mg lutein, 0.7 mg α-carotene, 4.3 mg lycopene); total diets: 36% fat, 3.4 g fibre/100 J | (After 3 wk)<br>β-carotene: 0.534-0.379;<br>lutein/zeaxanthin: 0.526-0.370;<br>α-carotene: 0.041-0.033;<br>other carotenoids: n.s. different | Retinol: 1.504-1.539 | A |
| Fotouhi et al. 1996 | 59 M (30 placebo, 29 treatment), 50-86 y; 50 mg β-carotene daily for 12 y | β-Carotene, placebo adjusted: 1.73-0.54 = 1.19;<br>lutein: 0.42-0.44 | | A |

| Study | | | | SLAMENGHI |
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| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | factor[d] |
| Friis et al. 1997 | 159 M + F pre-school (0.25-5.1 y), 695 primary school children (9.2-17 y) | | Lower serum retinol levels in pre-school children and in boys compared to girls; primary school children: age, sex and *S. mansoni* infection sign. predictors: 0.05 μmol/L retinol increase/y; boys: 0.12 lower levels than girls | H |
| Fuller et al. 1992 | 24 M, 19-39 y, non-smokers; 2 x 15 mg β-carotene daily (one capsule with each of the main meals) for 28 d or placebo | β-Carotene: 4.28-0.35; placebo: 0.26-0.48 | | A |
| Gärtner et al. 1996 | 5 M and 3 F; single dose of 5.6 μmol carotene per kg body weight from *Dunaliella salina* extract (0.5% lutein, 0.75% zeaxanthin, 3.6% α-carotene, 70.3% all-*trans*-β-carotene, 22.7% *cis*-β-carotene isomers), with 500 g milk | (In chylomicrons at 9, nmol/L) all-*trans*-β-carotene: 92.3-2.96; lutein: 9.59-2.49; zeaxanthin: 3.80-0.54; lycopene: 8.88-8.41; α-carotene: 5.44-0.53 | | A |
| Gärtner et al. 1997 | 2 M and 3 F, mean 32 y; single dose of 23 mg lycopene from 400 g fresh tomatoes or 40 g tomato paste with 15 g corn oil and 100 g bread (2 experimental d, 2 wk apart) | (Chylomicron fraction: fresh tomato, tomato paste) total lycopene: 0.0110, 0.0279; no differences in α- or β-carotene | | M |

| Study | | | | SLAMENGHI |
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| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | factor[d] |
| Gaziano et al. 1995 | 7 M and 17 F; 100 mg β-carotene daily for 6 d | β-Carotene: 1.889-0.271 = 1.618 | | A |
| Herbst et al. 1997 | 10 M and 8 F; single dose of 0.65 μmol lutein diesters 0.5 μmol lutein per kg body weight | Mean AUCs LD/L = 2103/1301 x 0.5/0.65 = 1.24 | | L |
| Hussein & El-Tohamy 1990 | 17 M; carrot juice and spinach for 2 wk | Carotenes from 3350 μg carrot juice = 12700 μg boiled spinach leaves (apparent digestibilities 47 and 81%) | | M |
| Jayarajan et al. 1980 | 26 and 22 and 22 M + F children, 2-6 y, vitamin A deficient; 1.2 mg β-carotene daily for 4 wk from 40 g spinach with 50 g cooked rice and no oil or 5 g or 10 g groundnut oil | | (No oil, 5 or 10 g oil) retinol: 0.893-0.744, 1.079-0.781 | A, E |
| Jensen et al. 1987 | 6 M and 10 F, 18-60 y; 24 mg β-carotene daily for 7 d from *Dunaliella salina* (all-*trans*:9-*cis*=40:60) or from carrots (98% all-*trans*-β-carotene) or placebo | (Alga, carrot) β-carotene: 0.882-0.179+0.031 = 0.734, 0.915-0.438+0.031 = 0.508 | | M |
| Jialal et al. 1991 | 39-43 M + F/group, 2-7 y, vitamin A deficient; 5.1 mg β-carotene daily for 24 d from sweet potato and dark green leafy vegetables and/or deworming and/or 25 g coconut fat; Indonesia | | (Vegetables+fat, veg.) placebo 0.037; retinol: 0.350, 0.197 (Veg.+fat+deworming, veg.+deworming) retinol: 0.331, 0.350 (initial retinol level < 0.744: 0.372, 0.197) | A, E, H |

| Study | | | | SLAMENGHI |
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| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | factor[d] |
| Johnson et al. 1992 | 11 M and 5 M (placebo); single dose of 120 mg β-carotene | β-Carotene at d 5: 0.22-0.19-(0.01) = 0.02 | n.s. | A |
| Johnson et al. 1995 | 8 F (placebo) and 7 F (treatment), > 60 y; 90 mg β-carotene daily for 23 d or placebo | β-Carotene at wk 3: 5.36-0.66 | | A |
| Kostic et al. 1995 | 4 M and 4 F, 24-38 y, 53-91 kg; single dose of 0.5 μmol β-carotene per kg body weight with breakfast | Peak concentration serum β-carotene (-baseline): 1.94-0.36;<br>lutein as separate dose: lutein: 2.90-0.30;<br>β-carotene/lutein: 54-61% reduction | | A |
| Kramer & Burri 1997 | 9 F, pre-menopausal; days 1-60: 0.5 mg β-carotene daily ($n$ = 5) or placebo ($n$ = 4), d 61-100: 0.5 mg β-carotene daily ($n$ = 9), d 101-120: 0.5 mg β-carotene daily plus vegetable complex (mg/d: 1.398 α-carotene, 3.306 β-carotene, 0.117 β-cryptoxanthin, 1.491 lutein/zeaxanthin, 0.663 lycopene) | (d 1, d 60, d 100, d 120)<br>β-carotene: 1.27, 0.66 (n.s.), 0.91 (n.s.), 3.39 | | A, M |
| Lala & Reddy 1970 | 29 M + F, 2-6 y, vitamin A deficient; 1.2 mg β-carotene daily for 15 d from amaranth plus chapati or placebo ($n$ = 6); daily diet contained 5-7 g fat | | (Initial retinol < 0.930 ($n$ = 17), > 0.930 ($n$ = 12), placebo)<br>retinol: 0.469, 0.231, 0 | A, M |
| Margetts & Jackson 1996 | 1483 M + F, 16-64 y; 7-d weighed inventory and blood sample | (Non-smokers, light smokers, heavy smokers)<br>β-carotene: 0.37, 0.31, 0.25;<br>for every 1000 μg change in diet intake there was a 0.01 μmol/L change in plasma β-carotene in smokers and a 0.04 μmol/L change in non-smokers | | H |

| Study | | Bioavailability[b] | Bioconversion[c] | SLAMENGHI factor[d] |
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| Author | Details[a] | | | |
| Martini et al. 1995 | 19 M + F; control diet (carotenoid-free); control with 42.2 mg carrots and spinach or control diet with 5.1 mg broccoli and cauliflower | Carrot/spinach diet: β-carotene: 0.58, lutein: 0.32; broccoli/cauliflower: β-carotene: 0.11, lutein: 0.27 | | A, M |
| Mayne et al. 1997 | 259 M + F; 50 mg β-carotene daily for 3 mo | β-Carotene: 2.235-0.224 = 2.011; α-carotene: 0.095-0.045 = 0.050; no effect on plasma concentrations of lycopene or lutein/zeaxanthin | | A |
| Meydani et al. 1994 | 12 F, 62-80 y; 90 mg β-carotene daily for 21 d or placebo | β-Carotene: 6.45-0.76; placebo: 0.54-0.75 | | A |
| Micozzi et al. 1992 | 6 x 5 M, 20-45 y, non-smokers; daily for 6 wk 12 and 30 mg pure β-carotene or carrots (29 mg β-carotene + 9 mg α-carotene or broccoli (3 mg β-carotene + 3 mg lutein) or tomato juice (12 mg lycopene); diets: 40% fat, 17% protein | (12 mg, 30 mg, carrots, broccoli, tomato juice) β-carotene (baseline 0.303, placebo = 0.021): 3.568, 7.880, 1.417, 0.172, -0.004; α-carotene (baseline 0.0785, placebo = -0.057): 0.155, 0.155, 1,028, 0.044, 0.021; lutein (baseline 0.410, placebo = 0.233): -0.385, -0.255, 0.148, 0.446, -0.143; lycopene (baseline: 0.8923): n.s., n.s., n.s., n.s., 0.646; other carotenoids n.s. | | A, M |
| Nierenberg et al. 1991a | 403 M and 179 F; 50 mg β-carotene daily for 1 y | β-Carotene: 3.163-0.335 = 2.828 (Smokers, non-smokers) β-carotene: 2.146, 3.100 (Men, women) β-carotene: 2.522, 3.420 | | A, H |
| Nierenberg et al. 1997 | 82 M and 20 F (placebo: *n* = 53; treatment: *n* = 49), 40-78 y, patients who had at least one adenoma removed from the large bowel; 25 mg β-carotene daily for 4 y or placebo | β-Carotene: 0.614 (adjusted 0.638); placebo: 0.383-0.415 = -0.032 | n.s. | A |

| Study | | | | SLAMENGHI |
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| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | factor[d] |
| Nierenberg et al. 1991b | 31 M + F skin cancer patients and 30 placebo; 50 mg β-carotene daily for 1 y | β-Carotene: 4.702-0.423-(0.472-0.462) = 4.267 | n.s. | A |
| Novotny et al. 1995 | 1 M, 53 y, 94 kg; single dose of 40 mg β-carotene | 22% Absorbed: 17.8% as intact β-carotene, 4.2% as retinoid | 1 μg dietary β-carotene = 0.054 μg retinol | A |
| Pamuk et al. 1994 | 91 F, 30-69 y, low-income African-American, 50 non-smokers, 41 smokers; | Smokers had adjusted serum concentrations for α-carotene, β-carotene, β-cryptoxanthin, lycopene: 0.71-0.79 x values in non-smokers | Current smokers: higher serum vitamin A concentrations | H |
| de Pee et al. 1995 | 57 F and 62 F and 54 F, breastfeeding with low haemoglobin concentrations; 3.5 mg β-carotene daily for 12 wk from stir-fried vegetables or pure β-carotene in an enriched wafer or placebo wafer | (Wafer, vegetables)<br>β-carotene: 0.92-0.19-(0.17-0.19) = 0.75, 0.05;<br>α-carotene: 0.09-0.06-(0.06-0.06), 0.01;<br>zeaxanthin: 0.09-0.10-(0.09-0.11), -0.01-(0.09-0.11);<br>lutein: 0.35-0.47-(0.37-0.43), 0.53-0.48-(0.37-0.43);<br>other carotenoids n.s. | (Wafer, vegetables)<br>retinol: 1.16-0.84-(0.83-0.81) = 0.30, 0.06-0.02 (n.s.) | A, M |
| de Pee et al. 1998 | 231 M + F, 7-11 y, *anaemic*; daily for 9 wk 44 RE from low-retinol, low-carotenoid diet (*n* = 46) or 684 RE from DGLV and carrots (*n* = 45) or 509 RE from fruits (*n* = 49) or 556 RE from retinol-rich foods (*n* = 48) | (Vegetables, fruits, retinol-rich, low-retinol low-carotenoid)<br>β-carotene: 0.14, 0.52, 0.06, 0.03;<br>β-cryptoxanthin: -, 0.96, -, -;<br>α-carotene: 0.05, 0.05, 0.04; 0.03;<br>lutein: 0.31, 0.07, 0.07, 0.04;<br>lycopene: -, 0.25, -, -;<br>zeaxanthin: -, 0.02, 0.04, - | (Vegetables, fruits, retinol-rich, low-retinol low-carotenoid)<br>retinol: 0.07, 0.12, 0.23, 0<br>1 RE = 26 μg, 11 μg β carotene | A, M |

| Study | | | | SLAMENGHI |
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| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | factor[d] |
| Peng et al. 1995 | 50 M and 46 F, 26-82 y, healthy Caucasians; 3 measurements over 1 mo period | (Smokers, non-smokers, adjusted differences) zeaxanthin: 0.033, 0.040; β-cryptoxanthin: 0.152, 0.199; α-carotene: 0.071, 0.136, -48%; β-carotene: 0.216, 0.495, -56%; *cis*-β-carotene: 0.017, 0.035, -53%; lutein and lycopene: n.s. | n.s. | H |
| Van Poppel et al. 1992 | 143 M (*n* = 73 placebo; *n* = 70 treatment), mean 39 y, heavy smokers (at least 15 cigarettes/d for over 2 y); 2 x 20 mg β-carotene daily for 2 wk followed by 20 mg β-carotene daily for 12 wk | β-Carotene group: 4.36-0.33; placebo: 0.28-0.30 | n.s. | A |
| Prince & Frisoli 1993 | 3 M and 2 F, 30-36 y; 51, 3 x 17, 3 x 34, 3 x 102 mg β-carotene daily for 20 d; 3 M and 2 F, 30-36 y; single dose of 51 mg β-carotene without dietary fat (*n* = 3) after period of fasting and with 200 g fat | (51 mg, 3 x 17 mg, 3 x 34 mg, 3 x 102 mg) β-Carotene: 2.12-0.671, 6.37-0.913 (sign.), 7.67-0.987 (sign.), 13.2-1.27 (sign.); lycopene: 0.391-0.596, 0.522-0.596, 0.484-0.596 (sign.), 0.466-0.596 (sign.); no fat: no change/no detectable accumulation in serum; plus 200 g fat: 2.5 fold increase (2.8% of dose absorbed) at 40 h | n.s. | A, E |
| Prince et al. 1991 | 5 M + F; 300 mg β-carotene daily for 21 d (3 divided doses with meals) | β-Carotene: 12.11-3.167 | | A |
| Rasmussen et al. 1991 | 12 M and 17 F (54-80 y), 14 M and 16 F (21-41 y), controls: 5 + 5; two test meals of 50% (372 μg vitamin A, 353 μg mixed carotenoids) and 100% RDA (882 μg vitamin A, 540 μg mixed carotenoids) for vitamin A | | (Elderly, young) retinyl esters (μg/dl): 50% RDA: 6.0, 5.0 (n.s.); 100% RDA: 9.0, 6.8 | A, H |

| Study | | | | SLAMENGHI |
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| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | factor[d] |
| Ribaya et al. 1995 | 2 x 5 F, 60-75 y; 90 mg β-carotene daily for 3 wk with high-fat breakfast or placebo | β-Carotene: 6.83-0.48;<br>lutein in supplemented group: -0.03 | 2.32-2.07 (n.s.) | A |
| Ringer et al. 1991 | 5 x 10 M+F (data for $n$ = 45), 18-54 y; 15 or 45 or 180 or 300 mg β-carotene daily for 28 d or placebo; 40% fat in diets for first 5 d, followed by usual diet | Baseline β-carotene: 0.47, lutein: 0.43; α-carotene: 0.09; lycopene: 0.66<br>(0 mg, 15 mg, 45 mg, 180 mg, 300 mg – estimates)<br>β-carotene: 0.5, 1.5, 4, 3, 6;<br>α-carotene increased 2-4 fold in each of the treated groups lycopene and lutein unchanged | n.s. (Baseline: 1.29) | A |
| Rock et al. 1992 | 7 F; single dose of 25 mg β-carotene with or without 12 g citrus fibre; cross-over design, 3 wk wash-out period between treatments, 500 kcal controlled meals | (With fibre, without fibre)<br>β-carotene at 30 h: 1.120-0.728 = 0.392, 1.638-0.697 = 0.941 | | A, E |
| Roels et al. 1958 | 22 M (4 boys/group), 9-16 y, 17 were vitamin A deficient; 14 mg pure β-carotene daily or 18.8 mg β-carotene daily for 31 d from grated carrots with or without 20 ml (18 g) olive oil or placebo | (Without oil, with oil)<br>β-carotene from carrots: 1.56-0.80+0.112, 6.24-0.89+0.112;<br>β-carotene oil only: 0.875-0.969 = -0.094;<br>β-carotene from pure β-carotene with oil: 9.33-1.19+0.112 | (Without oil, with oil)<br>carrots: 1.90-1.34, 1.97-1.19;<br>oil only: 0.07 (n.s.); pure β-carotene+oil: 2.08-1.30;<br>placebo: 0.18 | A, M, E |
| Ross et al. 1995 | 50 M smokers who had smoked for at least 10 y and 50 M who had never smoked, 50-59 y | (Smokers, never-smokers)<br>α-carotene: 0.078, 0.114 (sign.);<br>β-carotene: 0.484, 0.596 (sign.);<br>β-cryptoxanthin: 0.076, 0.114 (sign.);<br>lycopene, lutein/zeaxanthin, phytofluene: n.s. different | n.s. | H |

| Study | | | | SLAMENGHI |
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| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | factor[d] |
| Seddon et al. 1994 | 3 M + F; 9.5 mg Marigold extracts in olive oil daily for 9 or 18 d | (9, 18 d)<br>Lutein: 0.80, 0.83 | | A |
| Shiau et al. 1994 | 11 M (18-30 y) and 5 M (65-80 y); single dose of 15 mg β-carotene or placebo; total gut washout method (TGWM) | No meal: 83% of ingested β-carotene recovered in rectal effluent; with meals: 49-71% recovered in young men and 35-51% in elderly men; more fat: higher serum concentrations | | A, H |
| Stahl & Sies 1992 | 5 M + F; single dose of 2.5 μmol lycopene per kg body weight from tomato juice boiled with 1% corn oil for 1 h or unprocessed tomato juice or placebo | Control: 0;<br>unheated juice: 0;<br>processed tomato juice: lycopene in serum increased, dose-dependent but not linear with dose | | A |
| Stryker et al. 1988 | 137 M (mean 36 y) and 139 F (mean 35 y), 18-79 y; epidemiological study | (FFQ: geometric means for β-carotene smokers, non-smokers)<br>M: 0.158, 0.285; F: 0.322, 0.490<br>M: 72%, F: 79% of plasma levels of β-carotene as non-smokers | | H |
| Sugerman et al. 1991 | 7 M (73 y) and 6 M (24 y); single dose of 15 mg β-carotene, 3 x 500 kcal meals | β-Carotene young : old = 2-3 : 1 | | H |
| Tamai et al. 1995 | 30 M, 20-25 y; 60 mg β-carotene daily for 44 wk or placebo | β-Carotene: 1.49-0.279 (all-*trans*)+0.121-0.037; (*cis*) = 1.295 | n.s. | A |
| Tang et al. 1996 | 7 M and 5 F, 23-68 y; single dose of 120 mg β-carotene | (*Trans, cis*)<br>β-carotene: 0.780, 0.020<br>(Gastric pH 6.4, 1.3)<br>β-carotene: 6825, 3390 nmol.h | | A, H |
| Törrönen et al. 1996 | 38 F, 20-53 y, non-smoking; 12 mg β-carotene daily for 6 wk | (Low carotenoid run-in, habitual diet)<br>β-carotene: 1.75-0.548 = 1.20, 1.75-0.976<br>(Capsules, raw carrots, carrot juice)<br>β-carotene: 1.745-0.548, 0.842-0.529, 1.062-0.525 | | A, M |

| Study | | | | SLAMENGHI |
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| van Vliet et al. 1995 | 10 M and 2 M (placebo), young, non-smoking; 15 mg β-carotene on 2 experimental d, 16 d apart | TRL (triglyceride-rich lipoprotein) fraction:<br>AUC plasma β-carotene response d 6, d 1: 149, 100 mmol.h/L | (d 6, d 1 in TRL) AUC plasma retinyl palmitate: 245, 188 | A |
| Wahlqvist et al. 1994 | 139 M and 85 F with colorectal adenomas, 30-73 y; 20 mg β-carotene daily for 24 mo (78 M and 43 F) or placebo (*n* = 103) | (Significant differences for M, F)<br>β-carotene: 2.139-0.280-(0.270-0.258), 2.367-0.446-(0.235-0.236);<br>α-carotene: 0.085-0.036-0.041, 0.102-0.047-0;<br>lycopene: 0.530-0.255-0.056, n.s. | | A, H |
| Watson et al. 1991 | 10 M and 10 F (postmenopausal), in groups of 2 M and 2 F, mean age 56 y, non-smokers; 15 or 30 or 45 or 60 mg β-carotene daily for 3 mo or placebo | (0 mg, 15 mg, 30 mg, 45 mg, 60 mg after 2 mo)<br>β-Carotene: 0.330-0.286, 0.312-0.210, 0.443-0.225, 0.501-0.253, 0.592-0.256 | n.s. Only effect of 30 mg on retinyl palmitate: 0.099-0.0286 | A |
| Weststrate & van het Hof 1995 | 18 M and 3 F, 23-55 y; 12.4 g SPE daily for 4 wk in 31 g margarine added to mean mail (7.5 g margarine at main meal), cross-over design<br><br>24 M and 29 F, 19-64 y; 3 g SPE daily for 4 wk in 31 g margarine with a meal | (12.4 g SPE, 3 g SPE)<br>β-Carotene: -0.13 (45% reduction), -0.094 (20% reduction);<br>lycopene: -0.14, -0.12;<br>β-cryptoxanthin, lutein, and zeaxanthin sign. reduced | | E |
| White et al. 1994 | 2 F, 33 and 50 y, non-smoking; single doses of 25 mg β-carotene, 25 mg canthaxanthin or combined doses (2 x 25 mg) | (Canthaxanthin+placebo, canthaxanthin+β-carotene, β-carotene+placebo, β-carotene+canthaxanthin)<br>mean peak increments: 2.37, 1.43, 0.56, 0.67 | | A |
| Willett et al. 1983 | 59 M + F in 4 groups, 23-57 y; 25,000 IU retinyl palmitate or 30 mg β-carotene daily for 16 wk or placebo | (Placebo, retinyl palmitate, β-carotene group after 8 wk)<br>total carotenoid levels: 3.22-3.46, 3.32-3.17, 10.34-3.17 | n.s. | A |

| Study | | | | |
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| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | SLAMENGHI factor[d] |
| Wingerath et al. 1995 | 3 M and 4 F, 25-41 y; single dose of 0.34 μmol β-cryptoxanthin per kg body weight from tangerine concentrate, with milk | (Chylomicrons at 6 h, nmol/L)<br>β-cryptoxanthin: 13.8 to 91.6 nmol/L (range); no esters in chylomicrons | | L |
| Winklhofer-Roob et al. 1995 | 35 M + F patients with cystic fibrosis and controls; 0.5 mg β-carotene daily for 16 mo | (Patients, controls after 3 wk)<br>β-carotene: 0.89-0.09, 0.89-0.86 | | A, H |
| Winklhofer-Roob et al. 1997 | 208 M + F, Swiss individuals, 0.4-38.7 y | Age was significant predictor of plasma concentrations except for α-carotene; no sex-related differences (M/F = 1). Plasma β-carotene (μmol/L): log y = -0.56+0.0129 x age;<br>*cis*-β-carotene: log y = -1.45+0.0117 x age;<br>*trans*-β-carotene: log y = -0.60+0.0131 x age;<br>lycopene: log y = -1.00+0.016 x age | Age differences were 0.04 μmol/L per year;<br>Retinol: y = 0.81+0.037 x age (corrected for season) | H |
| Xu et al. 1992 | 45 M + F, 50-65 y; 15 or 30 or 45 or 60 mg β-carotene daily for 9 mo or placebo | (0 mg, 15 mg, 30 mg, 45 mg, 60 mg after 1 mo)<br>β-Carotene: 0.447, 2.09, 2.42, 2.31, 2.72 | | A |
| Yeum et al. 1996 | 36 M + F in 4 groups, 20-40 y and 60-80 y; 16 mg carotenoids daily in 3 x 15 d (2 portions) followed by broccoli (2.3 mg lutein in diet) for 5 d, cross-over design | F had higher baseline plasma carotenoid conc. than younger M; older M had highest values;<br>all-*trans* β-carotene, 13-*cis* β-carotene: young M: 320%; 187; young F: 280%; 195; older M: 152% (n.s); older F: 277%; 213;<br>α-carotene: young M: 557%; young F: 392%; older M: 244%; older F: 366%;<br>plasma lutein conc. increased for younger M, younger and older F; older M had highest baseline conc. and no sign. change; addition of broccoli (1.7 mg lutein): sign. effect, except for young F | n.s. | A, H |

| Study | | | | SLAMENGHI |
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| Author | Details[a] | Bioavailability[b] | Bioconversion[c] | factor[d] |
| Yong et al. 1994 | 98 F, pre-menopausal, non-smoking; dietary correlation study | (Geometric means of plasma concentrations) α-carotene: 0.08; β-carotene: 0.30; β-cryptoxanthin: 0.17; lutein: 0.43; lycopene: 0.54; | | A |
| Zhu et al. 1997 | 31 M, 51-81 y, non-smoking, moderate or non-drinkers; 2 x 15 mg β-carotene daily for 4 wk taken one with each of the two main meals or placebo | β-Carotene: 2.74-0.28-(0.20-0.43); α-carotene: 0.06-0.06-(0.04-0.09); lutein: 0.23-0.40-(0.23-0.42); lycopene: 0.35-0.83-(0.34-0.76); β-cryptoxanthin: 0.16-0.29-(0.16-0.29) | | A |

[a] M, male; F, female; h, hours; d, days; wk, weeks; mo, months; y, years; n.s., not statistically significant. Unless otherwise stated, the final minus initial values, minus difference in response in the control group, are given. Where this is not available only the difference is provided.
[b] Serum or plasma carotenoid concentration shown as micromoles/liter.
[c] Serum or plasma retinol concentration, shown as micromoles/liter.
[d] SLAMENGHI factors: Species, molecular Linkage, Amount in meal, Matrix, Effectors, Nutrient status, Genetic factor, Host-related factors, Interactions.

## REFERENCES

Abbey A, Noakes M, Nestel PJ. Dietary supplementation with orange and carrot juice in cigarette smokers lowers oxidation products in copper-oxidized low-density lipoproteins. J Am Diet Assoc 1995;95:671-5.

Albanes D, Virtamo J, Rautalahti M, Haukka J, Palmgren J, et al. β-Carotene before and after β-carotene supplementation. Eur J Clin Nutr 1992;46:15-24.

Albanes D, Virtamo J, Taylor PR, Rautalahti M, Pietinen P, Heinonen OP. Effects of supplemental β-carotene, cigarette smoking, and alcohol consumption on serum carotenoids in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. Am J Clin Nutr 1997;66:366-72.

Allard JP, Royall D, Kurian R, Muggli R, Jeejeebhoy K. Effects of β-carotene supplementation on lipid peroxidation in humans. Am J Clin Nutr 1994;59:884-90.

Baker H, DeAngelis B, Baker E, Khalil M, Frank O. Human plasma patterns during 14 days ingestion of vitamin E, beta-carotene, ascorbic acid, and their various combinations. J Am Coll Nutr 1996;15:159-63.

Ben-Amotz A, Levy Y. Bioavailability of a natural isomer mixture compared with synthetic all-*trans* β-carotene in human serum. Am J Clin Nutr 1996; 63:729-34.

Borel P, Grolier P, Mekki N, Simonet L, Rochette C, et al. Medium-chain triglycerides reduce β-carotene bioavailability in healthy humans as compared to long-chain triglycerides. In: Int Congr Nutr, 16th, Ottawa, pp 123. Ottawa, Canada: IUNS Secretariat, 1997.

Bos RP, van Poppel G, Theuws JLG, Kok FJ. Decreased excretion of thioethers in urine of smokers after the use of β-carotene. Int Arch Occup Environ Health 1992;64:189-93.

Brown ED, Micozzi MS, Craft NE, Bieri JG, Beecher G, et al. Plasma carotenoids in normal men after a single ingestion of vegetables or purified β-carotene. Am J Clin Nutr 1989;49:1258-65.

Bulux J, Quan de Serrano J, Giuliano A, Perez R, Lopez CY. Plasma response of children to short-term chronic β-carotene supplementation. Am J Clin Nutr 1994;59:1369-75.

Canfield LM, Giuliano AR, Neilson EM, Yap HH, Graver EJ, Cui HA, Blashill BM. β-Carotene in breast milk and serum is increased after a single β-carotene dose. Am J Clin Nutr 1997;66:52-61.

Carughi A, Hooper FG. Plasma carotenoid concentrations before and after supplementation with a carotenoid mixture. Am J Clin Nutr 1994;59:896-9.

Cooney RV, Bertram JS, Hankin JH, Kolonel LN, Miyake A, Billings K, Bourne W. Relationship between dietary, serum, and tissue levels of carotenoids. Cancer Lett 1991;61:81-8.

Costantino JP, Kuller LH, Begg L, Redmond CK, Bates MW. Serum level changes after administration of a pharmacologic dose of β-carotene. Am J Clin Nutr 1988;48:1277-83.

Das BS, Thurnham DI, Das DB. Plasma α-tocopherol, retinol, and carotenoids in children with falciparum malaria. Am J Clin Nutr 1996;64:94-100.

Dimitrov NV, Meyer C, Ullrey DE, Chenoweth W, Michelakis A, et al. Bioavailability of β-carotene in humans. Am J Clin Nutr 1988;48:298-304.

Drewnowski A, Rock CL, Henderson SA, Shore AB, Fischler C, et al. Serum β-carotene and vitamin C as biomarkers of vegetable and fruit intakes in a community-based sample of French adults. Am J Clin Nutr 1997;65:1796-802.

Forman MR, Beecher GR, Lanza E, Reichman ME, Graubard BI, et al. Effect of alcohol consumption on plasma carotenoid concentrations in premenopausal women: a controlled dietary study. Am J Clin Nutr 1995;62:131-5.

Forman MR, Beecher GR, Muesing R, Lanza E, Olson B, et al. The fluctuation of plasma carotenoid concentrations by phase of the menstrual cycle: a controlled diet study. Am J Clin Nutr 1996;64:559-65.

Fotouhi N, Meydani M, Santos MS, Meydani SN, Hennekens CH, Gaziano JM. Carotenoid and tocopherol concentrations in plasma, peripheral blood mononuclear cells, and red blood cells after long-term β-carotene supplementation in men. Am J Clin Nutr 1996;63:553-8.

Friis H, Mwaniki D, Omondi B, Muniu E, Magnussen P, et al. Serum retinol concentrations and *Schistosoma mansoni*, intestinal helminths, and malarial parasitemia: a cross-sectional study in Kenyan preschool and primary school children. Am J Clin Nutr 1997;66:665-71.

Fuller CJ, Faulkner H, Bendich A, Parker RS, Roe DA. Effect of β-carotene supplementation on photosuppression of delayed-type hypersensitivity in normal young men. Am J Clin Nutr 1992;56:684-90.

Gärtner C, Stahl W, Sies H. Lycopene is more bioavailable from tomato paste than from fresh tomatoes. Am J Clin Nutr 1997;66:116-22.

Gärtner C, Stahl W, Sies H. Preferential increase in chylomicron levels of the xanthophylls lutein and zeaxanthin compared to β-carotene in the human. Internat J Vit Nutr Res 1996;66:119-25.

Gaziano JM, Johnson EJ, Russell RM, Manson JE, Stampfer MJ, et al. Discrimination in absorption or transport of β-carotene isomers after oral supplementation with either all-*trans*- or 9-*cis*-β-carotene. Am J Clin Nutr 1995;61:1248-52.

Herbst S, Bowen P, Hussain E, Stacewicz-Sapuntzakis M, Damayanti B, Burns J. Evaluation of the bioavailability of lutein (L) and lutein diesters (LD) in humans. FASEB J 1997;11:A447 (Abstr).

Hussein L, El-Tohamy M. Vitamin A potency of carrot and spinach carotenes in human metabolic studies. Internat J Vit Nutr Res 1990;60:229-35.

Jayarajan P, Reddy V, Mohanram M. Effect of dietary fat on absorption of β-carotene from green leafy vegetables. J Med Ind Res 1980;71:53-6.

Jensen CD, Howes TW, Spiller GA, Pattison TS, Whittam JH, Scala J. Observations on the effects of ingesting *cis* and *trans*-beta-carotene isomers on human serum concentrations. Nutr Rep Int 1987;35:413-22.

Jialal I, Norkus EP, Cristol L, Grundy SM. β-Carotene inhibits the oxidative modification of low-density lipoprotein. Biochim Biophys Acta 1991;1086:134-8.

Johnson EJ, Russell RM. Distribution of orally administered β-carotene among lipoproteins in healthy men. Am J Clin Nutr 1992;56:128-35.

Johnson EJ, Suter PM, Sahyoun N, Ribaya-Mercado JD, Russell RM. Relation between β-carotene intake and plasma and adipose tissue concentrations of carotenoids and retinoids. Am J Clin Nutr 1995;62:598-603.

Kostic D, White WS, Olson JA. Intestinal absorption, serum clearance, and interactions between lutein and β-carotene when administered to human adults in separate or combined oral doses. Am J Clin Nutr 1995;62:604-10.

Kramer TR, Burri BJ. Modulated mitogenic proliferative responsiveness of lymphocytes in whole-blood cultures after a low-carotene diet and mixed-carotenoid supplementation in women. Am J Clin Nutr 1997;65:871-5.

Lala VR, Reddy V. Absorption of β-carotene from green leafy vegetables in undernourished children. Am J Clin Nutr 1970;23:110-3.

Margetts BM, Jackson AA. The determinants of plasma β-carotene: interaction between smoking and other lifestyle factors. Eur J Clin Nutr 1996;50:236-8.

Martini MC, Campbell DR, Gross MD, Grandits GA, Potter JD, Slavin JL. Plasma carotenoids as biomarkers of vegetable intake: the University of Minnesota Cancer Prevention Research Unit Feeding Studies. Cancer Epidemiol Biomarkers Prev 1995;4:491-6.

Mayne S, Cartmel B, Silva F, Fallon B, Briskin K, et al. Effect of supplemental beta-carotene on plasma concentrations of carotenoids, retinol and alpha-tocopherol in humans. FASEB J 1997;11:A448.

Meydani M, Martin A, Ribaya-Mercado JD, Gong J, Blumberg JB, Russell RM. β-Carotene supplementation increases antioxidant capacity of plasma in older women. J Nutr 1994;124:2397-2403.

Micozzi MS, Brown ED, Edwards BK, Bieri JG, Taylor PR, et al. Plasma carotenoid response to chronic intake of selected foods and β-carotene supplements in men. Am J Clin Nutr 1992;55:1120-5.

Nierenberg DW, Bayrd GT, Stukel TA. Lack of effect of chronic administration of oral β-carotene on serum cholesterol and triglyceride concentrations. Am J Clin Nutr 1991;53:652-4.

Nierenberg DW, Dain BJ, Mott LA, Baron JA, Greenberg ER. Effects of 4 y of oral supplementation with β-carotene on serum concentrations of retinol, tocopherol, and five carotenoids. Am J Clin Nutr 1997;66:315-9.

Nierenberg DW, Stukel TA, Baron JA, Dain BJ, Greenberg ER, The Skin Cancer Prevention Study Group. Determinants of increase in plasma concentration of β-carotene after chronic oral supplementation. Am J Clin Nutr 1991;53:1443-9.

Novotny JA, Dueker SR, Zech LA, Clifford AJ. Compartmental analysis of the dynamics of β-carotene metabolism in an adult volunteer. J Lipid Res 1995;36:1825-38.

Pamuk ER, Byers T, Coates RJ, Vann JW, Sowell AL, et al. Effect of smoking on serum nutrient concentrations in African-American women. Am J Clin Nutr 1994;59:891-5.

Pee S de, West CE, Muhilal, Karyadi D, Hautvast JGAJ. Lack of improvement in vitamin A status with increased consumption of dark-green leafy vegetables. Lancet 1995;346:75-81.

Pee S de, West CE, Permaesih D, Martuti S, Muhilal, Hautvast JGAJ. Orange fruit is more effective than are dark-green, leafy vegetables in increasing serum concentrations of retinol and β-carotene in schoolchildren in Indonesia. Am J Clin Nutr 1998;68:1058-67.

Peng YM, Peng YS, Lin Y, Moon T, Roe DJ, Ritenbaugh C. Concentrations and plasma-tissue-diet relationships of carotenoids, retinoids, and tocopherols in humans. Nutr Cancer 1995;23:233-46.

Poppel G van, Kok FJ, Hermus RJJ. Beta-carotene supplementation in smokers reduces the frequency of micronuclei in sputum. Br J Cancer 1992;66:1164-8.

Prince MR, Frisoli JK, Goetschkes MM, Stringham JM, LaMuraglia GM. Rapid serum carotene loading with high-dose β-carotene: clinical implications. J Cardiovasc Pharmacol 1991;17:343-7.

Prince MR, Frisoli JK. Beta-carotene accumulation in serum and skin. Am J Clin Nutr 1993;57:175-81.

Rasmussen HM, Dallal GE, Phelan E, Russell RM. Serum concentrations of retinol and retinyl esters in adults in response to mixed vitamin A and carotenoid containing meals. J Am Coll Nutr 1991;10:460-5.

Ribaya-Mercado JD, Ordovas JM, Russell RM. Effect of β-carotene supplementation on the concentrations and distribution of carotenoids, vitamin E, vitamin A, and cholesterol in plasma lipoprotein and non-lipoprotein fractions in healthy older women. J Am Coll Nutr 1995;14:614-20.

Ringer TV, DeLoof MJ, Winterrowd GE, Francom SF, Gaylor SK, et al. Beta-carotene's effects on serum lipoproteins and immunologic indices in humans. Am J Clin Nutr 1991;53:688-94.

Rock CL, Swendseid ME. Plasma β-carotene response in humans after meals supplemented with dietary pectin. Am J Clin Nutr 1992;55:96-9.

Roels OA, Trout M, Dujacquier R. Carotene balances on boys in Ruanda where vitamin A deficiency is prevalent. J Nutr 1958;65:115-27.

Ross MA, Crosley LK, Brown KM, Duthie SJ, Collins AC, Arthur JR, Duthie GG. Plasma concentrations of carotenoids and antioxidant vitamins in Scottish males: influences of smoking. Eur J Clin Nutr 1995;49:861-5.

Seddon JM, Ajani UA, Sperduto RD, Hiller R, Blair N, et al. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. J Am Med Assoc 1994;272:1413-20.

Shiau A, Mobarhan S, Stacewicz-Sapuntzakis M, Benya R, Liao Y, et al. Assessment of the intestinal

retention of β-carotene in humans. J Am Coll Nutr 1994;13:369-75.

Stahl W, Sies H. Uptake of lycopene and its geometrical isomers is greater from heat-processed than from unprocessed tomato juice in humans. J Nutr 1992;122:2161-6.

Stryker WS, Kaplan LA, Stein EA, Stampfer MJ, Sober A, Willett WC. The relation of diet, cigarette smoking, and alcohol consumption to plasma beta-carotene and alpha-tocopherol levels. Am J Epidemiol 1988;127:283-96.

Sugarman SB, Mobarhan S, Bowen PE, Stacewicz-Sapuntzakis M, Langenberg P, et al. Serum time curve characteristics of a fixed dose of β-carotene in young and old men. J Am Coll Nutr 1991;10:297-307.

Tamai H, Morinobu T, Murata T, Manago M, Mino M. 9-*cis* β-carotene in human plasma and blood cells after ingestion of β-carotene. Lipids 1995;30:493-8.

Tang G, Serfaty-Lacrosniere C, Camilo ME, Russell RM. Gastric acidity influences the blood response to a β-carotene dose in humans. Am J Clin Nutr 1996;64:622-6.

Törrönen R, Lehmusaho M, Häkkinen S, Hänninen O, Mykkänen H. Serum β-carotene response to supplementation with raw carrots, carrot juice or purified β-carotene in healthy non-smoking women. Nutr Res 1996;16:565-75.

Vliet T van, Schreurs WHP, van den Berg H. Intestinal β-carotene absorption and cleavage in men: response of β-carotene and retinyl esters in the triglyceride-rich lipoprotein fraction after a single oral dose of β-carotene. Am J Clin Nutr 1995;62:110-6.

Wahlqvist ML, Wattanapenpaiboon N, Macrae FA, Lambert JR, MacLennan R, Hsu-Hage BHH, Australian Polyp Prevention Project Investigators. Changes in serum carotenoids in subjects with colorectal adenomas after 24 mo of β-carotene supplementation. Am J Clin Nutr 1994;60:936-43.

Watson RR, Prabhala RH, Plezia PM, Alberts DS. Effect of β-carotene on lymphocyte subpopulations in elderly humans: evidence for a dose-response relationship. Am J Clin Nutr 1991;53:90-4.

Weststrate JA, van het Hof KH. Sucrose polyester and plasma carotenoid concentrations in healthy subjects. Am J Clin Nutr 1995;62:591-7.

White WS, Stacewicz-Sapuntzakis M, Erdman JW Jr, Bowen PE. Pharmacokinetics of β-carotene and canthaxanthin after ingestion of individual and combined doses by human subjects. J Am Coll Nutr 1994;13:665-71.

Willett WC, Stampfer MJ, Underwood BA, Taylor JO, Hennekens CH. Vitamins A, E, and carotene: effects of supplementation on their plasma levels. Am J Clin Nutr 1983;38:559-66.

Wingerath T, Stahl W, Sies H. β-Cryptoxanthin selectively increases in human chylomicrons upon ingestion of tangerine concentrate rich in β-cryptoxanthin esters. Arch Biochem Biophys 1995;324:385-90.

Winklhofer-Roob BM, van 't Hof MA, Shmerling DH. Reference values for plasma concentrations of vitamin E and A and carotenoids in a Swiss population from infancy to adulthood, adjusted for seasonal influences. Clin Chem 1997;43:146-53.

Winklhofer-Roob BM, van 't Hof MA, Shmerling DH. Response to oral β-carotene supplementation in patients with cystic fibrosis: a 16-month follow-up study. Acta Paediatr 1995;84:1132-6.

Xu MJ, Plezia PM, Alberts DS, Emerson SS, Peng YM. Reduction in plasma or skin alpha-tocopherol concentration with long-term oral administration of beta-carotene in humans and mice. J Natl Cancer Inst 1992;84:1559-65.

Yeum KJ, Booth SL, Sadowski JA, Liu C, Tang G, et al. Human plasma carotenoid response to the ingestion of controlled diets high in fruits and vegetables. Am J Clin Nutr 1996;64:594-602.

Yong LC, Forman MR, Beecher GR, Graubard BI, Campbell WS, et al. Relationship between dietary intake and plasma concentrations of carotenoids in premenopausal women: application of the USDA-NCI carotenoid food-composition database. Am J Clin Nutr 1994;60:223-30.

Zhu YI, Hsieh WC, Parker RS, Herraiz LA, Haas JD, Swanson JE, Roe DA. Evidence of a role for fat-free body mass in modulation of plasma carotenoid concentrations in older men: studies with hydrodensitometry. J Nutr 1997;127:321-6.

# 3

# The food matrix of spinach is a limiting factor in determining the bioavailability of β-carotene and to a lesser extent of lutein in humans

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## ABSTRACT

Carotenoid bioavailability depends, amongst other factors, on the food matrix and on the type and extent of processing. To examine the effect of variously processed spinach products and of dietary fibre on serum carotenoid concentrations, subjects received, over a 3-week period, a control diet ($n$ = 10) or a control diet supplemented with carotenoids or one of four spinach products ($n$ = 12 per group): whole-leaf spinach with an almost intact food matrix, minced spinach with the matrix partially disrupted, enzymatically liquefied spinach in which the matrix was further disrupted and the liquefied spinach to which dietary fibre (10 g/kg wet weight) was added. Consumption of spinach significantly increased serum concentrations of all-*trans*-β-carotene, *cis*-β-carotene, (and consequently total β-carotene), lutein, α-carotene and retinol and decreased the serum concentration of lycopene. Serum total β-carotene responses (changes in serum concentrations from the start to the end of the intervention period) differed significantly between the whole-leaf and liquefied spinach groups and between the minced and liquefied spinach groups. The lutein response did not differ among spinach groups. Addition of dietary fibre to the liquefied spinach had no effect on serum carotenoid responses. The relative bioavailability as compared to bioavailability of the carotenoid supplement for whole-leaf, minced, liquefied and liquefied spinach plus added dietary fibre for β-carotene was 5.1, 6.4, 9.5 and 9.3%, respectively, and for lutein 45, 52, 55 and 54%, respectively. We conclude that the bioavailability of lutein from spinach was higher than that of β-carotene and that enzymatic disruption of the matrix (cell wall structure) enhanced the bioavailability of β-carotene from whole-leaf and minced spinach, but had no effect on lutein bioavailability.

**KEY WORDS:** carotenoids, bioavailability, processing spinach, dietary fibre, humans

## INTRODUCTION

Carotenoids have many functions in the human body, including being precursors of retinol and retinoids, and acting as antioxidants (Krinsky 1993). They are found in dark-green, leafy vegetables; in yellow and orange coloured fruits and vegetables; and, to a lesser extent, in animal products. Carotenoids are found in the chloroplasts of all green plant tissues, where they occur in the photosynthetic pigment-protein complexes, and in the chromoplasts of fruits. Some carotenoids, including β-carotene, lycopene, and some oxycarotenoids such as lutein and zeaxanthin, exert antioxidant activity in lipid phases by quenching oxygen or free radicals (Sies & Stahl 1995). This may explain why a higher dietary intake of carotenoids has been found to be associated with a lower risk for age-related macular degeneration (Seddon et al. 1994) and some forms of cancer and cardiovascular disease (van Poppel 1996).

Bioavailability is defined as the fraction of an ingested nutrient that is available to the body for utilisation in normal physiological functions or for storage (Jackson 1997). We have developed the mnemonic SLAMENGHI, which includes all factors that could affect bioavailability, especially bioavailability of β-carotene and other carotenoids (Castenmiller & West 1998, de Pee & West 1996). The SLAMENGHI factors are as follows: *S*pecies of carotenoids, molecular *L*inkage, *A*mount of carotenoids consumed in a meal, *M*atrix in which the carotenoid is incorporated, *E*ffectors of absorption and bioconversion, *N*utrient status of the host, *G*enetic factors, *H*ost-related factors, and *I*nteractions. Current information on carotenoid bioavailability is limited, fragmentary, and often conflicting. Apart from the many factors, which determine bioavailability, the lack of adequate indicators has made it difficult to establish the bioavailability of carotenoids in food.

Carotenes dissolved in oil are more readily absorbed than when they are incorporated in foods such as fruits and vegetables. The plasma response to ingestion of β-carotene dissolved in oil was found to be about five times the response to a similar amount of β-carotene in carrots (Brown et al. 1989, Micozzi et al. 1992). Recent studies in Indonesia have shown that feeding β-carotene from dark-green, leafy vegetables produced a lower plasma response than similar quantities of β-carotene in a fat matrix (de Pee et al. 1995). The serum response to β-carotene from fruits was four times higher than that from vegetables (de Pee et al. 1998). Cooking or fine grinding of foods could increase the bioavailability of carotenes by disrupting or softening plant cell walls and disrupting carotenoid-protein complexes (Hussein & El-Tohamy 1990, van Zeben & Hendriks 1948).

The greater ease with which carotenoids in thermally treated foods can be extracted during analysis may imply that they are also biologically more available.

We describe a controlled dietary intervention study with differently processed spinach products to examine the effect of the food matrix on carotenoid concentrations in human serum. Different processing techniques were used as a means of testing the role of cellular structure. One of the spinach products was treated with a mixture of enzymes, which causes disruption of the cell wall structure as well as depolymerisation of the cell wall components, reducing the dietary fibre content of the spinach product. Dietary pectin added to meals reduced plasma responses to β-carotene in humans (Rock & Swendseid 1992). Thus, by adding sugar beet fibre, we also examined the effect of restoring fibre to the enzyme-treated spinach on serum carotenoid concentrations. Although the fibre added replaces that lost during enzyme treatment, it did not restore cell wall structure or cellular integrity.

## MATERIALS AND METHODS

### Subjects

The subjects were 72 healthy, non-smoking, normolipidemic volunteers: 42 women and 30 men, aged 18-58 y. The subjects, students of Wageningen Agricultural University and other residents of the Wageningen area, were recruited through local advertisements and all gave their written informed consent. None of the subjects were taking oral medication, apart from oral contraceptives, nor supplements of any kind during the last 3 mo before the study started and during the study period. Subjects were screened for fasting elevated glucose and protein levels in urine and for low haemoglobin and abnormal haematology. All subjects completed a medical and a general questionnaire and food frequency questionnaires to estimate their habitual intakes of energy, carotenoids and vitamin A. All subjects had normal body mass indices (18-28 $kg/m^2$), fasting serum cholesterol concentrations $< 6.5$ mmol/L and fasting triacylglycerol concentrations $< 2.8$ mmol/L. During the study, two male volunteers withdrew from the study for personal reasons. Characteristics of the subjects are presented in Table 1. The protocol for this study was approved by the Medical-Ethical Committee of the Division of Human Nutrition and Epidemiology of Wageningen Agricultural University.

**Table 1. Characteristics of subjects participating in a study to determine the bioavailability of β-carotene and lutein from differently processed spinach products[1]**

| | Group | | | | | |
|---|---|---|---|---|---|---|
| | Control | Carotenoid supplement | Whole-leaf spinach | Minced spinach | Liquefied spinach | Liquefied spinach plus dietary fibre |
| Men, women; *n, n* | 3, 7 | 5, 7 | 5, 7 | 5, 7 | 5, 7 | 5, 7 |
| Age, *y* | 21 (18, 25)[2] | 21 (18, 24) | 20 (18, 58) | 21 (18, 42) | 21 (18, 38) | 20 (18, 54) |
| Energy (estimated), *MJ/d* | 11.4 ± 2.6 | 12.8 ± 3.4 | 11.5 ± 2.3 | 11.3 ± 2.1 | 11.4 ± 2.1 | 11.4 ± 2.3 |
| Serum cholesterol, *mmol/L* | 4.14 ± 0.65 | 4.07 ± 0.61 | 4.11 ± 0.79 | 4.23 ± 0.74 | 3.93 ± 0.47 | 4.17 ± 0.87 |
| Serum triacylglycerol, *mmol/L* | 0.88 ± 0.41 | 0.87 ± 0.32 | 0.92 ± 0.32 | 1.00 ± 0.39 | 0.89 ± 0.27 | 1.00 ± 0.34 |
| Body mass index, *kg/m²* | 21 ± 2.1 | 22 ± 2.3 | 22 ± 2.0 | 22 ± 2.4 | 23 ± 2.1 | 22 ± 1.8 |

[1] Data collected at beginning of study except for energy intake which is the mean over the intervention period. Values are means ± SD.
[2] Medians (ranges).

### Study design

The study started with a 3-wk run-in period followed by a dietary intervention period of 3 wk. During the intervention period, subjects were allocated to one of six experimental groups. Blood was taken by venipuncture from fasting subjects between 7.15 and 10.00 h at the start of the run-in period and at days 0, 1, 8, 15, 21 and 22 of the dietary intervention period. Blood samples, to which no anticoagulant was added, were left to clot and were centrifuged within 1 h after being drawn. The serum was separated and stored at -80°C until analysis.

During the first 3 wk of the study (run-in period), subjects chose their own diets but were instructed to avoid foods rich in carotenoids and vitamin A. During the dietary intervention period, subjects were supplied with total diets, except for a limited choice of free products (approximately 10 energy%), which did not contain carotenoids or retinol. Subjects received a hot meal at the Division of Human Nutrition and Epidemiology and foods for their other meals and snacks were packed to be taken home. A maximum of two alcoholic consumptions per day was allowed but was not permitted to be consumed together with the hot meal. Duplicate portions of diets were collected for chemical analysis. The daily

selection of free choice foods was recorded in a diary, and the nutrient content was calculated (Stichting NEVO 1995). Individual body weights throughout the study were maintained at ± 2 kg.

**Diet**

All subjects in the six treatment groups were fed the same control diet throughout the study, and the menu was changed daily on a weekly cycle. The control diet consisted of foods, other than fruits and vegetables with moderate or high amounts of carotenoids, and met the requirements of Dutch Recommended Daily Allowances (Netherlands Food and Nutrition Council 1992). Four groups received a spinach product, one control group received no additional source of carotenoids and one group received a carotenoid supplement. The carotenoid supplement was a suspension in vegetable oil of microcrystalline β-carotene (40 g/kg; Hoffmann-La Roche, Basel, Switzerland) and crystalline lutein and zeaxanthin derived from marigold flowers (60 g/kg and 3 g/kg, respectively; FloraGLO, Kemin Industries, Des Moines, IA), added to salad dressing. The energy content of the diets of the control group and carotenoid supplement group was adjusted to that of the spinach groups by providing extra amounts of appropriate foods. The spinach groups received 20 g whole-leaf spinach, minced spinach, liquefied spinach or liquefied spinach to which dietary fibre was added/MJ. All spinach products originated from one batch and were prepared and provided by Langnese-Iglo in Wunstorf (Germany) for Unilever Research Vlaardingen (The Netherlands). Four different spinach products were prepared: whole-leaf spinach with an almost intact food matrix; minced spinach in which the matrix is partially disrupted; enzymatically liquefied spinach in which the matrix is disrupted; and the enzymatically liquefied spinach to which dietary fibre was added. The whole-leaf spinach was washed and subsequently blanched for 90 s and cooled down quickly. The minced spinach was minced to 5 mm after blanching. The liquefied spinach was prepared by treating minced spinach with an enzymatic preparation with pectinase, hemicellulase and cellulase activities (Rapidase LIQ plus, Gist-brocades, Seclin, France) during 2.5 h at 35°C. After the enzyme treatment, the spinach was boiled for 5-10 min to inactivate the enzymes. The spinach products were frozen immediately after processing. Liquefaction resulted in a breakdown of cell wall material. Therefore, the fourth group received the liquefied spinach plus fibre prepared from sugar beet (10 g/kg wet weight, Fibrex 600, TEFCO Food Ingredients b.v., Bodegraven, The Netherlands) to compensate for the loss of dietary fibre. This fibre product contains per 100 g of product, 73 g of dietary fibre of which one third is soluble and 22 g is pectin. All frozen spinach was thawed

and heated by microwave before consumption. The spinach products contained no measurable nitrite, and the content of nitrate was less than 1000 mg/kg, thus ensuring that the nitrate intake of the subjects was below the Acceptable Daily Intake for nitrate (FAO/WHO 1995). Microbiological counts showed normal values and confirmed that the spinach products were safe for human consumption.

**Serum measurements**

Serum carotenoids and retinol were measured by high-performance liquid chromatography (HPLC) (Craft et al. 1992). To avoid day-to-day analytic variations, all samples from an individual were analysed sequentially as a set. After precipitation with ethanol, extraction followed with hexane twice; samples were evaporated under nitrogen and injected into the HPLC system described below. Serum cholesterol and triacylglycerol concentrations were analysed with the Abbott Spectrum high performance diagnostic system following a standard procedure (Siedel et al. 1983, Sullivan et al. 1985).

**Food measurements**

The duplicate portions of the daily food intake of one subject collected throughout the study were mixed thoroughly as weekly portions, and subsequently pooled samples were stored at -20°C until analysis. The moisture level and the ash content in each weekly portion were determined (Osborne & Voogt 1978) using a vacuum oven at 85°C and a muffle furnace at 550°C. The protein concentration was determined by the Kjeldahl method using a conversion factor of 6.25. The Folch method (Folch et al. 1957) was used to extract fat. Digestible carbohydrate was calculated by difference. Dietary fibre was analysed according to the AOAC Official Method 992.16 for total dietary fibre (AOAC 1996). The dietary fibre content of the various spinach products was determined as methanol-ether insoluble solids (MEIS) and the amount of pectin (polygalacturonic acid) in the spinach products was measured by hydrolysing pectic substances to water-soluble galacturonic acid using an enzyme preparation (Katan & van de Bovenkamp 1981). Carotenoids and retinol were determined in the pooled samples and in spinach products and salad dressings containing the carotenoid supplement. Carotenoids and retinol were extracted from wet material after homogenisation, using tetrahydrofuran (THF) and redissolved in THF/methanol (1:1 v/v) and injected into the HPLC system described below.

**Chemicals and instrumentation for carotenoid and retinol analysis**

Pure grades of all-*trans*-α-carotene, all-*trans*-β-carotene and lycopene were

obtained from Sigma Chemical Co. (St Louis, MO) and all-*trans*-lutein, all-*trans*-zeaxanthin, and all-*trans*-β-cryptoxanthin from Hoffmann-La Roche Ltd (Basel, Switzerland). The carotenoid concentrations of the standards were measured in a Zeiss M4 QIII spectrophotometer (Carl Zeiss, Oberkochen/Württemberg, Germany). The HPLC system, manufactured by Thermo Separation Products (San Jose, CA), was equipped with a pump (P4000), a solvent degasser (SCM1000), a temperature controlled autosampler (AS3000), a UV-visible forward optical scanning detector (Spectra Focus UV3000), interface (SN4000), and control and integration software (PC1000, version 3.0). A reversed phase Vydac 218 TP 54 column containing silica polymerically modified with C18 from The Separations Group (Hesperia, CA) was used, and the metal frits in the column were replaced by PAT (Peek Alloyed with Teflon) frits from the same manufacturer to minimise carotenoid degradation on the column. The mobile phase consisted of a mixture of methanol and tetrahydrofuran (98:2 v/v). For each series of analyses of serum samples, a control serum sample was analysed. The coefficients of variation (CV) within runs for serum analysis of α-carotene, β-carotene, lutein, zeaxanthin, β-cryptoxanthin, lycopene and retinol in control pools averaged 7.4, 3.9, 3.6, 8.7, 4.5, 10.4 and 1.6%, respectively. For each series of analyses of food samples, a control sample (homogenised baby food) was extracted in duplicate and injected into the HPLC system for monitoring the stability of the analytical procedure over time. The CV within runs for food analysis of α-carotene, β-carotene and lutein in control pools averaged 5.7, 6.8 and 8.8%, respectively. All sample preparations and extractions were carried out in duplicate and under subdued yellow light with minimal exposure to oxygen (Hulshof et al. 1997).

**Statistical analysis**

Serum concentrations were averaged for d 0 and 1 and for d 21 and 22 for each subject. For each person the response to treatment was calculated as the change in serum concentrations from the start to the end of the intervention period. Two-tailed *t*-tests for independent samples were performed to evaluate differences in serum responses between the control and treatment groups and between the carotenoid supplement and pooled spinach groups. To compare differences in responses among the groups receiving various spinach products, after significant F-tests (ANOVA) the Tukey method for multiple comparisons was used (Godfrey 1985). Analyses were carried out using general linear models to compare differences in response among intervention groups, controlling for several factors and covariables, including sex, vegetarian diet, age, body mass index, change in

cholesterol and triacylglycerol concentrations. Spearman correlation coefficients for the entire study group were calculated when relevant. Differences associated with $P < 0.05$ were regarded as significant (SPSS/PC 7.5, 1997, SPSS Inc., Chicago, IL).

The relative bioavailability of β-carotene and lutein was calculated by dividing the serum response (μmol/L) relative to the intake of the respective carotenoid in each spinach group (mg/MJ) by the serum response to the carotenoid in the supplement group relative to the intake of carotenoid in the carotenoid supplement (mg/MJ). The serum carotenoid responses were adjusted for serum responses in the control group, and the amount of carotenoid consumed (mg/MJ) by the control group was subtracted from the amount consumed by each spinach group.

## RESULTS

### Carotenoids

The composition of the diets is given in Table 2. Consumption of spinach significantly increased the serum concentrations of all-*trans*-β-carotene, *cis*-β-carotene (and consequently total β-carotene), lutein (Table 3, Fig. 1 and Fig. 2), α-carotene and retinol ($P < 0.05$); decreased the serum concentration of lycopene as compared to the control group ($P < 0.05$); and had no effect on serum concentrations of zeaxanthin and β-cryptoxanthin. The serum responses (changes in serum concentrations from the start to the end of the intervention period) of total β-carotene and lutein were significantly related ($r_s = 0.51$; $P < 0.001$). The α-carotene response was significantly correlated to the total β-carotene response ($r_s = 0.69$; $P < 0.001$) and the all-*trans*-β-carotene and *cis*-β-carotene responses were significantly correlated ($r_s = 0.79$; $P < 0.001$).

There were significant differences only among the spinach groups for serum responses of β-carotene: total β-carotene responses were significantly different between the whole-leaf and liquefied spinach group ($P = 0.03$) and between the minced and liquefied spinach groups ($P = 0.05$), and all-*trans*-β-carotene responses were significantly different between the whole-leaf and liquefied spinach groups ($P = 0.03$). The serum *cis*-β-carotene and lutein responses were similar among spinach groups. In a multiple linear regression model with all spinach groups and with serum total β-carotene response as dependent variable, treatment (group) and body mass index were significant in the model, whereas for

**Table 2. Total daily intake of carotenoids, dietary fibre and energy of subjects participating in a study to examine the bioavailability of β-carotene and lutein from various spinach products[1]**

| | Group | | | | | |
|---|---|---|---|---|---|---|
| | Control | Carotenoid supplement | Whole-leaf spinach | Minced spinach | Liquefied spinach | Liquefied spinach plus dietary fibre |
| Total β-carotene, *mg/d* | 0.5 | 9.8 | 10.4 | 8.8 | 9.0 | 8.8 |
| All-*trans*-β-carotene, *mg/d* | 0.4 | 8.9 | 8.8 | 7.4 | 7.6 | 7.4 |
| *Cis*-β-carotene, *mg/d* | 0.1 | 0.9 | 1.7 | 1.4 | 1.4 | 1.4 |
| Lutein, *mg/d* | 0.5 | 6.6 | 12.6 | 11.2 | 11.3 | 10.9 |
| Dietary fibre, *g/d* | 27.4 | 25.2 | 32.4 | 32.3 | 28.2 | 31.3 |
| Energy, *MJ* | 10.6 | 10.7 | 10.8 | 10.8 | 10.8 | 10.8 |

[1] Results are based on the analysis of duplicate portions for one subject per group consuming approximately 11 MJ together with an estimate calculated from the composition of the free items consumed. The free items did not contain carotenoids or retinol. There were no significant differences in nutrient intakes among the 3 wk of dietary intervention. The mean daily intake of macronutrients and alcohol was as follows (energy%): protein, 13.9; fat, 34.1; carbohydrate, 51.0; alcohol, 1.0.

the serum lutein response, only the serum cholesterol response was significant (data not shown).

The carotenoid supplement group had significantly higher responses of serum concentrations of all-*trans*-β-carotene, *cis*-β-carotene, (and consequently total β-carotene), lutein (Table 3), zeaxanthin and α-carotene than the control group, and significantly higher responses of all-*trans*-β-carotene, *cis*-β-carotene, (and consequently total β-carotene) (Table 3), zeaxanthin, α-carotene, lycopene and β-cryptoxanthin than the pooled spinach groups. Thus, the food matrix (cellular structure) played an important role in the uptake of β-carotene from spinach. Processing spinach in various ways did not affect lutein bioavailability.

### Dietary fibre

The all-*trans*-β-carotene, *cis*-β-carotene, total β-carotene and lutein serum responses in the liquefied spinach group were not different from the responses in the liquefied spinach plus added dietary fibre group. Adding 10 g of sugar beet fibre per kg wet weight of spinach had no effect on serum carotenoid response. The enzyme-treated spinach contained 13% less MEIS (methanol-ether insoluble solids) and 17% less polygalacturonic acid than the whole-leaf spinach, which

contained per 100 g dry matter 71 g MEIS and 11 g polygalacturonic acid, respectively. The composition of the sugar beet fibre added to spinach is comparable to the fibre present in the whole-leaf spinach, but had a higher amount of pectin.

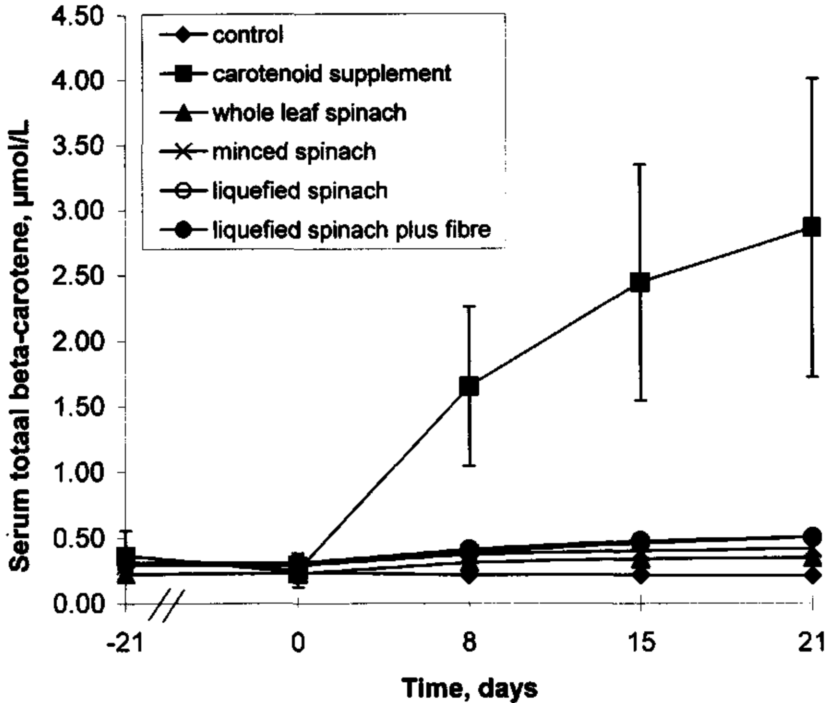


**Figure 1.** Serum β-carotene concentrations in groups of healthy subjects fed a control diet or a control diet with a carotenoid supplement or with various spinach products. Values are expressed as means and as means ± SD for the carotenoid supplement group; $n = 12$, for control group: $n = 10$. Serum concentrations were averaged for d 0 and 1 (d 0) and for d 21 and 22 (d 21) for each subject. For significant differences among the study groups, see Table 3.

## Retinol

The decrease in serum concentration of retinol was significantly less ($P = 0.04$) in the pooled spinach groups than in the control group. Serum retinol concentrations decreased in the control group (7.6%), in the minced spinach group (5.1%) and liquefied spinach plus dietary fibre group (2.9%), but increased in the carotenoid supplement group (2.9%), whole-leaf spinach group (1.4%) and liquefied spinach group (0.4%).

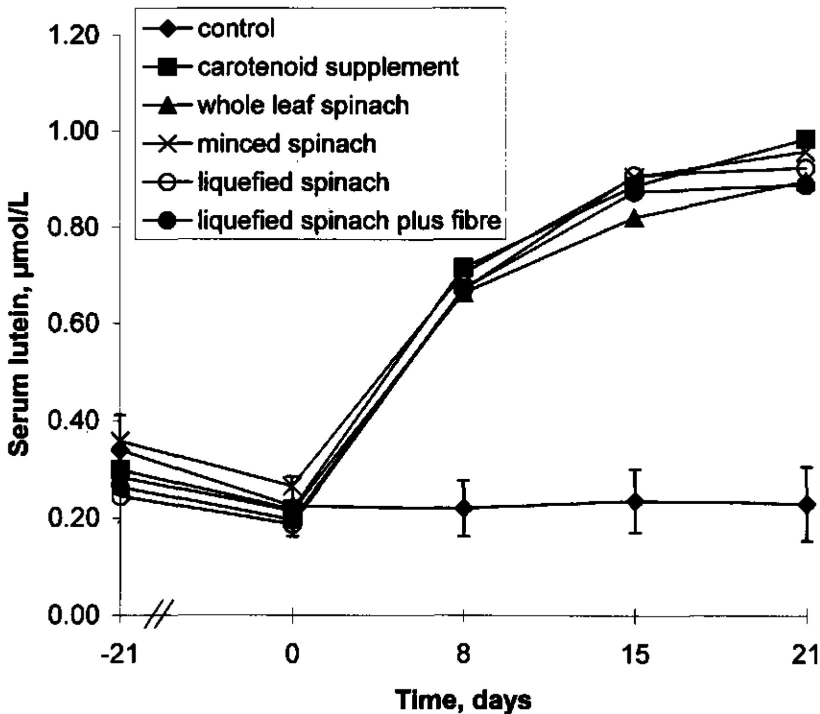


**Figure 2.** Serum lutein concentrations in groups of healthy subjects fed a control diet or a control diet with a carotenoid supplement or with various spinach products. Values are expressed as means and as means ± SD for the control group; $n$ = 12, for control group: $n$ = 10. Serum concentrations were averaged for d 0 and 1 (d 0) and for d 21 and 22 (d 21) for each subject. For significant differences among the study groups, see Table 3.

**Cholesterol and triacylglycerol**

The changes in serum cholesterol and triacylglycerol concentrations after 3 wk of dietary intervention (serum cholesterol and triacylglycerol responses) of the carotenoid supplement group and pooled spinach groups were not significantly different from the response in the control group. Normalised serum concentrations of total β-carotene and lutein were calculated as follows: carotenoid concentration/(cholesterol + triacylglycerol concentration). Adjustment of serum carotenoid concentrations for concentrations of cholesterol and other lipids may provide a better reflection of dietary intake of carotenoids (Willett et al. 1983). Analysis of the normalised serum carotenoid concentrations of total β-carotene and lutein showed significant differences of the normalised β-carotene responses

**Table 3. Serum concentrations of β-carotene and lutein 3 wk before dietary intervention (wk -3), at the start of the dietary intervention period (wk 0), after 3 wk of dietary intervention (wk 3) and responses from begin to end of the dietary intervention period in healthy subjects fed various spinach products[1]**

| | Group | | | | | |
|---|---|---|---|---|---|---|
| | Control | Carotenoid supplement | Whole-leaf spinach | Minced spinach | Liquefied spinach | Liquefied spinach plus dietary fibre |
| | | | μmol/L | | | |
| *n* | 10[2] | 12 | 12 | 12 | 12 | 12 |
| Total β-carotene | | | | | | |
| Wk −3 | 0.368 ± 0.141 | 0.362 ± 0.193 | 0.376 ± 0.172 | 0.436 ± 0.220 | 0.447 ± 0.194 | 0.456 ± 0.215 |
| Wk 0 | 0.234 ± 0.115 | 0.253 ± 0.130 | 0.229 ± 0.091 | 0.291 ± 0.139 | 0.301 ± 0.124 | 0.310 ± 0.193 |
| Wk3 | 0.213 ± 0.075 | 2.862 ± 1.141 | 0.350 ± 0.104 | 0.419 ± 0.176 | 0.505 ± 0.157 | 0.505 ± 0.206 |
| Response | -0.021 ± 0.103$^{3,a}$ | 2.608 ± 1.056$^{3,a}$ | 0.121 ± 0.072$^{b}$ | 0.127 ± 0.074$^{c}$ | 0.204 ± 0.079$^{b,c}$ | 0.195 ± 0.059 |
| All-*trans*-β-carotene | | | | | | |
| Wk −3 | 0.349 ± 0.132 | 0.343 ± 0.179 | 0.354 ± 0.158 | 0.413 ± 0.205 | 0.421 ± 0.185 | 0.434 ± 0.205 |
| Wk 0 | 0.217 ± 0.100 | 0.239 ± 0.015 | 0.216 ± 0.087 | 0.275 ± 0.130 | 0.286 ± 0.119 | 0.293 ± 0.184 |
| Wk 3 | 0.201 ± 0.071 | 2.692 ± 1.078 | 0.330 ± 0.100 | 0.397 ± 0.164 | 0.480 ± 0.149 | 0.478 ± 0.192 |
| Response | -0.015 ± 0.085$^{3,d}$ | 2.453 ± 1.000$^{3,d}$ | 0.114 ± 0.069$^{e}$ | 0.122 ± 0.070 | 0.194 ± 0.076$^{e}$ | 0.185 ± 0.057 |
| *Cis*-β-carotene | | | | | | |
| Wk −3 | 0.020 ± 0.009 | 0.020 ± 0.014 | 0.022 ± 0.014 | 0.023 ± 0.016 | 0.026 ± 0.013 | 0.022 ± 0.012 |
| Wk 0 | 0.017 ± 0.019 | 0.014 ± 0.009 | 0.013 ± 0.006 | 0.017 ± 0.010 | 0.015 ± 0.005 | 0.017 ± 0.010 |
| Wk 3 | 0.011 ± 0.005 | 0.169 ± 0.067 | 0.019 ± 0.008 | 0.022 ± 0.013 | 0.025 ± 0.010 | 0.027 ± 0.014 |
| Response | -0.006 ± 0.020$^{4,f}$ | 0.155 ± 0.060$^{3,f}$ | 0.007 ± 0.008 | 0.005 ± 0.007 | 0.011 ± 0.007 | 0.010 ± 0.006 |
| Lutein | | | | | | |
| Wk −3 | 0.340 ± 0.071 | 0.299 ± 0.079 | 0.284 ± 0.094 | 0.358 ± 0.134 | 0.245 ± 0.058 | 0.263 ± 0.109 |
| Wk 0 | 0.224 ± 0.061 | 0.218 ± 0.064 | 0.215 ± 0.061 | 0.266 ± 0.090 | 0.187 ± 0.046 | 0.197 ± 0.066 |
| Wk 3 | 0.229 ± 0.075 | 0.983 ± 0.287 | 0.895 ± 0.336 | 0.957 ± 0.280 | 0.922 ± 0.191 | 0.887 ± 0.291 |
| Response | 0.005 ± 0.041$^{3,g}$ | 0.765 ± 0.240$^{g}$ | 0.680 ± 0.318 | 0.691 ± 0.228 | 0.735 ± 0.181 | 0.691 ± 0.232 |

[1] Means ± SD.
[2] *n* = 10, for wk -3: *n* = 9.
[3,4] Significantly different from pooled spinach groups, $P < 0.001$ and $P < 0.05$, respectively.
[a,d,f,g; b,c,e] Means with common superscript letter are significantly different, $P < 0.001$ and $P < 0.05$, respectively.

between the whole-leaf spinach and liquefied spinach group ($P = 0.05$) and between the whole-leaf spinach group and liquefied spinach plus added dietary fibre group ($P < 0.02$).

**Diets**

The chemical analysis of the diets supplied showed consistent data for the 3 wk and among the treatment groups. Surprisingly, the enzyme-treated spinach products, which were treated for 2.5 h at 35°C, did not have a higher concentration of *cis*-β-carotene than the whole-leaf spinach. Chemical analysis of the salad dressings showed that the carotenoid supplement should provide daily per 11 MJ energy intake: β-carotene, 10.9 mg (all-*trans*-β-carotene, 10.5 mg; *cis*-β-carotene, 0.4 mg); lutein, 12.2 mg; and zeaxanthin, 1.0 mg. However, from the analysis of the duplicate portions of ~ 11 MJ of the carotenoid supplement group, the daily intake of lutein was 46% lower (6.6 ± 0.2 mg) than was to be expected from the lutein concentrations in the salad dressings, whereas the daily intake of β-carotene in the duplicate food portions was 10% lower than expected based on the amounts analysed in the salad dressing (Table 2). Prepared spinach products, as consumed during the dietary intervention period, contained 15-18% of the β-carotene as *cis* isomers. The amounts of total β-carotene and lutein in the duplicate food portions were 0-9% and 31-43%, respectively, lower than those expected to be present in the daily food from analysis of the spinach products. The amount of carotenoids measured in the duplicate portions was used to calculate relative bioavailability.

**Estimate of relative bioavailability**

Based on the analysis of the duplicate portions, the bioavailability of β-carotene from spinach as compared to the carotenoid supplement was 5.1, 6.4, 9.5 and 9.3% for whole-leaf, minced, liquefied and liquefied spinach plus added dietary fibre, respectively. The bioavailability of β-carotene from whole-leaf spinach was calculated as follows:

$[(0.121 - -0.021)/(2.61 - -0.021)] \times [(9.8/10.7 - 0.5/10.6)/(10.4/10.8 - 0.5/10.6)] \times 100\%$.

The relative bioavailability of lutein from spinach was 45, 52, 55 and 54% for whole-leaf, minced, liquefied and liquefied spinach plus added dietary fibre, respectively. Thus, enzymatic treatment increased the relative bioavailability of β-carotene in spinach by about half. The relative bioavailability of lutein in spinach was more than five times higher than that of β-carotene and was not affected by the enzymatic treatment.

## DISCUSSION

Consumption of processed spinach products significantly increased serum concentrations of all-*trans*-β-carotene, *cis*-β-carotene, (and consequently total β-carotene), lutein, β-carotene and retinol and decreased serum concentrations of lycopene. For β-carotene, but not for lutein, there was a significant effect of processing: liquefied spinach, in which the vegetable food matrix is disrupted, produced a higher serum response than whole-leaf spinach, where the food matrix is still intact, and minced spinach. Addition of dietary fibre to liquefied spinach does not restore the cellular structure but did compensate for the fibre that was broken down and had no effect on serum responses of carotenoids as compared to serum responses after consumption of liquefied spinach.

There are three possible explanations for the low bioavailability of β-carotene (5.1-9.5%) from spinach as compared to the carotenoid supplement. First is the food matrix in which the carotenoids are embedded. Other investigators also found a low bioavailability of β-carotene from vegetables as compared to pure β-carotene: stir-fried vegetables, 7% (de Pee et al. 1995); carrots, 18-26% (Brown et al. 1989, Micozzi et al. 1992, Törrönen et al. 1996). Rock et al. (1998) found a trend for a greater percentage increase in plasma concentrations of total β-carotene in the period when thermally processed and pureed carrots and spinach were fed to healthy women as compared to the period when these women were fed raw carrots and spinach.

Second is the isomeric form of β-carotene in spinach. The carotenoid supplement dissolved in oil contained mainly all-*trans*-β-carotene (approximately 96%), whereas in the spinach products 15-18% of the β-carotene was present as *cis* isomers. Several investigators have shown that all-*trans*-β-carotene is more readily absorbed than its *cis* isomers (Ben-Amotz & Levy 1996, Gaziano et al. 1995, Jensen et al. 1987, Tamai et al. 1995). Rock et al. (1998) recently found that feeding subjects thermally processed and pureed spinach that provided an increased proportion of *cis*-β-carotene was not associated with a significant increase in the plasma *cis*-β-carotene concentration after 4 wk. They concluded that isomerisation of β-carotene in foods by heat treatment does not negate the enhanced β-carotene uptake associated with consuming processed vegetables compared with raw vegetables. In our study, a significant increase in the serum concentrations of the *cis*-β-carotene was observed in the treatment groups at the end of the dietary intervention period. The relative bioavailability of all-*trans*-β-carotene for whole-leaf, minced, liquefied, and liquefied spinach plus added

dietary fibre was 6.2, 6.8, 10.1, and 9.9% and of *cis*-β-carotene was 4.1, 4.2, 6.6, and 6.2%, respectively. These results provide some support for more ready absorption of all-*trans*-β-carotene than *cis*-β-carotene. However, the issue needs further research. The ratio of serum all-*trans*- to *cis*-β-carotene was strongly correlated in our study. It is not known whether this is due to an artefact produced by the chemical analysis because measuring *cis*-β-carotene isomers in chlorophyll-containing foods is extremely difficult or whether β-carotene isomers are in some sort of equilibrium in human serum.

Third, there may be an effect of other carotenoids on the bioavailability of β-carotene. Kostic et al. (1995) found that when β-carotene and lutein were given to subjects in the same dose, β-carotene significantly reduced the serum area-under-the-curve values for lutein. Also Micozzi et al. (1992) found that oral doses of β-carotene (12 or 30 mg daily for 6 wk) lowered serum lutein concentrations in men. In contrast, a preferential increase in chylomicron concentrations of lutein compared to β-carotene in the presence of high amounts of β-carotene and small amounts of lutein was described by Gärtner et al. (1996). Although our study was not designed to examine the interaction between carotenoids, we found no evidence to conclude that β-carotene and lutein interact with each other.

The relative bioavailability of lutein from spinach products ranged from 45 to 55%. Thus, we conclude that the relative bioavailability of lutein from spinach is greater than that of β-carotene and less affected by the food matrix. Lutein, which is a dihydroxycarotenoid, is about 0.1% as lipophylic as β-carotene. This may explain why the matrix in which lutein is embedded in spinach does not reduce lutein absorption as it does the absorption of β-carotene. The amount of lutein found in the duplicate portions was much lower than was to be expected from addition of the results of the separate analyses of the control diet and either the carotenoid supplement or the spinach products. Lutein from spinach products and from the carotenoid supplement was apparently lost when added to a complete, daily diet. The composition of the carotenoid supplement was based on analysis of the various spinach products and experiments to examine the preferred preparation of the spinach for this study. Unexpectedly, at the time of the intervention study, the lutein content of the spinach products was found to be higher than those analysed several months before the study, which partly explains why the carotenoid supplement group received less lutein than the spinach groups.

One of the effects of dietary fibre on lipid metabolism centres upon its interactions with bile acids, resulting in their increased loss by faecal excretion,

disturbance of micelle formation and thus a decreased absorption of fats and fat-soluble substances (Olson 1994). Addition of dietary fibre to liquefied spinach had no effect on the serum β-carotene responses. Rock and Swendseid (1992) reported earlier that adding 12 g dietary citrus pectin to controlled meals with 25 mg synthetic β-carotene (0.48 g pectin/mg β-carotene) reduced the increase in plasma β-carotene. In our study, where the proportion of dietary fibre added (0.23 g fibre/mg β-carotene) was half that used earlier (Rock & Swendseid 1992), we were not able to demonstrate an effect of dietary fibre.

In all groups, intake of carotenoids other than β-carotene and lutein was rather low. We found that the β-carotene response was significantly correlated to the β-carotene response, which is in line with the finding of others that β-carotene supplementation increases concentrations of α-carotene (Castenmiller & West 1998).

In conclusion, this study confirmed that the bioavailability of β-carotene, and to a lesser extent of lutein, was affected by the food matrix and that processing spinach had an effect on the matrix (disruption of cell wall structure and loss of cellular integrity), and thus on the bioavailability of β-carotene from spinach. The bioavailability of β-carotene from liquefied spinach was higher than from whole-leaf or minced spinach. We could not demonstrate an effect on serum β-carotene or lutein responses from the addition of dietary fibre to liquefied spinach. This suggests that once the cell wall components are broken down, addition of dietary fibre in amounts previously present in the food, has no effect on bioavailability of carotenoids. A limitation of the present study may be that subjects in the control group were fed low-carotenoid diets. The effects of feeding carotenoids in this type of study may result in greater apparent increases and differences in serum carotenoid responses. To obtain estimates of absolute bioavailability and to understand the mechanisms involved, it will be necessary to carry out studies using other experimental approaches including those involving isotopically labelled carotenoids.

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## REFERENCES

AOAC Official Method 992.16 for total dietary fiber. In: Cunniff P, ed. Official Methods of Analysis of AOAC International, vol. II, 16th ed. Association of Official Analytical Chemists, Gaithersburg, Maryland, 1996.

Ben-Amotz A, Levy Y. Bioavailability of a natural isomer mixture compared with synthetic all-*trans* β-carotene in human serum. Am J Clin Nutr 1996;63:729-34.

Brown ED, Micozzi MS, Craft NE, Bieri JG, Beecher G, Edwards BK, Rose A, Taylor PR, Smith JC Jr. Plasma carotenoids in normal men after a single ingestion of vegetables or purified β-carotene. Am J Clin Nutr 1989;49:1258-65.

Castenmiller JJM, West CE. Bioavailability and bioconversion of carotenoids. Annu Rev Nutr 1998;18:19-38.

Craft NE, Wise SA, Soares JH Jr. Optimization of an isocratic high-performance liquid chromotographic separation of carotenoids. J Chromatogr 1992;589:171-6.

FAO/WHO. Evaluation of certain food additives and contaminants. Forty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series 859. Geneva, Switzerland: World Health Organization, 1995.

Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497-509.

Gärtner C, Stahl W, Sies H. Preferential increase in chylomicron levels of the xanthophylls lutein and zeaxanthin compared to β-carotene in the human. Internat J Vit Nutr Res 1996;66:119-25.

Gaziano JM, Johnson EJ, Russell RM, Manson JE, Stampfer MJ, Ridker PM, Frei B, Hennekens CH, Krinsky NI. Discrimination in absorption or transport of β-carotene isomers after oral supplementation with either all-*trans*- or 9-*cis*-β-carotene. Am J Clin Nutr 1995;61:1248-52.

Godfrey K. Statistics in practice. Comparing the means of several groups. N Engl J Med 1985;313:1450-6.

Hulshof PJM, Chao X, van de Bovenkamp P, Muhilal, West CE. Application of a validated method for the determination of provitamin A carotenoids in Indonesian foods of different maturity and origin. J Agric Food Chem 1997;45:1174-9.

Hussein L, El-Tohamy M. Vitamin A potency of carrot and spinach carotenes in human metabolic studies. Internat J Vit Nutr Res 1990;60:229-35.

Jackson MJ. The assessment of bioavailability of micronutrients: introduction. Eur J Clin Nutr 1997;51:S1-S2.

Jensen CD, Howes TW, Spiller GA, Pattison TS, Whittam JH, Scala J. Observations on the effects of ingesting *cis* and *trans*-beta-carotene isomers on human serum concentrations. Nutr Rep Int 1987;35:413-22.

Katan MB, van de Bovenkamp P. Determination of total dietary fiber by difference and of pectin by colorimetry or copper titration. In: James WPT, Theander O, eds). The analysis of dietary fiber in

food, Ch 14, pp 217-39. New York, NY: Marcel Dekker Inc., 1981.

Kostic D, White WS, Olson JA. Intestinal absorption, serum clearance, and interactions between lutein and β-carotene when administered to human adults in separate or combined oral doses. Am J Clin Nutr 1995;62: 604-10.

Krinsky NI. Actions of carotenoids in biological systems. Annu Rev Nutr 1993;13:561-87.

Micozzi MS, Brown ED, Edwards BK, Bieri JG, Taylor PR, Khachik F, Beecher GR, Smith JC Jr. Plasma carotenoid response to chronic intake of selected foods and β-carotene supplements in men. Am J Clin Nutr 1992;55:1120-5.

Netherlands Food and Nutrition Council. Recommended Dietary Allowances 1989 in The Netherlands. The Hague, The Netherlands: Netherlands Food and Nutrition Council, 1992.

Olson JA. Absorption, transport, and metabolism of carotenoids in humans. Pure Appl Chem 1994;66:1011-6.

Osborne DR, Voogt P. The analysis of nutrients in foods. London, UK: Academic Press, 1978.

Pee S de, West CE, Muhilal, Karyadi D, Hautvast JGAJ. Lack of improvement in vitamin A status with increased consumption of dark-green leafy vegetables. Lancet 1995;346:75-81.

Pee S de, West CE. Dietary carotenoids and their role in combating vitamin A deficiency: a review of the literature. Eur J Clin Nutr 1996;50:S38-S53.

Pee S de, West CE, Permaesih D, Martuti S, Muhilal, Hautvast JGAJ. Orange fruit is more effective than are dark-green, leafy vegetables in increasing serum concentrations of retinol and β-carotene in schoolchildren in Indonesia. Am J Clin Nutr 1998;68:1058-67.

Poppel, G van. Epidemiological evidence for β-carotene in prevention of cancer and cardiovascular disease. Eur J Clin Nutr 1996;50:S57-S61.

Rock CL, Swendseid ME. Plasma β-carotene response in humans after meals supplemented with dietary pectin. Am J Clin Nutr 1992;55:96-9.

Rock CL, Lovalvo JL, Emenhiser C, Ruffin MT, Flatt SW, Schwartz SJ. Bioavailability of β-carotene is lower in raw than in processed carrots and spinach in women. J Nutr 1998;128:913-6.

Seddon JM, Ajani UA, Sperduto RD, Hiller R, Blair N, Burton TC, Farber MD, Gragoudas ES, Haller J, Miller DT, et al. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. Eye Disease Case-Control Study Group. J Am Med Assoc 1994;272:1413-20.

Siedel J, Hägele EO, Ziegenhorn J, Wahlefeld AW. Reagent for the enzymatic determination of serum total cholesterol with improved lipolytic efficiency. Clin Chem 1983;29:1075-80.

Sies H, Stahl W. Vitamins E and C, β-carotene, and other carotenoids as antioxidants. Am J Clin Nutr 1995;62:1315S-1321S.

Stichting NEVO. Dutch nutrient data base. The Hague, The Netherlands: Voorlichtingsbureau voor de Voeding, 1995.

Sullivan DR, Kruijswijk Z, West CE, Kohlmeier M, Katan MB. Determination of serum triglycerides by an accurate enzymatic method not affected by free glycerol. Clin Chem 1985;31:1227-8.

Tamai H, Morinobu T, Murata T, Manago M, Mino M. 9-*cis* β-carotene in human plasma and blood cells after ingestion of β-carotene. Lipids 1995;30:493-8.

Törrönen R, Lehmusaho M, Häkkinen S, Hänninen O, Mykkänen H. Serum β-carotene response to supplementation with raw carrots, carrot juice or purified β-carotene in healthy non-smoking women. Nutr Res 1996;16:565-75.

Willett WC, Stampfer MJ, Underwood BA, Speizer FE, Rosner B, Hennekens CH. Validation of a dietary questionnaire with plasma carotenoid and α-tocopherol levels. Am J Clin Nutr 1983;38:631-9.

Zeben W van, Hendriks TF. The absorption of carotene from cooked carrots. Zschr Vitamin Forsch 1948;19:265-6.

# 4

# The bioavailability of folate from processed spinach in humans: effect of food matrix and interaction with carotenoids

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**ABSTRACT**

**Objective**: To examine the effect of the food matrix and dietary fibre on the bioavailability of folate as measured by plasma folate response.

**Design and subjects**: In a controlled, three-week dietary intervention study, 28 men and 42 women were divided into six groups to receive either a control diet ($n$ = 10), or the control diet plus 20 g/MJ per day ($n$ = 12 per group) of whole-leaf spinach, minced spinach, liquefied spinach, or liquefied spinach to which dietary fibre (10 g/kg wet weight) was added. The sixth group received the control diet plus a synthetic carotenoid supplement with similar amounts of β-carotene and lutein as found in spinach.

**Results**: A significantly higher plasma folate response was found for the pooled spinach groups than for the control group. Among the spinach groups no significant differences were detected. However, the plasma folate response of the pooled minced and liquefied spinach groups was greater than that of the whole-leaf spinach group ($P$ = 0.03). Re-addition of dietary fibre to the liquefied spinach to compensate for the fibre broken down during liquefaction did not reduce the plasma folate response. The consumption of the carotenoid supplement did not have an effect on plasma folate concentrations compared with the control group.

**Conclusions**: Consumption of spinach in a mixed diet increased folate status and the food matrix in which the folate is entrapped plays a role in folate bioavailability. There is no interaction between carotenoid and folate responses after consumption of spinach.

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**KEY WORDS**: dietary fibre, plasma folate concentration, food processing, dietary carotenoids

## INTRODUCTION

The term folate refers to a group of compounds that exhibit vitamin activity similar to that of pteroyl-L-glutamic acid (folic acid) and which are conjugated to one glutamyl residue (monoglutamyl folates) or more (polyglutamyl folates). A low intake of folate is associated with increased plasma homocysteine levels (Selhub et al. 1993), cardiovascular disease (Rimm et al. 1998) and colon cancer (Giovannucci et al. 1995). An elevated plasma homocysteine concentration is a risk factor for neural tube defects (Steegers-Theunissen et al. 1991) and cardiovascular disease (Graham et al. 1997, Kang et al. 1992, Nygård et al. 1995, Pancharuniti et al. 1994, Verhoef et al. 1996). The relationship between low folate intake and elevated homocysteine levels can be attributed to impaired one-carbon metabolism (Allen et al. 1993). Analysis of data from the Framingham study showed that an increased frequency of consumption of fruits and vegetables, the dominant source of total folate in the human diet, was associated with both higher levels of plasma folate and lower levels of plasma homocysteine (Tucker et al. 1996). Many studies have been directed toward determining folate bioavailability in individual foods but data on the bioavailability of folate from foods in a mixed diet is scarce. Based on plasma folate response, Sauberlich et al. (1987) found that bioavailability of endogenous food folate from such a diet was no more than 50% of that of folic acid added to the diet. Recently, this finding was confirmed by Cuskelly et al. (1996) who also demonstrated that the bioavailability of folic acid in fortified cereal-grain foods was similar to that of folic acid supplements.

Factors that are considered to influence bioavailability of nutrients, such as folate in plants are: chemical species; molecular linkage; amount of folate in a meal; food matrix, effectors and inhibitors of absorption; nutritional status; genetic factors; and host-related factors (Castenmiller & West 1998, Gregory 1995, Gregory 1997). The complex food matrix of leafy vegetables may inhibit folate absorption, as was observed in the case of carotenoid bioavailability (de Pee et al. 1995). If so, chopping or otherwise destroying the matrix of vegetables during food processing, might increase the bioavailability of folate. Folate absorption may also be affected by the presence of dietary fibre. Data on the effect of dietary fibre in humans are limited to the effect of wheat bran on folate bioavailability (Bailey et al. 1988, Brodribb & Humphreys 1976, Keagy et al. 1988, Russell et al. 1976), and little is known about the effect of dietary fibre, other than from wheat bran, on folate absorption in humans.

In this controlled dietary human intervention study we examined whether the consumption of spinach as part of a meal increased plasma folate concentrations

and whether the partial or complete destruction of the spinach food matrix had an effect on the bioavailability of folate. One of the spinach products was treated with a mixture of enzymes, which changed the spinach food matrix by causing disruption of the cell wall and loss of dietary fibre. We examined the effect of adding dietary fibre back to spinach in which some of the fibre had been broken down by enzymatic means. This fibre replaced the fibre that was lost but did not restore the cell wall structure. We also evaluated the effect of carotenoids per se on the plasma folate concentration.

## METHODS

### Subjects

We recruited subjects through advertisements in local newspapers and posters in university and public buildings in Wageningen. Initially 72 subjects were included in the study. They were apparently healthy, non-smoking men and women, aged 18-58 y, who did not use vitamin or yeast tablets or anti-malaria medication during the 3 mo preceding the study. Women who were planning to become pregnant were not allowed to participate in the study. The experiment was originally designed to study the bioavailability of carotenoids, for which reason a control group receiving a carotenoid supplement was included. Because no data are available on possible interactions of carotenoids and folic acid, it was decided that the carotenoid supplement group should not receive folic acid in addition to the carotenoid supplement. The results on the bioavailability of carotenoids are reported separately (Castenmiller et al. 1999). During the experimental period two men withdrew for personal reasons. Therefore, 70 persons (28 men and 42 women) participated in this study.

### Design

The study had a parallel design and consisted of a 3-wk run-in period, in which participants chose their own diets but were instructed to avoid foods rich in carotenoids and folates. The run-in period was followed by a 3-wk intervention period in February - March 1997. All subjects were free-living. At the end of the run-in period subjects were stratified according to age and sex, and subsequently randomised over six treatment groups. All subjects received a control diet and four groups received 20 g/MJ of one of the following frozen spinach products: whole-leaf spinach, with an almost intact food matrix; minced spinach, in which the food matrix was disrupted; liquefied spinach, in which the matrix was further

disrupted; and the liquefied spinach above to which dietary fibre was added. The control group received the control diet, which was adjusted for energy and macronutrients to compensate for the energy and macronutrients provided by the spinach products. The carotenoid supplement group received the control diet and a synthetic carotenoid supplement, containing 0.8 mg β-carotene/MJ and 1.2 mg lutein/MJ (see Castenmiller et al. 1999).

To estimate energy intake, participants had recorded their habitual diet of the past four weeks, using a validated food frequency questionnaire (Feunekes et al. 1993). During the intervention period, almost all foods were supplied according to each person's energy requirement. In addition to the food supplied, each subject had a limited free choice of products. A maximum of two alcoholic consumptions per day was allowed, provided they were not consumed with the hot meal. On weekdays at noon hot meals were served at the Division of Human Nutrition and Epidemiology in Wageningen. All other foods were provided and packed for consumption elsewhere. On Fridays, food for the weekend and instructions for its preparation were given. The portion of spinach was consumed entirely as part of the hot meal. Menus were changed daily with a one-week cycle. Body weight was recorded twice weekly and energy intake was adjusted, when necessary, to limit changes in weight to less than 2 kg. Subjects recorded in diaries illness, medication used, the free choice items selected, and any deviations from their diets. Inspection of the diaries did not reveal any deviations from the protocol, which were thought to affect the results.

The frozen spinach used for the study was provided by Unilever Research Vlaardingen (The Netherlands). The whole-leaf spinach was washed and subsequently blanched for 90 s and cooled down quickly. The minced spinach was minced to 5 mm after blanching. To prepare liquefied spinach, chopped spinach was warmed to 35°C and an enzyme mixture containing pectinase, hemicellulase and cellulase activities (10 g/kg wet weight of spinach; Rapidase® LIQ plus, kindly supplied by Gist-brocades, Delft, The Netherlands) was added. The spinach was then maintained at 35-40°C and stirred for 2.5 h. After this, the spinach was boiled for 5 - 10 min to destroy all enzyme activity. The cooking water was not discarded after boiling the spinach, so that the loss of folate due to leaching of folate into the cooking water was minimal. To study the effect of fibre, a mixture of dietary fibre derived from sugar beet (10 g/kg; Fibrex 600®, kindly supplied by TEFCO Food Ingredients Inc., Bodegraven, The Netherlands) was added to the liquefied spinach to compensate for the fibre that was digested by the enzymes. This sugar beet fibre product was selected because it is comparable to the fibre present in the whole-leaf spinach and contained per 100 g

product, 73 g dietary fibre of which one third is soluble and 22 g pectin.

During the intervention period, complete duplicate portions of the control diet for an imaginary subject with a daily energy intake of 11 MJ were collected. Duplicate portions for analysis of macronutrients and fibre were pooled as weekly portions, mixed thoroughly and stored at -20°C until analysis. Duplicate portions for the analysis of folate were blended daily and stored at -80°C until analysis. The different spinach products were sampled during the study and frozen at -80°C to determine the content of folate. The total daily intake of energy, macronutrients and fibre was based on analysis of the pooled weekly duplicate portions and an estimate of the energy and nutrient content of the free choice items, which was calculated using data from the Dutch food composition database (NEVO Foundation 1995). To estimate the total folate intake of subjects, values for the chemically analysed mean folate content of the control diet on week days were added to those of the spinach products consumed and the estimated folate intake from the free choice foods in each group (Brants & Hulshof 1995).

### Blood collection

Fasting blood samples were drawn at days 0, 1, 8, 15, 21 and 22 of the intervention period, in EDTA vacutainer tubes (Venoject II, Terumo, Madrid, Spain). Samples were immediately placed on ice and centrifuged within 30 min at 3000 x g for 10 min. Plasma was separated and stored at -35°C until analysis.

### Measurements

Plasma folate concentration was assessed in all blood samples. Plasma vitamin $B_{12}$ concentration was determined only at day 0 to check for deviations from the normal level. Folate and vitamin $B_{12}$ concentrations in plasma were determined with the Abbott IMx folic acid and vitamin $B_{12}$ assays (IMx automated microparticle enzyme immunoassay system, Abbott Laboratories, North Chicago, IL, USA). The intra-assay coefficient of variation (CV) of the folate assay varied between 3 and 6% and the inter-assay CV between 6 and 10%, depending on the folate concentration. For vitamin $B_{12}$, both the intra- and inter-assay CV were lower than 5%. All samples from each subject were analysed in the same assay.

The moisture level and the ash content in each weekly food portion were determined using a vacuum oven at 85°C and a muffle furnace at 550°C. The protein concentration was assessed by the Kjeldahl method using a conversion factor of 6.25; fat was measured by the method of Folch et al. (1957) and digestible carbohydrate was calculated by difference. Dietary fibre was analysed according to the AOAC Official Method for total dietary fibre (AOAC 1996). The

dietary fibre content of the various spinach products was determined as methanol-ether insoluble solids (MEIS) and the amount of pectin (polygalacturonic acid) in the spinach products was measured by hydrolysing pectic substances to water-soluble galacturonic acid using an enzyme preparation (Katan & van de Bovenkamp 1981). Total folate in food samples of the control group and the various spinach products was measured by a *Lactobacillus casei* microbiological assay as described by Horne & Patterson (1988) and modified by Tamura et al. (1990). Before extraction, 2% sodium ascorbate was added to the sample, followed by suspending the sample in 10 volumes of a buffer containing 5 mmol/L 2-mercaptoethanol and 50 mmol/L potassium tetraborate (Seyoum & Selhub 1993). A chicken pancreas conjugase preparation was used to convert all the polyglutamates to their corresponding monoglutamate derivatives. The intra-assay CV of the analysis was 9.3%.

**Statistical analysis**

The response to treatment was calculated for each subject as the change in plasma folate from the start (average value for day 0 and day 1) to the end (average value for day 21 and day 22) of the dietary intervention period. The differences in mean plasma folate response between the pooled spinach groups and the control group and between the carotenoid supplement and the control group were compared using Student's t-test. The Tukey method for multiple comparisons among the various spinach groups was used after a significant F-test. A general linear model was used to control for covariables, including age, sex, vegetarianism, and body mass index. Because differences among spinach groups were not significantly different, the plasma folate response in further processed spinach products (pooled minced and liquefied spinach groups) was compared with whole-leaf spinach. The effect of adding fibre back to liquefied spinach and the effect of a pure carotenoid supplement on plasma folate responses was tested using a t-test for independent samples. Differences with $P < 0.05$ were regarded as statistically significant (SPSS Advanced Statistics 7.5., 1997, SPSS Inc., Chicago, IL). Results are expressed as means ± SD. Assuming a SD of 3.0, the power of the study to detect a difference in plasma folate concentration of 2.9 nmol/L was > 80%.

**Ethical considerations**

The protocol and aims of the study were fully explained to the subjects who all gave their written consent. Approval of the study was obtained from the Medical Ethical Committee of the Division of Human Nutrition and Epidemiology.

## RESULTS

Characteristics of the subjects are given in Table 1. At baseline the experimental groups did not differ in age, body mass index, plasma vitamin $B_{12}$ concentration and plasma folate concentration (see Table 3). Folate and vitamin $B_{12}$ status were within the normal range in all subjects (Herbert & Das 1994). The total daily intake of energy, macronutrients, dietary fibre and folate for a subject in the energy group providing 11 MJ is described in Table 2. There were no significant differences in macronutrient and fibre intake among the groups or among the three weeks of dietary intervention. The control diet, excluding the free choice products, provided on average daily 22.4 ± 3.9 ($n$ = 5) µg folate per MJ on average daily 22.4 ± 3.9 ($n$ = 5) µg folate per MJ. The daily folate intake in the spinach groups was approximately twice that of the control group. There were no significant differences in folate intake among the spinach groups. The whole-leaf, minced, liquefied, and liquefied plus added dietary fibre spinach products contained 113.5 ± 0.1, 118.8 ± 1.4, 109.1 ± 2.0, and 122.7 ± 18.0 µg folate per 100 g spinach, respectively. Plasma folate concentrations increased in all groups, including the control group, during the first week of intervention (Table 3). The

**Table 1. Characteristics of subjects participating in a study to determine the bioavailability of folate from differently processed spinach products***

| | Group | | | | | |
|---|---|---|---|---|---|---|
| | Control | Carotenoid supplement | Whole-leaf spinach | Minced spinach | Liquefied spinach | Liquefied spinach plus dietary fibre |
| Men, women; *n, n* | 3, 7 | 5, 7 | 5, 7 | 5, 7 | 5, 7 | 5, 7 |
| Vegetarians, *n* | 3 | 1 | 3 | 7 | 3 | 3 |
| Age, *years*† | 21 (18, 25) | 21 (18, 24) | 20 (18, 58) | 21 (18, 42) | 21 (18, 38) | 20 (18, 54) |
| Energy intake, *MJ/d* | 11.4 ± 2.6 | 12.8 ± 3.4 | 11.5 ± 2.3 | 11.3 ± 2.1 | 11.4 ± 2.1 | 11.4 ± 2.3 |
| Body mass index, *kg/m²* | 21 ± 2.1 | 22 ± 2.3 | 22 ± 2.0 | 22 ± 2.4 | 23 ± 2.1 | 22 ± 1.8 |
| Plasma vitamin $B_{12}$, *pmol/L* | 250 ± 91 | 230 ± 83 | 206 ± 57 | 250 ± 49 | 268 ± 76 | 215 ± 93 |

* Data collected at beginning of study except for energy intake, which is the estimated mean over the intervention period. Values are means ± SD.

† Values are expressed as medians (ranges).

**Table 2. Mean total daily intake of energy, fibre and folate of subjects participating in a study to examine the bioavailability of folate from various spinach products***

| | Group | | | | | |
|---|---|---|---|---|---|---|
| | Control ($n$ = 10) | Carotenoid supplement ($n$ = 12) | Whole-leaf spinach ($n$ = 12) | Minced spinach ($n$ = 12) | Liquefied spinach ($n$ = 12) | Liquefied spinach plus dietary fibre ($n$ = 12) |
| Energy (*MJ/d*) | 10.6 | 10.7 | 10.8 | 10.8 | 10.8 | 10.8 |
| Dietary fibre (*g/d*) | 27.4 | 25.2 | 32.4 | 32.3 | 28.2 | 31.3 |
| Folate (*μg/d*)† | 258 | 258 | 510 | 518 | 495 | 527 |

* Results are based on the analysis of duplicate portions for a subject consuming approximately 11 MJ together with an estimate calculated from the composition of the free items consumed. There were no significant differences in nutrient intakes among the groups or among the three weeks of dietary intervention. The mean daily intake was as follows: protein, 13.9 energy%; fat, 34.1 energy%; carbohydrate, 51.0 energy%; alcohol, 1.0 energy%.

† Results are based on the analysis of duplicate portions of five week days for a subject in the control group consuming approximately 11 MJ together with an estimate calculated from the composition of the free choice food items consumed in each group and the analysed amounts of folate in each of the spinach products.

pooled spinach groups had a statistically significant higher plasma folate response than the control group (2.4 nmol/L and -0.04 nmol/L, respectively; $P < 0.01$). Multiple comparisons among the spinach groups revealed no significant differences between the various spinach products. When comparing the folate responses of the whole-leaf and the pooled minced and liquefied spinach groups, controlling for vegetarianism, the plasma folate response was higher in the pooled minced and liquefied spinach group than in the whole-leaf spinach group ($P < 0.03$). Vegetarians ($n$ = 20) had higher baseline (wk 0) plasma folate concentrations than non-vegetarians (18.0 ± 5.7, and 14.5 ± 3.2 nmol/L, respectively; $P = 0.01$), and their mean response was lower than that of the non-vegetarians (0.9 ± 2.9 and 2.3 ± 2.6 nmol/L, respectively; $P = 0.004$). The response of the whole-leaf spinach group was small. If the trial was carried out with the whole-leaf spinach group and the control group only, we would not have been able to demonstrate a higher plasma folate response in the whole-leaf spinach group as compared to the control group ($P = 0.3$).

Adding dietary fibre (10 g/kg wet weight) to the liquefied spinach group did not significantly reduce the plasma folate response relative to the response of the liquefied spinach group ($P = 0.39$; Table 3). The enzyme-treated spinach contained per 100 g dry matter 62 g MEIS and 9 g polygalacturonic acid as

compared to 71 g and 11 g, respectively, in the whole-leaf spinach.

The plasma folate response did not differ between the carotenoid supplement group and the control group ($P = 0.25$), suggesting that the absorption of folates and carotenoids are independent processes and that there is no interaction between these nutrients.

**Table 3. Plasma folate concentrations in nmol/L (mean ± SD) at the start of the dietary intervention period (wk 0), after one week (wk 1), after two weeks (wk 2) and after three weeks of dietary intervention (wk 3) and response from begin to end of the dietary intervention period in healthy subjects fed various spinach products***

| | Group | | | | | |
|---|---|---|---|---|---|---|
| | Control ($n$ = 10) | Carotenoid supplement ($n$ = 12) | Whole-leaf spinach ($n$ = 12) | Minced spinach ($n$ = 12) | Liquefied spinach ($n$ = 12) | Liquefied spinach plus dietary fibre ($n$ = 12) |
| Wk 0 | 15.4 ± 3.1 | 16.6 ± 4.9 | 17.3 ± 4.8 | 16.2 ± 3.7 | 13.1 ± 4.1 | 14.4 ± 4.5 |
| Wk 1 | 17.6 ± 3.3 | 18.7 ± 4.6 | 20.8 ± 5.5 | 19.8 ± 3.7 | 16.9 ± 3.7 | 18.3 ± 4.2 |
| Wk 2 | 16.6 ± 2.2 | 17.7 ± 4.1 | 19.9 ± 4.0 | 21.2 ± 4.9 | 17.2 ± 4.2 | 18.0 ± 4.7 |
| Wk 3 | 15.3 ± 2.4 | 17.9 ± 4.0 | 18.6 ± 3.4 | 19.4 ± 4.1 | 16.1 ± 4.3 | 16.5 ± 3.6 |
| Response | -0.04 ± 2.4* | 1.3 ± 2.7 | 1.3 ± 3.4† | 3.3 ± 2.7 | 2.9 ± 1.7 | 2.2 ± 2.6 |

* Significantly different from pooled spinach groups, $P < 0.01$ (independent samples t-test).
† Significantly different, controlling for vegetarian diet, from pooled minced and liquefied spinach groups, $P < 0.03$ (ANOVA).

## DISCUSSION

We found that consumption of 20 g/MJ of spinach daily for 3 wk significantly increased plasma folate concentration. Consumption of minced or liquefied spinach led to higher plasma folate responses than consumption of whole-leaf spinach, which suggests that the food matrix of spinach plays a role with regard to bioavailability of folate. Re-addition of dietary fibre did not significantly reduce the folate response in the liquefied spinach plus dietary fibre group compared to the liquefied spinach group.

Our study suggests that folate from spinach consumed as part of a meal and mixed diet was absorbed and that further processing of spinach improved bioavailability. However, it should be noted that, perhaps due to the limited power of the study and the relative small amount of spinach served, the increase in plasma folate concentration in the whole-leaf spinach was small (8% or 1.3 nmol/L).

Few studies have examined the bioavailability of endogenous (polyglutamate) folate in foods. In an experiment with ten adult, non-pregnant women, who were maintained in a metabolic unit for 92 d, dietary folates appeared to be no more than 50% available when compared to pteroylmonoglutamic acid added to the diets (Sauberlich et al. 1987). This was confirmed by Cuskelly et al. (1996) in a long-term study, who found that the bioavailability of added folic acid was higher than the bioavailability of food folate. To our knowledge, there are no studies published that have examined the effect of food processing on improving folate bioavailability. We conclude that the bioavailability of folate in spinach products can be improved by further processing of the spinach, suggesting that the food matrix plays a role in folate bioavailability. Disruption of the food matrix by mincing or liquefying spinach resulted in a higher folate bioavailability.

The plasma folate concentrations increased in all study groups, including the control group, after 1 wk and decreased after wk 2 and 3. We have no explanation for this finding. The plasma folate responses after wk 1 showed small increases in plasma folate concentrations for all spinach groups compared to the control group. Responses after wk 2 and 3 were consistent.

In our study, 20 vegetarians participated and they were fed the same foods, except for the meat products, which were replaced by a vegetarian substitute with similar nutrient composition. The vegetarians had higher baseline plasma folate concentrations and smaller folate responses, which suggests that the vegetarians had higher intakes of folate before the start of the intervention period. During the run-in period subjects were instructed to avoid foods with a high folate content, but no restriction was given for cereal or meat products. To our knowledge, effects of baseline plasma folate concentrations on plasma folate responses to treatment with folate-rich foods have not been reported before. Lower plasma folate responses could also be due to differences in folate body stores, but we did not measure folate concentrations in erythrocytes.

The difference in plasma folate response between liquefied spinach and liquefied spinach with added dietary fibre (0.7 nmol/L) was not statistical significant ($P = 0.39$). This suggests that dietary fibre (0.3 g/MJ) had no effect on folate bioavailability of spinach consumed as part of a mixed diet. Russell and co-workers (1976) found no effect of cellulose or dietary fibre from wheat bran on folate absorption in humans of meals (breads) with increasing fibre contents (2.3 to 8.2 g dietary fibre per meal). Addition of 30 g wheat bran to a formula meal had no effect on the absorption of heptaglutamyl folate whereas monoglutamyl folic acid absorption was increased (Keagy et al. 1988). On the other hand, Bailey et al. (1988) demonstrated in a short-term study that 34 g wheat bran, containing 19

g of dietary fibre, reduced the plasma folate response of polyglutamate relative to monoglutamyl or polyglutamyl folates consumed alone in solution. Brodribb & Humphreys (1976) showed that chronic ingestion of 24 g wheat bran daily to diverticular patients was associated with a small, but significant, decrease in serum folate concentrations. The mechanism behind a possible inhibitory effect of dietary fibre is not clear. In vitro data show a lack of effect of dietary fibre on brush border conjugase activity (Bhandari & Gregory 1990) and little or no effect on bioavailability of folic acid monoglutamate in a chick bioassay (Ristow et al. 1982). However, whether polyglutamyl folate binds to dietary fibre has not yet been investigated. In the present study, with the addition of 0.3 g/MJ dietary fibre to spinach no significant reduction of the plasma folate response was observed, but the intake of dietary fibre in the liquefied spinach plus dietary fibre group was only slightly higher than that in the liquefied spinach group (2.6 and 2.9 g dietary fibre/MJ, respectively). With the addition of higher amounts of dietary fibre the plasma folate response may further decrease and the difference between the two treatment groups may become statistically significant.

Naturally occurring folates in foods are mainly in the form of polyglutamates, which are cleaved in the intestine by pteroylpolyglutamyl hydrolase (conjugase) to monoglutamates before absorption. Spinach and other vegetables contain endogenous folate conjugase (Leichter et al. 1979). When cell membranes are disrupted, endogenous folate conjugase, if still active, can act on polyglutamyl folates in spinach cells. Different ratios of polyglutamates to monoglutamates in the various spinach products could cause differences in folate bioavailability. However, all spinach products originated from one batch and all spinach was blanched to destroy all enzyme activity. Therefore, we assume that the inactivation of folate conjugase in all spinach products was complete.

A limitation of the present study may be that plasma folate response was used as the criterion of response to bioavailable dietary folate. Although the plasma folate concentrations are often considered to be insensitive and highly variable, we think that measuring plasma folate concentrations after three weeks of fully controlled diets is a valuable method to examine the folate bioavailability. However, further research on the variability and sensitivity of plasma folate concentrations is needed. Another limitation to this study was the lack of a positive control group receiving synthetic folic acid. Therefore, we cannot relate the plasma folate responses of the spinach groups to the plasma folate response of a control group fed folic acid.

We conclude that consumption of spinach (20 g/MJ) added to a mixed diet increased folate status in healthy volunteers. Our data suggest that the way foods

are processed influences folate bioavailability: consumption of minced and liquefied spinach increased plasma folate response more than consumption of whole-leaf spinach. The response to treatment may depend on the baseline plasma folate concentration, and the addition of dietary fibre (0.3 g/MJ added to 2.6 g/MJ) did not reduce the plasma folate response. Consumption of a synthetic carotenoid supplement with a similar composition of carotenoids as present in spinach had no effect on the plasma folate concentration.

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## REFERENCES

Allen RH, Stabler SP, Savage DG, Lindenbaum J. Metabolic abnormalities in cobalamin (vitamin B-12) and folate deficiency. FASEB J 1993;7:1344-53.

AOAC Official Method 992.16 for total dietary fiber. In: Cunniff P, ed. Official Methods of Analysis of AOAC International, vol. II, 16th ed, 1995. Gaithersburg, Maryland: Association of Official Analytical Chemists, 1996.

Bailey LB, Barton LE, Hillier SE, Cerda JJ. Bioavailability of mono and polyglutamyl folate in human subjects. Nutr Rep Int 1988;38:509-18.

Bhandari SD, Gregory JF III. Inhibition by selected food components of human and porcine intestinal pteroylpolyglutamate hydrolase activity. Am J Clin Nutr 1990;51:87-94.

Brants HAM, Hulshof KFAM. De ontwikkeling van een voedingsmiddelentabel met foliumzuurgehalten [Development of a folate food composition table]. Zeist, The Netherlands: TNO-Nutrition, 1995.

Brodribb AJM, Humphreys DM. Diverticular disease: three studies. Br Med J 1976;1:424-30.

Brouwer IA, van Dusseldorp M, West CE, Meyboom S, Thomas CMG, Duran M, van het Hof KH, Eskes TKAB, Hautvast JGAJ, Steegers-Theunissen RPM. Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J Nutr 1999;129:1135-9.

Castenmiller JJM, West CE. Bioavailability and bioconversion of carotenoids. Annu Rev Nutr 1998;18:19-38.

Castenmiller JJM, West CE, Linssen JPH, van het Hof KH, Voragen AGJ. The food matrix of spinach is a limiting factor in determining the bioavailability of β-carotene and to a lesser extent of lutein in humans. J Nutr 1999;129:349-55.

Cuskelly GJ, McNulty H, Scott JM. Effect of increasing dietary folate on red-cell folate: implications for prevention of neural tube defects. Lancet 1996;347:657-9.

Feunekes GIJ, Staveren WA, de Vries JHM, Burema J, Hautvast JGAJ. Relative and biomarker-based validity of a food frequency questionnaire estimating intake of fats and cholesterol. Am J Clin Nutr 1993;58:489-96.

Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497-509.

Giovannucci E, Rimm EB, Ascherio A, Stampfer MJ, Colditz GA, Willett WC. Alcohol, low-methionine - low folate diets, and risk of colon cancer in men. J Natl Cancer Inst 1995;87:265-73.

Graham IM, Daly LE, Refsum HM, Robinson K, Brattström LE, Ueland PM, Palma-Reis RJ, Boers GHJ, Sheahan RG, Israelsson B, Uiterwaal CS, Meleady R, McMaster D, Verhoef P, Witteman J, Rubba P, Bellet H, Wautrecht JC, de Valk HW, Sales Lúis AC, Parrot-Pouland FM, Tan KS, Higgins I, Garcon D, Medrano MJ, Candito M, Evans AE, Andria G (European Concerted Action Project). Plasma homocysteine as a risk factor for vascular disease. J Am Med Assoc 1997;277:1775-81.

Gregory JF III. The bioavailability of folate. In: Bailey LB, ed. Folate in health and disease, pp 195-235. New York: Marcel Dekker, Inc., 1995.

Gregory JF III. Bioavailability of folate. Eur J Clin Nutr 1997;51(suppl.1):S54-S59.

Herbert V, Das CK. Folic acid and vitamin B12. In: Shils ME, Olson JA, Shike M, eds. Modern Nutrition in Health and Disease 1, pp 402-26. Philadelphia: Lea & Febiger, 1994.

Horne DW, Patterson D. Lactobacillus casei microbiological assay of folic acid derivatives in 96-well microtiter plates. Clin Chem 1988;34:2357-9.

Kang S, Wong PW, Malinow MR. Hyperhomocysteinemia as a risk factor for occlusive vascular disease. Annu Rev Nutr 1992;12:279-98.

Katan MB, van de Bovenkamp P. Determination of total dietary fiber by difference and of pectin by colorimetry or copper titration. In: James WPT, Theander O, eds. The analysis of dietary fiber in foods, Ch 14, pp 217-39. New York: Marcel Dekker, Inc., 1981.

Keagy PM, Shane B, Oace SM. Folate bioavailability in humans: effects of wheat bran and beans. Am J Clin Nutr 1988;47:80-8.

Leichter J, Landymore AF, Krumdieck CL. Folate conjugase activity in fresh vegetables and its effect on the determination of free folate content. Am J Clin Nutr 1979;32:92-5.

NEVO Foundation. Dutch Nutrient Data Base 1995. The Hague, The Netherlands: Voorlichtingsbureau voor de Voeding, 1995.

Nygård O, Vollset SE, Refsum H, Stensvold I, Tverdal A, Nordrehaug JE, Ueland M, Kvale G. Total plasma homocysteine and cardiovascular risk profile. The Hordaland Homocysteine Study. J Am Med Assoc 1995;274:1526-33.

Pancharuniti N, Lewis CA, Sauberlich HE, Perkins LL, Go RCP, Alvarez JO, Macaluso M, Acton RT, Copeland RB, Cousins AL, Gore TB, Cornwell PE, Roseman JM. Plasma homocyst(e)ine, folate, and vitamin B-12 concentrations and risk for early-onset coronary artery disease. Am J Clin Nutr 1994;59:940-8.

Pee S de, West CE, Muhilal, Karyadi D, Hautvast JGAJ. Lack of improvement in vitamin A status with increased consumption of dark-green leafy vegetables. Lancet 1995;346:75-81.

Rimm EB, Willett WC, Hu FB, Sampson L, Colditz GA, Manson JE, Hennekens C, Stampfer MJ. Folate and vitamin B6 from diet and supplements in relation to risk of coronary heart disease

among women. J Am Med Assoc 1998;279:359-64.

Ristow KA, Gregory JF III, Damron BL. Effects of dietary fiber on the bioavailability of folic acid monoglutamate. J Nutr 1982;112:750-8.

Russell RM, Ismail-Beigi F, Reinhold JG. Folate content of Iranian breads and the effect of their fiber content on the intestinal absorption of folic acid. Am J Clin Nutr 1976;29:799-802.

Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr 1987;46:1016-28.

Selhub J, Jacques PF, Wilson PWF, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. J Am Med Assoc 1993;270:2693-8.

Seyoum E, Selhub J. Combined affinity and ion pair column chromatographies for the analysis of food folate. J Nutr Biochem 1993;4:488-94.

Steegers-Theunissen RPM, Boers GHJ, Trijbels JMF, Eskes TKAB. Neural tube defects and derangement of homocysteine metabolism (letter). N Engl J Med 1991;324:199-200.

Tamura T, Freeberg LE, Cornwell PE. Inhibition of EDTA of growth of Lactobacillus casei in the folate microbiological assay and its reversal by added manganese or iron. Clin Chem 1990;36:1993.

Tucker KL, Selhub J, Wilson PWF, Rosenberg IH. Dietary intake pattern relates to plasma folate and homocysteine concentrations in the Framingham Heart Study. J Nutr 1996;126:3025-31.

Verhoef P, Stampfer MJ, Buring JE, Gaziano JM, Allen RH, Stabler SP, Reynolds RD, Kok FJ, Hennekens CH, Willett WC. Homocysteine metabolism and risk of myocardial infarction: relation with vitamins B6, B12, and folate. Am J Epidemiol 1996;143:845-59.

# 5

# Beta-carotene does not change markers of enzymatic and non-enzymatic antioxidant activity in human blood

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## ABSTRACT

In vitamin A-replete populations, increased concentrations of serum carotenoids have been associated with a decreased risk of degenerative diseases. The mechanism of action of carotenoids in determining antioxidant activity is largely unknown. The aim of the study was to examine the effect of carotenoid supplementation and spinach intake on erythrocyte enzyme antioxidant activities, serum or plasma non-enzymatic antioxidant concentrations, and concentrations of oxidatively damaged amino acids in plasma. Subjects received for 3 wk a basic diet ($n$ = 10), a basic diet with a carotenoid supplement ($n$ = 12) or with a spinach product ($n$ = 12 per group), i.e., whole-leaf, minced, liquefied or liquefied spinach plus added dietary fibre. After 3 wk of dietary intervention, changes in serum or plasma concentrations of ascorbic acid, α-tocopherol, FRAP (ferric reducing ability of plasma) and uric acid and erythrocyte enzyme activities were assessed and differences among experimental groups were tested. Consumption of spinach resulted in greater ($P < 0.01$) erythrocyte glutathione reductase activity and lower ($P < 0.05$) erythrocyte catalase activity and serum α-tocopherol concentration compared to the control group. Consumption of the carotenoid supplement led to lower α-tocopherol responses ($P = 0.02$) compared with the basic diet only. Our data suggest that the short-term changes in erythrocyte glutathione reductase activity and serum α-tocopherol concentration can be attributed to an increased carotenoid (lutein and zeaxanthin) intake, but β-carotene is unlikely to be a causative factor. Lower erythrocyte catalase activity after intervention with spinach products may be related to other constituents in spinach such as flavonoids.


**KEY WORDS**: carotenoids, antioxidants, α-tocopherol, humans, spinach

## INTRODUCTION

Carotenoids are naturally occurring compounds in plants, but only a limited number of carotenoids are found in human plasma and tissues. The major ones are β-carotene, lutein, α-carotene, zeaxanthin, cryptoxanthin and lycopene. A number of carotenoids are precursors of retinol and retinoids, but carotenoids also have several other functions in humans, including protecting against oxidation by quenching singlet oxygen (Stahl et al. 1997). In addition, β-carotene reacts chemically with peroxyl radicals to produce epoxide and apocarotenal products (Canfield 1992). A low level of carotenoids is associated with poor cognitive performance (Berr et al. 1998), and higher plasma β-carotene levels are associated with better memory performance in elderly people (Perrig et al. 1997). On the basis of intake or biomarkers of intake, carotenoids have been postulated to play a protective role in angina pectoris (Riemersma et al. 1991), cardiovascular disease (Gaziano et al. 1995, Kardinaal et al. 1993, Knekt et al. 1994, Street et al. 1994) and cancer (Stahelin et al. 1991, van Poppel 1996), particularly cancer of lung (Dartigues et al. 1990, Knekt et al. 1990) and stomach (Chen et al. 1992). However, intervention studies have not borne this out (The ATBC Cancer Prevention Study Group 1994, Blot et al. 1993, Blot 1997, Greenberg et al. 1996, Omenn et al. 1996, Rapola et al. 1997). These intervention studies used β-carotene supplements rather than a mixture of carotenoids as present in fruits and vegetables. The positive effects on health postulated for the carotenoids have been attributed largely to their antioxidant actions. However, studies linking higher carotenoid intakes to better antioxidant defence and to a decrease in oxidative damage in the body are very few and have generally used indirect methods (Esterbauer 1996, Miller & Rice-Evans 1997, Puhl et al. 1994). Therefore, we conducted a dietary controlled intervention study to investigate the effects of intake of a carotenoid supplement dissolved in oil and three differently processed spinach products on a range of enzymatic and non-enzymatic antioxidant parameters in human blood. The effect of consumption of the differently processed spinach products on the bioavailability of carotenoids has been published separately (Castenmiller et al. 1999). The primary aim of this study was to evaluate whether a spinach intervention had an effect on markers of antioxidative status and whether this effect could be contributed to carotenoids present in spinach.

## MATERIALS AND METHODS

### Subjects

The subjects were 72 healthy, non-smoking, normolipidemic volunteers; they were 42 women and 30 men, aged 18-58 y. The subjects, students of Wageningen Agricultural University and other inhabitants in the Wageningen area, were recruited through local advertisements. None of the subjects were taking oral medications, except for oral contraceptives, or supplements of any kind during the last three mo before the study started or during the study. The screening procedures included a test for elevated glucose and protein levels in urine and a check for abnormal haematology or low haemoglobin concentrations. All subjects completed a medical and a general questionnaire in addition to food frequency questionnaires to estimate their intakes of energy, and carotenoids and vitamin A. The subjects had normal body mass indices (18-28 $kg/m^2$); their fasting serum cholesterol concentrations were lower < 6.5 mmol/L and triacylglycerol

**Table 1. Characteristics of subjects participating in a study to determine the effect of consumption of a carotenoid supplement or spinach products on antioxidant activity in human blood**[1]

| | Group | | | | | |
|---|---|---|---|---|---|---|
| | Control | Carotenoid supplement | Whole-leaf spinach | Minced spinach | Liquefied spinach | Liquefied spinach plus dietary fibre |
| *n* | 10 | 12 | 12 | 12 | 12 | 12 |
| Men, women; *n, n* | 3, 7 | 5, 7 | 5, 7 | 5, 7 | 5, 7 | 5, 7 |
| Vegetarians, *n* | 3 | 1 | 3 | 7 | 3 | 3 |
| Age, *y* | 21 (18, 25)[2] | 21 (18, 24) | 20 (18, 58) | 21 (18, 42) | 21 (18, 38) | 20 (18, 54) |
| Energy (estimated), *MJ/d* | 11.4 ± 2.6 | 12.8 ± 3.4 | 11.5 ± 2.3 | 11.3 ± 2.1 | 11.4 ± 2.1 | 11.4 ± 2.3 |
| Serum cholesterol, *mmol/L* | 4.14 ± 0.65 | 4.07 ± 0.61 | 4.11 ± 0.79 | 4.23 ± 0.74 | 3.93 ± 0.47 | 4.17 ± 0.87 |
| Serum triacylglycerol, *mmol/L* | 0.88 ± 0.41 | 0.87 ± 0.32 | 0.92 ± 0.32 | 1.00 ± 0.39 | 0.89 ± 0.27 | 1.00 ± 0.34 |
| Body mass index, *$kg/m^2$* | 21 ± 2.1 | 22 ± 2.3 | 22 ± 2.0 | 22 ± 2.4 | 23 ± 2.1 | 22 ± 1.8 |

[1] Data collected at beginning of study except for energy intake, which is the mean over the intervention period. Values are means ± SD.

[2] Medians (ranges).

concentrations were < 2.8 mmol/L. During the study, two male subjects withdrew from the study for personal reasons. For characteristics of the subjects, see Table 1. The protocol for this study was approved by the Medical-Ethical Committee of Wageningen Agricultural University and all subjects gave their written informed consent.

**Study design**

The study started in January 1997 with a 3-wk run-in period, during which subjects chose their own diets but were instructed to avoid foods rich in carotenoids and retinol. Then, subjects were stratified according to age, sex, cholesterol concentration and energy intake and assigned to six experimental groups. The six treatment groups were fed the same basic diet throughout the study and the menu was changed daily on a weekly cycle. The basic diet did not include fruits and vegetables with moderate or high amounts of carotenoids and met the requirements of the Dutch Recommended Daily Allowances (Netherlands Food and Nutrition Council 1992). In addition to the basic diet, four groups received daily a spinach product; one group received a supplement of a suspension in vegetable oil of microcrystalline β-carotene (40 g/kg; Hoffmann-La Roche, Basel, Switzerland) and crystalline lutein and zeaxanthin derived from marigold flowers (60 g/kg and 3 g/kg, respectively; FloraGLO, kindly supplied by Kemin Industries Inc., Des Moines, IA). The carotenoid supplement was suspended in sunflower oil; for the carotenoid supplement group, some of the sunflower oil used in salad dressing fed to the control and spinach groups was replaced by the carotenoid supplement suspension. The spinach groups received 20 g/MJ of whole-leaf spinach, minced spinach, liquefied spinach or liquefied spinach to which dietary fibre was added. All spinach products originated from one batch and were provided, prepared and subsequently frozen by Langnese-Iglo (Wunstorf, Germany) for Unilever Research (Vlaardingen, The Netherlands). Whole-leaf spinach was washed and blanched for 90 s and cooled down quickly; minced spinach was minced to 5 mm after blanching. An enzymatic preparation with pectinase, hemicellulase and cellulase activities (Rapidase LIQ+, kindly supplied by Gist-Brocades, Seclin, France) was used for the liquefaction of minced spinach. After the enzyme treatment, the spinach was boiled for 5-10 min to inactivate the enzymes. One group received the liquefied spinach plus fibre prepared from beetroot pulp (10 g/kg, Fibrex 600, kindly supplied by TEFCO Food Ingredients b.v., Bodegraven, The Netherlands). The energy content of the diets of the control group and carotenoid supplement group was adjusted to that of the spinach groups by extra amounts of appropriate foods. All frozen spinach was

thawed and heated by microwave before consumption. The spinach products contained no measurable nitrite and < 1000 mg nitrate/kg, thus ensuring that the nitrate intake of the subjects was below the Acceptable Daily Intake for nitrate (Joint FAO/WHO Expert Committee on Food Additives 1995). Microbiological counts showed normal values and confirmed that the spinach products were safe for human consumption. Subjects were supplied with total diets, except for a limited choice of free products (~10 energy%). Twenty vegetarians took part in the experiment. They received the same foods as the non-vegetarian subjects ($n$ = 50), except for the meat, which was replaced by a vegetarian substitute with similar nutrient composition. On week days, subjects received a hot meal at the Division of Human Nutrition and Epidemiology; foods for their other meals and snacks were packed to be taken home. For weekend days, all foods were packed together with instructions and suggestions for preparation and consumption. The daily selection of free-choice foods was recorded in a diary and the nutrient content was calculated (NEVO Foundation 1995). The free items did not contain carotenoids or retinol. A maximum of two alcoholic consumptions per day was allowed but was not to be consumed together with the hot meal. Individual body weights throughout the study were maintained at ± 2 kg. Subjects were asked not to change their usual pattern of activities.

**Blood measurements**

Blood was taken from fasting subjects at 715-1000 h by venipuncture on d 0, 1, 8, 15, 21 and 22 of the intervention period for serum analyses of carotenoids and α-tocopherol concentrations, on d 0, 8, 15, and 22 for plasma analysis of ferric reducing ability (FRAP)[1], uric acid, and vitamin C concentrations and at d 0 and 22 for analysis of erythrocyte enzyme activities and oxidatively modified amino acids in plasma proteins.

**Carotenoids and α-tocopherol**

Samples, to which no anticoagulant was added, were left to clot; within 1 h after being drawn, were centrifuged and stored at -80°C. Serum carotenoids and α-tocopherol were measured by HPLC. To avoid day-to-day analytical variations, all samples from an individual were analysed as a set. After precipitation with ethanol, extraction followed with hexane twice, samples were evaporated under nitrogen and injected into the HPLC system (Craft et al. 1992). All sample

[1] Abbreviations used: AAS, adipic semialdehyde; CAT, catalase; FRAP, ferric reducing ability of plasma; GPx, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase.

preparations and extractions were carried out in duplicate and under subdued yellow light with minimal exposure to oxygen. The intra-assay CV for serum analysis of α-carotene, β-carotene, lutein, and zeaxanthin in control pools averaged 7.4, 3.9, 3.6 and 8.7%, respectively.

**Erythrocyte antioxidative enzymes**

Antioxidant enzyme activities were determined in erythrocyte lysates. Heparinised blood samples were centrifuged at 1500 x g for 10 min and plasma was removed. The erythrocytes were washed twice in 4 vol sterile physiological buffered saline, resuspended in 1 vol of sterile, deionised water for lysis and immediately frozen at -80°C. Automated assays were performed on a Cobas Mira analyser (Roche, Basel, Switzerland) to determine the activity of the antioxidant enzymes glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT). The activities of the enzymes were related to the amount of haemoglobin in the blood haemolysates. SOD (Randox cat. no. SD 125, Ardmore, UK), GPx (Randox cat. no. RS 05) and haemoglobin (Randox cat. no. HG 980) were determined using commercially available kits. GR activity and CAT activity were determined according to methods described previously (Wheeler et al. 1990). All analyses were conducted within 20 d. The intraday CV of SOD, CAT, GR and GPx averaged 1.9, 1.4, 3.1 and 1.9%, respectively. NADPH, glutathione, FAD, purpald and potassium periodate were purchased from Sigma Chemical (St. Louis, MO).

**Determination of adipic semialdehyde (AAS)**

Oxidatively modified amino acids in plasma proteins were analysed by HPLC (Daneshvar et al. 1997). The protein fractions were reacted with fluoresceinamine before the hydrolysis, and the decarboxylated fluoresceinamine derivatives of adipic and glutamic semialdehydes were measured by HPLC using a diode array detector. The intraday CV for this determination was 5.2%.

**FRAP, uric acid and vitamin C**

The antioxidant activity of samples, to which EDTA was added as an anticoagulant, was assessed as their ferric reducing ability (FRAP) (Benzie & Strain 1996). Uric acid concentration was measured in plasma samples using enzymatic colorimetric methods (Boehringer Mannheim, Germany). Vitamin C concentration in plasma treated with trichloroacetic acid was determined fluorimetrically as ascorbate plus dehydroascorbate (Vuilleumier & Keck 1996). The intra-assay, interday CV for plasma analysis of FRAP, uric acid and vitamin C

was 3.5, 2.0 and 5.6%, respectively.

**Food measurements**

The duplicate portions of the daily food intake of one subject collected throughout the study were mixed thoroughly as weekly portions; subsequently pooled samples were stored at -20°C until analysis. The moisture level and the ash content in each weekly portion were determined using a vacuum oven at 85°C and a muffle furnace at 550°C. The protein concentration was determined by the Kjeldahl method using a conversion factor of 6.25. The method of Folch et al. (1957) was used to extract fat. Dietary fibre was analysed according to the AOAC (1996) Official Method 992.16 for total dietary fibre. Digestible carbohydrate was calculated by difference. Carotenoids were extracted from wet material after homogenisation using tetrahydrofuran (THF), redissolved in THF/methanol (1:1 v/v) and injected into the HPLC system (Hulshof et al. 1997). The CV within runs of α-carotene, β-carotene and lutein in control pools averaged 5.7, 6.8 and 8.8%, respectively. Samples for vitamin C determination, to which metaphophoric acid was added, were collected from a number of daily-homogenised duplicate portions and immediately frozen. Vitamin C was determined fluorimetrically, after extraction with metaphosphoric acid/acetic acid (60:80, wt/v), as ascorbate plus dehydroascorbate (CV was 5.6%, Vuilleumier & Keck 1996).

**Statistical analysis**

Responses to the dietary intervention were averaged for carotenoids and α-tocopherol for d 0 and 1 and for d 21 and 22 for each subject. All other variables were measured once at the beginning and end of the dietary intervention period. The response to treatment was calculated for each person as the change in serum or plasma concentration or enzyme activity from the start to the end of the dietary intervention period. ANOVA was used to test equality of mean elevation for the different treatment groups, thereby controlling for several factors and covariables (SPSS Advanced Statistics, Chicago, IL). When all significant predictors of antioxidant levels are included in a model, it can be shown whether a particular variable is an independent predictor of the response after treatment. For example, for the ferric reducing ability of plasma (FRAP), uric acid should be included as a covariable because the uric acid concentration determines ~60% of the antioxidant activity of plasma (Benzie & Strain 1996). To evaluate the effect of one of the erythrocyte enzyme activities, all other activity responses were included in the model as covariables. Because initial values may have affected the magnitude of changes, effects of treatment were also assessed after

adjustment for baseline values by analysis of covariance, where applicable. Significant *F*-tests were followed by Tukey studentised range tests of pair-wise differences and Least Significant Difference (LSD) tests to evaluate differences among the four means of each spinach treatment. Pearson's correlations were calculated for the responses of several variables. *P*-values < 0.05 were regarded as significant. Values are means ± SD.

## RESULTS

Results of the composition of the diets are presented in Table 2 and results of analyses of various erythrocyte enzymatic activities and non-enzymatic, antioxidant concentrations in serum or plasma are described in Table 3 for the control, carotenoid supplement and pooled spinach groups. At baseline (wk 0), there were no significant differences among the erythrocyte antioxidant enzyme activities or serum or plasma antioxidant concentrations of the various experimental groups, except for erythrocyte SOD and GR activities. Results of the consumption of differently processed spinach products on the bioavailability of

**Table 2. Total daily intake of energy and nutrients of subjects participating in a study to examine the effect of consumption of a carotenoid supplement or spinach products on antioxidant activity in human blood[1]**

| | Group | | |
|---|---|---|---|
| | Control | Carotenoid supplement | Spinach products |
| Energy, *MJ/d* | 10.6 | 10.7 | 10.8 |
| β-Carotene, *mg/d* | 0.5 | 9.8 | 9.3 |
| Lutein, *mg/d* | 0.5 | 6.6 | 11.5 |
| Zeaxanthin, *mg/d* | 0.1 | 0.3 | 0.1 |
| Retinol, *mg/d* | 0.4 | 0.3 | 0.4 |
| Vitamin C, *mg/d*[2] | 100 | 83 | 63 |
| Dietary fibre, *g/d*[3] | 27.4 | 25.2 | 31.1 |

[1] Results are based on the analysis of duplicate portions for a subject consuming ~11 MJ together with an estimate calculated from the composition of the free items consumed. The free items did not contain carotenoids or retinol. There were no significant differences in nutrient intake among the groups or the 3 wk of dietary intervention. The daily intake was as follows: protein, 14 energy%; fat, 34 energy%; carbohydrate, 51 energy%; and alcohol, 1 energy%.

[2] Includes estimated amounts of vitamin C from free choice foods (2.0 - 3.1 mg/d). The vitamin C content of whole-leaf, minced, liquefied, and liquefied spinach plus added dietary fibre groups was 75, 61, 68 and 48 mg/d, respectively.

[3] The dietary fibre content of whole-leaf, minced, liquefied and liquefied spinach plus added dietary fibre groups was 32.4, 32.3, 28.2 and 31.3 g/d, respectively.

**Table 3. Variables of antioxidant activity in human blood: concentrations and activities of the control, carotenoid supplement and pooled spinach groups at baseline (wk 0), after 3 wk of dietary intervention (wk 3) and responses from begin to end of the dietary intervention period[1]**

| | Group | | |
|---|---|---|---|
| | Control | Carotenoid supplement | Pooled spinach |
| *n* | 10 | 12 | 48 |
| Adipic semialdehyde, *pmol/mg protein* | | | |
| Wk 0 | 23.8 ± 3.9 | 25.7 ± 4.0 | 24.8 ± 4.5 |
| Wk 3 | 27.3 ± 4.3 | 28.1 ± 8.8 | 27.2 ± 4.6 |
| Response | 3.5 ± 6.5 | 2.4 ± 9.1 | 2.3 ± 6.1 |
| Glutathione peroxidase, *U/g Hb* | | | |
| Wk 0 | 61.3 ± 27.7 | 54.6 ± 8.5 | 51.7 ± 10.8 |
| Wk 3 | 61.8 ± 26.5 | 59.2 ± 9.2 | 54.3 ± 9.7 |
| Response | 0.5 ± 8.9 | 4.6 ± 7.9 | 2.5 ± 9.3 |
| Glutathione reductase, *U/g Hb* | | | |
| Wk 0 | 9.7 ± 2.1 | 7.7 ± 1.4 | 8.5 ± 1.7 |
| Wk 3 | 8.8 ± 1.8 | 8.1 ± 1.9 | 8.7 ± 2.1 |
| Response | -0.9 ± 1.3 | 0.4 ± 1.6[2] | 0.2 ± 1.9[3] |
| Superoxide dismutase, *U/g Hb* | | | |
| Wk 0 | 1299 ± 312 | 1082 ± 155 | 1152 ± 180 |
| Wk 3 | 1295 ± 248 | 1143 ± 101 | 1190 ± 130 |
| Response | -4 ± 181 | 61 ± 133 | 38 ± 162 |
| Catalase, *U/g Hb* | | | |
| Wk 0 | 17.2 ± 3.1 | 17.5 ± 8.5 | 16.2 ± 3.3 |
| Wk 3 | 22.1 ± 9.0 | 20.1 ± 5.5 | 18.1 ± 4.5 |
| Response | 4.8 ± 7.1 | 2.6 ± 5.0 | 1.9 ± 5.2[3] |
| FRAP, *mmol/L* | | | |
| Wk 0 | 1.01 ± 0.13[4] | 1.03 ± 0.22 | 1.06 ± 0.17 |
| Wk 3 | 1.02 ± 0.28 | 1.01 ± 0.19 | 1.05 ± 0.12 |
| Response | 0.02 ± 0.22 | -0.02 ± 0.10 | -0.01 ± 0.12 |
| Uric acid, *mmol/L* | | | |
| Wk 0 | 242 ± 42[4] | 253 ± 70 | 266 ± 55 |
| Wk 3 | 247 ± 102 | 244 ± 63 | 254 ± 43 |
| Response | 5 ± 73 | -9 ± 21 | -12 ± 36 |
| Vitamin C, *μmol/L* | | | |
| Wk 0 | 57.0 ± 11.5 | 58.3 ± 14.5 | 60.5 ± 14.6 |
| Wk 3 | 67.8 ± 4.0 | 69.8 ± 12.7 | 70.2 ± 10.3 |
| Response | 11.8 ± 11.9 | 11.4 ± 15.2 | 9.7 ± 13.6 |
| α-Tocopherol, *μmol/L* | | | |
| Wk 0 | 18.8 ± 3.9 | 21.1 ± 4.5 | 20.5 ± 4.2 |
| Wk 3 | 21.9 ± 4.5 | 22.6 ± 4.1 | 21.5 ± 4.4 |
| Response | 3.1 ± 2.5 | 1.5 ± 2.1[2] | 1.0 ± 2.9[3] |

[1] Values are means ± SD. U/g Hb: units per gram haemoglobin. FRAP: ferric reducing ability of plasma.
[2] Significant difference between control group and carotenoid supplement group, $P = 0.02$.
[3] Significant difference between control group and pooled spinach groups, $P < 0.05$.
[4] $n = 9$.

carotenoids are described elsewhere (Castenmiller et al. 1999). The serum concentration of β-carotene increased from 0.253 ± 0.130 μmol/L to 2.608 ± 1.06 μmol/L in the carotenoid supplement group and from 0.283 ± 0.141 μmol/L to 0.445 ± 0.172 μmol/L in the pooled spinach groups. For lutein, the increase was from 0.218 ± 0.064 μmol/L to 0.983 ± 0.287 μmol/L in the carotenoid supplement group and from 0.216 ± 0.072 μmol/L to 0.915 ± 0.272 μmol/L in the pooled spinach groups. The serum zeaxanthin response was higher in the carotenoid supplement group than in the control group ($P = 0.006$) or the pooled spinach groups ($P < 0.001$; independent $t$ test) but was not different between the pooled spinach and control groups ($P = 0.07$). The serum triacylglycerol and cholesterol concentrations did not change over the experimental period or among the experimental groups (data not shown).

**Diets**

Duplicate portions of the food provided to the participants were analysed as pooled weekly portions; results of chemical analysis are shown in Table 2. For macronutrients and β-carotene, the concentrations measured were consistent among the treatment groups and for the 3 wk; the intake of lutein in the carotenoid supplement group (0.6 mg/MJ) was lower than in the spinach groups (1.1 mg/MJ). The vitamin C content of the diets with a carotenoid supplement, whole-leaf spinach, minced spinach, liquefied spinach and liquefied spinach plus added dietary fibre were 83, 75, 61, 68 and 48%, respectively, that of the control group. The α-tocopherol content of the diets was not measured. However, we expected that the supply of α-tocopherol would have been similar for all treatment groups, but may have been different for the vegetarian diet, in which meat was replaced by a vegetarian product. Therefore, when analysing the α-tocopherol responses, an adjustment was made for the factor vegetarian diet.

**Spinach products and carotenoid supplement vs. control diet**

The erythrocyte GR activity response, adjusted for responses of SOD and CAT, was higher ($P < 0.01$) whereas the CAT, adjusted for responses of GR, GPx and SOD, and α-tocopherol responses, adjusted for vegetarian diet ($P = 0.02$ and $P = 0.04$, respectively), were lower in the pooled spinach groups than in the control group. The response of erythrocyte GR activity, adjusted for responses of CAT and SOD, was higher ($P = 0.02$), whereas the serum α-tocopherol response was lower ($P = 0.02$) in the carotenoid supplement group than in the control group. Introducing the baseline value into the model, the difference between GR activity of the control and carotenoid supplement group was no longer significant,

whereas the difference between the control and spinach groups remained significant. The change in serum α-tocopherol concentration in the control group, which had the lowest mean baseline (wk 0) serum α-tocopherol concentration, was greater than that for the carotenoid and pooled spinach groups. These data are difficult to interpret. Adjustment of serum α-tocopherol concentrations for concentrations of cholesterol and triacylglycerol may provide a better reflection of dietary intake (Willett et al. 1983). The relative serum α-tocopherol concentration is the concentration of α-tocopherol in serum related to that of the sum of the concentrations of cholesterol and triacylglycerol in serum. The relative serum α-tocopherol responses also showed significant differences between the control and pooled spinach groups ($P < 0.001$) and tended to be different between the control and carotenoid supplement group ($P = 0.06$; $P = 0.006$ when adjusted for vegetarian diet). Analysis of covariance with baseline values (wk 0) and vegetarian diet as covariable and the serum concentration at wk 3 as dependent variable showed that the difference between the pooled spinach group and the control group was no longer significant, but the difference between the control and carotenoid supplement group remained significant. This study could not demonstrate any effect of intake of spinach or the carotenoid supplement on plasma concentrations of AAS, vitamin C, FRAP or uric acid.

**Carotenoid supplement vs. spinach products**

Although the bioavailability of β-carotene from the carotenoid supplement was much higher than that from the spinach products, no significant differences could be detected for any of the responses in enzymatic erythrocyte activities, plasma AAS, FRAP, uric acid and vitamin C concentrations or serum α-tocopherol concentration between carotenoid supplement and pooled spinach groups.

**Results among various spinach products**

The differences among the four spinach groups tended to be different ($P = 0.07$) only for uric acid responses (see Table 4). Whole-leaf spinach resulted in a higher uric acid response than consumption of liquefied spinach (Tukey method, $P = 0.06$; LSD method, $P = 0.01$). These results indicate that consumption of whole-leaf spinach, which resulted in lower serum carotenoid responses than consumption of other spinach products, may enhance oxidative defence more than consumption of liquefied spinach.

**Relationship between responses**

Pearson correlation coefficients for significant correlations of responses among

**Table 4. Variables of antioxidant activity in human blood: concentrations and activities at baseline (wk 0), after 3 wk of dietary intervention (wk 3) and responses from begin to end of the dietary intervention period for each spinach group[1]**

| | Group | | | |
|---|---|---|---|---|
| | Whole-leaf spinach | Minced spinach | Liquefied spinach | Liquefied spinach plus dietary fibre |
| Adipic semialdehyde, *pmol/mg protein* | | | | |
| Wk 0 | 25.5 ± 4.8 | 25.2 ± 4.2 | 23.8 ± 4.6 | 24.8 ± 4.7 |
| Wk 3 | 27.3 ± 4.4 | 27.0 ± 4.7 | 25.3 ± 3.9 | 29.0 ± 5.2 |
| Response | 1.8 ± 7.3 | 1.8 ± 5.8 | 1.6 ± 6.0 | 4.2 ± 5.6 |
| Glutathione peroxidase, *U/g Hb* | | | | |
| Wk 0 | 50.1 ± 8.5 | 50.6 ± 13.2 | 52.5 ± 13.4 | 53.7 ± 8.0 |
| Wk 3 | 52.5 ± 10.4 | 52.2 ± 9.6 | 57.3 ± 8.0 | 55.0 ± 11.0 |
| Response | 2.4 ± 6.8 | 1.6 ± 9.1 | 4.8 ± 10.6 | 1.3 ± 11.1 |
| Glutathione reductase, *U/g Hb* | | | | |
| Wk 0 | 9.4 ± 1.6 | 8.2 ± 1.2 | 7.6 ± 1.8 | 8.7 ± 2.0 |
| Wk 3 | 9.9 ± 2.9 | 8.4 ± 1.5 | 8.0 ± 1.2 | 8.4 ± 2.0 |
| Response | 0.6 ± 2.6 | 0.2 ± 1.3 | 0.4 ± 1.8 | -0.3 ± 1.8 |
| Superoxide dismutase, *U/g Hb* | | | | |
| Wk 0 | 1167 ± 150 | 1131 ± 194 | 1096 ± 187 | 1213 ± 187 |
| Wk 3 | 1173 ± 86 | 1211 ± 180 | 1133 ± 120 | 1242 ± 102 |
| Response | 6 ± 182 | 80 ± 170 | 37 ± 172 | 29 ± 132 |
| Catalase, *U/g Hb* | | | | |
| Wk 0 | 16.5 ± 3.2 | 16.7 ± 3.2 | 15.1 ± 2.7 | 16.6 ± 4.0 |
| Wk 3 | 20.5 ± 6.5 | 16.3 ± 2.6 | 17.3 ± 3.7 | 18.2 ± 3.7 |
| Response | 4.0 ± 6.6 | -0.4 ± 1.1[2] | 2.3 ± 4.0 | 1.6 ± 5.7 |
| FRAP, *mmol/L* | | | | |
| Wk 0 | 1.04 ± 0.14 | 1.02 ± 0.21 | 1.11 ± 0.16 | 1.07 ± 0.17 |
| Wk 3 | 1.07 ± 0.12 | 1.04 ± 0.12 | 1.05 ± 0.10 | 1.03 ± 0.15 |
| Response | 0.03 ± 0.10 | 0.02 ± 0.13 | -0.06 ± 0.12 | -0.04 ± 0.12 |
| Uric acid, *mmol/L* | | | | |
| Wk 0 | 264 ± 46 | 249 ± 63 | 284 ± 50 | 268 ± 61 |
| Wk 3 | 268 ± 43 | 244 ± 45 | 252 ± 40 | 253 ± 46 |
| Response | 5 ± 34 | -5 ± 32 | -32 ± 31[2] | -15 ± 39 |
| Vitamin C, *μmol/L* | | | | |
| Wk 0 | 67.3 ± 15.2 | 60.4 ± 14.9 | 54.7 ± 12.0 | 59.6 ± 15.0 |
| Wk 3 | 72.4 ± 9.0 | 71.4 ± 11.2 | 66.4 ± 7.9 | 70.8 ± 12.8 |
| Response | 5.0 ± 14.3 | 11.0 ± 12.3 | 11.7 ± 12.7 | 11.2 ± 15.6 |
| α-Tocopherol, *μmol/L* | | | | |
| Wk 0 | 21.0 ± 4.7 | 21.1 ± 4.5 | 20.4 ± 3.4 | 19.8 ± 4.3 |
| Wk 3 | 22.6 ± 5.4 | 21.3 ± 4.3 | 21.6 ± 3.9 | 20.7 ± 3.9 |
| Response | 1.6 ± 3.1 | 0.2 ± 4.0 | 1.3 ± 3.4 | 0.9 ± 1.2 |

[1] Values are means ± SD, $n$ = 12. U/g Hb: units per gram haemoglobin. FRAP: ferric reducing ability of plasma.

[2] Significant differences for uric acid response between whole-leaf spinach and liquefied spinach; Tukey studentised range test after ANOVA ($P$ = 0.07), $P$ = 0.06.

variables of antioxidant activities and concentrations were calculated. Significant and meaningful correlations ($r > 0.30$, $P < 0.05$) were observed among the responses of erythrocyte enzymatic antioxidant activities of GPx, GR, SOD and CAT (all positive), with an exception for the correlation between SOD and CAT. Positive correlations existed between plasma responses of FRAP and uric acid, plasma FRAP responses and serum α-tocopherol concentrations, plasma AAS and vitamin C responses. The serum zeaxanthin responses were positively correlated with responses in serum concentrations of α-tocopherol ($r = 0.39$), and a positive correlation was observed between erythrocyte GR responses and serum lutein responses ($r = 0.29$). There were no significant correlations between the serum β-carotene response and that of any of the other markers of antioxidative status measured.

## DISCUSSION

The present study showed that consumption of spinach resulted in greater responses of erythrocyte GR activity and lower erythrocyte CAT and serum α-tocopherol responses compared with the control group. Consumption of the carotenoid supplement increased erythrocyte GR activity response and decreased the serum α-tocopherol response compared with the control group. However, evidence for the lower α-tocopherol response in the spinach groups and greater GR response in the carotenoid supplement group is not conclusive. Because the diets supplied to the carotenoid supplement group and the control group were similar except for the carotenoid supplement, differences between these groups, as were found for responses of erythrocyte GR activity and serum α-tocopherol, can be attributed to the intake of β-carotene, lutein and a small amount of zeaxanthin as a carotenoid supplement dissolved in oil. It should be noted that the lutein is obtained from a natural source (marigold) and may thus contain some flavonoids and other phytochemicals. A small but significant correlation was observed between the responses of GR activity and serum lutein concentrations (Pearson correlation coefficient: 0.29; $P < 0.05$), suggesting that the greater responses of GR in the carotenoid supplement and spinach groups compared with the control group were related to the greater responses of serum lutein concentrations. The antioxidant responses in the pooled spinach group were not significantly different from those in the carotenoid supplement group. This suggests that the observed changes in antioxidant activities or concentrations were not caused by the increased absorption of β-carotene

because the β-carotene response, but not that of lutein, was much higher in the carotenoid supplement group than in the spinach groups. In cases in which significant differences between the pooled spinach and control group were present but not between the carotenoid supplement and the control group, as we found for the response in erythrocyte CAT activity, the difference was probably due to constituents other than carotenoids present in spinach (Dragsted et al. 1997).

**Antioxidant enzyme activities**

GR activity increased after 3 wk of dietary intervention with carotenoids. Diminished degradation of the antioxidant enzymes because of a protective role of carotenoids, known to deactivate singlet oxygen, could explain the relative increase in enzyme activity in the carotenoid supplement and spinach groups compared with the control group. Alternatively, carotenoids might act by inducing the enzymes. Although induction of an antioxidant enzyme theoretically cannot occur in erythrocytes, induction may occur during erythropoiesis. Moreover, activity of enzymes located in erythrocytes has been reported to change within hours after eating (Saghir et al. 1997).

GPx catalyses the degradation of peroxides with concomitant oxidation of glutathione. In the presence of GR and NADPH, the oxidised glutathione is immediately converted to the reduced form. Thus, it is not surprising that we found that the activities of GPx and GR were correlated. SOD catalyses the dismutation of superoxide anion radicals and CAT catalyses the reduction of hydrogen peroxide to water. From a kinetic point of view, CAT and GPx are both able to destroy hydrogen peroxide, but GPx has a much higher affinity for hydrogen peroxide than does CAT, suggesting that hydrogen peroxide is mainly degraded by GPx under normal conditions (Delmas-Beauvieux et al. 1996).

In a study conducted in France, elderly hospitalised subjects were supplied daily with a placebo; 20 mg zinc plus 100 μg selenium (mineral group); 120 mg vitamin C and 6 mg β-carotene and 15 mg vitamin E (vitamin group); or 20 mg zinc, 100 μg selenium, 120 mg vitamin C, 6 mg β-carotene and 15 mg vitamin E (mineral and vitamin group). After 6 mo of supplementation (Monget et al. 1996), significant effects of vitamin supplementation on GPx and SOD activities were reported whereas after 1 y of supplementation (Galan et al. 1997), significant increases were observed in GPx activity in the groups receiving minerals alone or in combination with vitamins, but there was no effect on SOD activity or thiobarbituric acid-reactive substances production. Omaye and co-workers (1996) fed nine women a low-carotenoid diet, followed by the same diet supplemented

with 15 mg β-carotene daily for 28 d and found a positive correlation between CAT and GPx activities; they concluded that β-carotene deficiency does have an effect on erythrocyte antioxidant status. In our study, a correlation between CAT and GPx activities was also found. Dixon et al. (1994) reported that erythrocyte SOD activity was depressed in carotene-depleted women, but it recovered with repletion. In a later study (Delmas-Beauvieux et al. 1996), supplementation of 60 mg β-carotene daily for 1 y did not result in a significant difference in SOD activity compared with baseline, whereas GPx activity slightly increased, and a significant increase in glutathione status values was observed after 12 mo compared with baseline. Thus, there is some previous evidence that β-carotene might influence the activity of antioxidant enzymes. Our study now suggests that not only β-carotene but also lutein has an effect on antioxidant enzyme activities.

**Antioxidant vitamins**

After 3 wk of dietary intervention, the serum α-tocopherol response was lower in the groups given the carotenoid supplement or spinach products than in the control group. The evidence for this was not completely conclusive for the difference between the pooled spinach and control groups. The lower response of α-tocopherol may reflect its increased utilisation as an antioxidant, in combination with the lower vitamin C content of the diets in the spinach groups. Furthermore, a significant correlation was found between serum α-tocopherol and zeaxanthin responses.

Xu and co-workers (1992) supplied subjects with a placebo or 15 to 60 mg β-carotene daily for 9 mo and found that all β-carotene doses resulted in similar decreases in plasma levels of α-tocopherol after 6 mo. On the other hand, the CARET study, after up to 6 y of supplementation, found a small but significant increase in the serum concentration of α-tocopherol in the participants taking 30 mg β-carotene and 7.5 mg retinyl palmitate (as retinol) per day (Goodman et al. 1994). Other studies have suggested that oral supplements of β-carotene do not change serum levels of α-tocopherol. The Polyp Prevention Study Group (Nierenberg et al. 1994) supplied 505 patients with a placebo or 25 mg β-carotene daily and found serum concentrations of vitamin E were not altered after 9 mo. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group (Albanes et al. 1997) studied 491 men in Helsinki. After an average of 6.7 y, the group supplemented with 20 mg β-carotene/d did not have different α-tocopherol concentrations than the control group. Nierenberg et al. (1997) conducted a supplementation study in which subjects received a placebo (*n* = 54) or 25 mg β-

carotene daily (*n* = 54) for 4 y and found that supplementation with β-carotene given orally did not alter serum concentrations of α-tocopherol. In an experiment in New Zealand (Zino et al. 1997), increased dietary intake of fruit and vegetables in the intervention group for 8 wk did not change concentrations of α-tocopherol. We conclude that most studies in which synthetic β-carotene is given as a supplement observe no effect on serum concentrations of α-tocopherol. Our study now suggests a relationship between serum zeaxanthin and α-tocopherol responses.

**Other antioxidants**

The higher response of plasma uric acid in the whole-leaf spinach group compared with the liquefied spinach group is noteworthy. Urate not only behaves as a *radical scavenger but also stabilises ascorbate in biological fluids*, e.g., human serum. This effect is due largely to iron chelation by urate. Unlike radical-scavenging reactions, this protective effect of urate is not associated with its depletion because a stable, non-catalytic urate-iron complex is formed (Sevanian et al. 1991). This phenomenon may explain why we did not find changes in responses of plasma vitamin C. The antioxidative effect of spinach and carotenoid supplement consumption could not be observed by measurements of FRAP. A study on the effect of juice intervention on markers of antioxidative status found that after 1 wk of intervention with increasing amounts of black currant and apple juice, only GPx activity had significantly increased with dose and FRAP remained unchanged (Young et al. 1999).

**Conclusions**

Consumption of a supplement of β-carotene, lutein and zeaxanthin, and spinach products resulted in changes in erythrocyte enzyme activities of GR and CAT and serum α-tocopherol concentrations. Antioxidants and antioxidant enzyme systems play key roles in the protection of biological membranes, lipoproteins, and DNA and in the prevention of protein damage. However, these defence systems are very complex, and changes in one antioxidant may result in changes in concentrations of other antioxidants or erythrocyte enzymes. We have documented the changes that occur after intake of a pure carotenoid supplement containing β-carotene and lutein, and a small amount of zeaxanthin, during a 3-wk controlled human dietary intervention study. The results were compared with those obtained after consumption of spinach products during the 3-wk study. The consumption of spinach resulted in greater responses of erythrocyte GR activity and lower erythrocyte CAT and serum α-tocopherol responses. Consumption of

the carotenoid supplement increased erythrocyte GR activity and lowered the serum α-tocopherol response. Our data suggest that carotenoid intake of lutein and zeaxanthin, but not β-carotene, is positively associated with erythrocyte GR activity and negatively with serum α-tocopherol concentration, respectively. The effect of spinach consumption on CAT activity is most likely not related to its carotenoid content. If analytical assays are improved in the near future and more information on antioxidant enzyme activity becomes available, we may be able to achieve a better understanding of the kinetics and mechanisms of the complex antioxidant defence systems.

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## REFERENCES

Albanes D, Virtamo J, Taylor PR, Rautalahti M, Pietinen P, Heinonen OP. Effects of supplemental β-carotene, cigarette smoking, and alcohol consumption on serum carotenoids in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. Am J Clin Nutr 1997;66:366-72.

Anonymous. AOAC Official Method 992.16 for total dietary fiber. In: Cunniff P, ed. Official Methods of Analysis of AOAC International, vol. II, 16th ed. Gaithersburg, MD: AOAC, 1996.

Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay. Anal Biochem 1996;239:70-6.

Berr C, Richard MJ, Roussel AM, Bonithon Kopp C. Systematic oxidative stress and cognitive performance in the population-based EVA study. Free Radic Biol Med 1998;24:1202-8.

Blot WJ. Vitamin/mineral supplementation and cancer risk: International chemoprevention trials. Proc Soc Exp Biol Med 1997;216:291-6.

Blot WJ, Li JY, Taylor PR, Guo W, Dawsey S, Wang GQ, Yang CS, Zheng SF, Gail M, Li GY, Yu Y, Liu B, Tangrea J, Sun Y, Fraumeni JF Jr, Zhang JH, Li B. Nutrition intervention trials in Linxian, China: Supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific

mortality in the general population. J Natl Cancer Inst 1993;85:1483-91.

Canfield LM. Carotenoids as cellular antioxidants. Proc Soc Exp Biol Med 1992;200:260-5.

Castenmiller JJM, West CE, Linssen JPH, van het Hof KH, Voragen AGJ. The food matrix of spinach is a limiting factor in determining the bioavailability of β-carotene and to a lesser extent of lutein in humans. J Nutr 1999;129:349-55.

Chen J, Geissler C, Parpia B, Li J, Campbell TC. Antioxidant status and cancer mortality in China. Int J Epidemiol 1992;21:625-35.

Craft NE, Wise SA, Soares JH Jr. Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids. J Chromatogr 1992;589:171-6.

Daneshvar B, Frandsen H, Autrup H, Dragsted LO. γ-Glutamyl semialdehyde and 2-amino-adipic semialdehyde: Biomarkers of oxidative damage to proteins. Biomarkers 1997;2:117-23.

Dartigues JF, Dabis F, Gros N, Moise A, Bois G, Salamon R, Dilhuydy JM, Courty G. Dietary vitamin A, beta carotene and risk of epidermoid lung cancer in south-western France. Eur J Epidemiol 1990;6:261-5.

Delmas-Beauvieux MC, Peuchant E, Couchouron A, Constans J, Sergeant C, Simonoff M, Pellegrin JL, Leng B, Conri C, Clerc M. The enzymatic antioxidant system in blood and glutathione status in human immunodeficiency virus (HIV)-infected patients: Effects of supplementation with selenium or beta-carotene. Am J Clin Nutr 1996;64:101-7.

Dixon ZR, Burri BJ, Clifford A, Frankel EN, Schneeman BO, Parks E, Keim NL, Barbieri T, Wu MM, Fong AKH, Kretsch MJ, Sowell AL, Erdman JW Jr. Effects of a carotene-deficient diet on measures of oxidative susceptibility and superoxide dismutase activity in adult women. Free Radic Biol Med 1994;17:537-44.

Dragsted LO, Strube M, Leth T. Dietary levels of plant phenols and other non-nutritive components: could they prevent cancer? Eur J Cancer Prev 1997;6:522-8.

Esterbauer H. Estimation of peroxidative damage. A critical review. Pathol Biol 1996;44:25-8.

Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497-509.

Galan P, Preziosi P, Monget AL, Richard MJ, Arnaud J, Lesourd B, Girodon F, Alferez MJ, Bourgeois C, Keller H, Favier A, Hercberg S. Effects of trace element and/or vitamin supplementation on vitamin and mineral status, free radical metabolism and immunological markers in elderly long term hospitalized subjects. The Geriatric/Min Vit Aox Network. Internat J Vit Nutr Res 1997;67:450-60.

Gaziano JM, Manson JE, Branch LG, Colditz GA, Willett WC, Buring JE. A prospective study of consumption of carotenoids in fruits and vegetables and decreased cardiovascular mortality in the elderly. Ann Epidemiol 1995;5:255-60.

Goodman GE, Metch BJ, Omenn GS. The effect of long-term beta-carotene and vitamin A administration on serum concentrations of alpha-tocopherol. Cancer Epidemiol Biomark Prev 1994;3:429-32.

Greenberg ER, Baron JA, Karagas MR, Stukel TA, Nierenberg DW, Stevens MM, Mandel JS, Haile RW. Mortality associated with low plasma concentration of beta carotene and the effect of oral supplementation. J Am Med Assoc 1996;275:699-703.

Hulshof PJM, Chao X, van de Bovenkamp P, Muhilal, West CE. Application of a validated method for the determination of provitamin A carotenoids in Indonesian foods of different maturity and origin. J Agric Food Chem 1997;45:1174-9.

Joint FAO/WHO Expert Committee on Food Additives. Evaluation of certain food additives and contaminants. Forty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series 859. Geneva, Switzerland: World Health Organization, 1995. [ISBN 92-4-120859-7].

Kardinaal AFM, Kok FJ, Ringstad J, Gomez-Aracena J, Mazaev VP, Kohlmeier L, Martin BC, Aro A, Kark JD, Delgado-Rodriguez M, Riemersma RA, van 't Veer P, Huttunen JK, Martin-Moreno JM. Antioxidants in adipose tissue and risk of myocardial infarction: the EURAMIC study. Lancet 1993;342:1379-84.

Knekt P, Aromaa A, Maatela J, Aaran RK, Nikkari T, Hakama M, Hakulinen T, Peto R, Teppo L. Serum vitamin A and subsequent risk of cancer: Cancer incidence follow-up of the Finnish Mobile Clinic Health Examination Survey. Am J Epidemiol 1990;132:857-70.

Knekt P, Reunanen A, Jarvinen R, Seppanen R, Heliovaara M, Aromaa A. Antioxidant vitamin intake and coronary mortality in a longitudinal population study. Am J Epidemiol 1994;139:1180-9.

Miller NJ, Rice-Evans CA. Factors influencing the antioxidant activity determined by the ABTS+ radical cation assay. Free Radic Res 1997;26:195-9.

Monget AL, Richard MJ, Cournot MP, Arnaud J, Galan P, Preziosi P, Herbeth B, Favier A, Hercberg S. Effect of 6 month supplementation with different combinations of an association of antioxidant nutrients on biochemical, parameters and markers of the antioxidant defense system in the elderly. The Geriatrie/Min Vit Aox Network. Eur J Clin Nutr 1996;50:443-9.

Netherlands Food and Nutrition Council. Recommended Dietary Allowances 1989 in The Netherlands. The Hague, The Netherlands: Netherlands Food and Nutrition Council, 1992.

NEVO Foundation. Dutch Nutrient Data Base. The Hague, The Netherlands: Voorlichtingsbureau voor de Voeding, 1995.

Nierenberg DW, Dain BJ, Mott LA, Baron JA, Greenberg ER. Effects of 4 y of oral supplementation with β-carotene on serum concentrations of retinol, tocopherol, and five carotenoids. Am J Clin Nutr 1997;66:315-9.

Nierenberg DW, Stukel TA, Mott LA, Greenberg ER. Steady-state serum concentration of alpha-tocopherol not altered by supplementation with oral beta-carotene. J Natl Cancer Inst 1994;86:117-20.

Omaye ST, Burri BJ, Swendseid ME, Hanning SM, Briggs LA, Bowen HT, Ota RB. Blood antioxidant changes in young women following beta-carotene depletion and repletion. J Am Coll Nutr 1996;15:469-74.

Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart , Hammar S. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. N Engl J Med 1996;334:11505.

Perrig WJ, Perrig P, Stahelin HB. The relation between antioxidants and memory performance in the old and very old. J Am Geriatr Soc 1997;45:718-24.

Poppel G van. Epidemiological evidence for β-carotene in prevention of cancer and cardiovascular disease. Eur J Clin Nutr 1996;50 (suppl.3):S57-S61.

Puhl H, Waeg G, Esterbauer H. Methods to determine oxidation of low-density lipoproteins. Methods Enzymol 1994;233:425-41.

Rapola JM, Virtamo J, Ripatti S, Huttunen JK, Albanes D, Taylor PR, Heinonen OP. Randomised trial of alpha-tocopherol and beta-carotene supplements on incidence of major coronary events in men with previous myocardial infarction. Lancet 1997;349:1715-20.

Riemersma RA, Wood D, Macintyre C, Elton R, Gay K, Oliver M. Risk of angina pectoris and plasma concentrations of vitamins A, C, and E and carotene. Lancet 1991;337:1-5.

Saghir AN, Rickards H, Pall HS. Postprandial changes in superoxide dismutase activity in subjects with Gilles de la Tourette syndrome and controls. Exp Neurol 1997;144:420-2.

Sevanian A, Davies KJ, Hochstein P. Serum urate as an antioxidant for ascorbic acid. Am J Clin Nutr 1991;54 (suppl.6):1129S-1134S.

Stahelin HB, Gey KF, Eichholzer M, Ludin E, Bernasconi F, Thurneysen J, Brubacher G. Plasma antioxidant vitamins and subsequent cancer mortality in the 12-year follow-up of the prospective

Basel (Switzerland) study. Am J Epidemiol 1991;133:766-75.

Stahl W, Nicolai S, Briviba K, Hanusch M, Broszeit G, Peters M, Martin H-D, Sies H. Biological activities of natural and synthetic carotenoids: induction of gap junctional communication and singlet oxygen quenching. Carcinogenesis 1997;18:89-92.

Street DA, Comstock GW, Salkeld RM, Schuep W, Klag M. Serum antioxidants and myocardial infarction. Are low levels of carotenoids and alpha-tocopherol risk factors for myocardial infarction? Circulation 1994;90:1154-61.

The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. N Engl J Med 1994;330:1029-35.

Vuilleumier JP, Keck E. Fluorometric assay of vitamin C in biological materials using a centrifugal analyser with fluorescence attachment. J Micronutr Anal 1996;5:25-34.

Wheeler CR, Salzman JA, Elsayed NM, Omaye ST, Korte DW Jr. Automated assays for superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activity. Anal Biochem 1990;184:193-9.

Willett WC, Stampfer MJ, Underwood BA, Speizer FE, Rosner B, Hennekens CH. Validation of a dietary questionnaire with plasma carotenoid and α–tocopherol levels. Am J Clin Nutr 1983;38:631-9.

Xu MJ, Plezia PM, Alberts DS, Emerson SS, Peng YM. Reduction in plasma or skin alpha-tocopherol concentration with long-term oral administration of beta-carotene in humans and mice. J Natl Cancer Inst 1992;84:1559-65.

Young JF, Nielsen SE, Haraldsdóttir J, Daneshvar B, Lauridsen ST, Knuthsen P, Crozier A, Sandström B, Dragsted LO. Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. Am J Clin Nutr 1999;69:87-94.

Zino S, Skeaff M, Williams S, Mann J. Randomised controlled trial of effect of fruit and vegetable consumption on plasma concentrations of lipids and antioxidants. Br Med J 1997;314:1787-91.

# 6

# Antioxidant properties of differently processed spinach products

**ABSTRACT**

The antioxidant activity of differently processed spinach products (whole-leaf, minced and enzymatically liquefied spinach) and its relation with carotenoids (β-carotene and lutein) and total phenolic compounds were determined. In an autoxidative methyl linoleate (MeLo) system the inhibition of formation of hydroperoxides, measured by HPLC after three days of oxidation at a level of 10 ppm was in descending order: whole-leaf > liquefied > minced spinach. The inhibition of formation of thiobarbituric acid-reactive substances (TBARS) and hexanal by spinach was determined in cooked meat with added spinach or water after two days of storage at 4°C. The formation of TBARS was inhibited by liquefied spinach at 200 g/kg meat; all other tested spinach products at 100 and 200 g/kg were prooxidative. The formation of hexanal was inhibited by both minced and liquefied spinach at 100 and 200 g/kg meat. The differently processed spinach products behaved differently when tested for their antioxidant activity (MeLo) or oxidative stability (meatballs). For the inhibition of TBARS formation, differences between whole-leaf and liquefied spinach were significant. For the inhibition of hexanal formation, activities among all spinach products were significantly different. We conclude that the antioxidant capacity of spinach is dose-dependent and affected by processing.

**KEY WORDS**: antioxidant; β-carotene; lutein; phenolics; food processing

## INTRODUCTION

In vitamin A replete populations, the incidence of cancer and of coronary heart disease has been found to be correlated negatively with the consumption of fruits and vegetables (Ness et al. 1997, World Cancer Research Fund/American Institute for Cancer Research 1997). This correlation has been attributed in part to the antioxidant properties of β-carotene. However, recent trials indicate that β-carotene supplements are not effective in disease prevention and raise questions about the biological significance of carotenoid antioxidant actions (Kritchevsky 1999). The beneficial effect of a high intake of fruits and vegetables may rely not only on the effect of the well characterised antioxidants, such as vitamins C and E and β-carotene, but rather on some other antioxidants or non-antioxidant constituents or on a concerted action of various compounds present in these foods (Cao et al. 1998). In general, more than 80% of the total antioxidant capacity in fruits and vegetables comes from ingredients other than vitamin C, indicating the presence of other potentially important antioxidants in these foods (Cao et al. 1996, Wang et al. 1996). In those studies, total antioxidant capacity was quantitated by using the oxygen radical absorbance capacity (ORAC) assay. Spinach is one of the vegetables that showed high ORAC activity (Cao et al. 1996, Wang et al. 1996). Flavonoids and other phenolic compounds appear to be antioxidants that contribute to the high antioxidant capacity observed in certain fruits and vegetables (Guo et al. 1997, Singleton et al. 1981). The antioxidant capacity of a food per se is important because it provides protection against loss of organoleptic properties.

The antioxidant activity of several plant materials has been reported (Al-Saikhan et al. 1995, Kanner et al. 1994, Mallet et al. 1994, Pietta et al. 1998, Prior et al. 1998, Velioglu et al. 1998), however information on the effect of processing on the antioxidant activity of plant foods is not available. Haila (1999) reviewed recently the antioxidant/prooxidant chemistry of carotenoids and summarised the available data on the action of carotenoids in different lipid systems.

The purpose of this work was to determine the effect of differently processed spinach products (whole-leaf, minced and enzymatically liquefied spinach) on inhibiting oxidation of food lipids. The antioxidant activity was tested using two oxidation systems: a methyl linoleate system and cooked meat. In the methyl linoleate system, the inhibition of formation of hydroperoxides was measured by HPLC after three days of oxidation. Inhibition of the formation of thiobarbituric acid-reactive substances (TBARS) and hexanal were determined in cooked meat after two days of storage at 4°C. Furthermore, the amounts of carotenoids, total

phenolics and some catechins, flavonols and flavones were measured in the spinach products tested.

## EXPERIMENTAL PROCEDURES

### Spinach products

Three differently processed spinach products were tested: whole-leaf spinach, minced spinach, and enzymatically liquefied spinach. All spinach products originated from one batch and were provided, prepared and subsequently frozen by Langnese-Iglo, Wunstorf (Germany) for Unilever Research Vlaardingen (The Netherlands). Whole-leaf spinach was washed and blanched for 90 sec and cooled down quickly and the blanched spinach was minced to 5 mm. An enzymatic preparation with pectinase, hemicellulase and cellulase activities (Rapidase® LIQ plus, kindly supplied by Gist-brocades, Seclin, France) was used for the liquefaction of minced spinach. After the enzyme treatment, the spinach was boiled for 5-10 min to inactivate enzymatic activities.

### Chemicals

Pure grades of all-*trans*-α-carotene, all-*trans*-β-carotene, all-*trans*-lycopene and gallic acid were obtained from Sigma Chemical Co. (St Louis, MO) and all-*trans*-lutein, all-*trans*-zeaxanthin, and all-*trans*-β-cryptoxanthin from Hoffmann-La Roche Ltd (Basel, Switzerland). Myricetin, quercetin dihydrate, kaempferol and apigenin were obtained from Fluka (Buchs, Switzerland). Methyl linoleate (MeLo) was purchased from Nu-Check-Prep, Inc. (Elysian, MI). All organic solvents and other chemicals used were of HPLC or reagent grades.

### Analysis of phenolics

The concentration of total phenolics in spinach was determined by the method described by Satué et al. (1995). Spinach samples (2 g) were extracted with 70% acetone (2 x 10 mL), evaporated to dryness and taken up to 10 mL in methanol. The concentrations of flavonols and flavones of the various spinach products were analysed by reversed phase HPLC, after acid hydrolysis of glycosides (Hertog et al. 1992). Catechins were measured by gradient reversed phase HPLC after methanol/water extraction (Arts et al. 1998).

### Analysis of dry matter, dietary fibre and carotenoids

The moisture level and the ash content in each spinach product were determined

using a vacuum oven at 85°C and a muffle furnace at 550°C. The dietary fibre content of the various spinach products was determined as methanol-ether insoluble solids (MEIS) and the amount of pectin (polygalacturonic acid) in the spinach products was measured by hydrolysing pectic substances to water-soluble galacturonic acid using an enzyme preparation (Katan & van de Bovenkamp, 1981). Carotenoids and retinol were extracted from wet material after homogenisation, using tetrahydrofuran (THF) and redissolved in THF/methanol (1:1 v/v) and injected into the HPLC system (Craft et al. 1992). The coefficients of variation (CV) within runs for food analysis of α-carotene, β-carotene and lutein in control pools averaged 5.7, 6.8 and 8.8%, respectively. All sample preparations and extractions were carried out in duplicate and under subdued yellow light with minimal exposure to oxygen (Hulshof et al. 1997).

**Methyl linoleate (MeLo) model**

Spinach extracts in methanol containing either 10 or 40 ppm (10 or 40 mg of total phenolics/kg spinach) were added to MeLo (1 g). Subsequently methanol was evaporated under nitrogen. Oxidation of MeLo was carried out in the dark at 40°C (Hopia et al. 1999). After 3 days of oxidation, the amount of hydroperoxides was measured as the formation of conjugated diene hydroperoxides by HPLC (Hopia et al. 1996a). The antioxidant activity of the spinach extract was expressed as inhibition (%) of the formation of MeLo hydroperoxides in the control sample. α-Tocopherol (20 ppm) was used as a reference antioxidant.

**Meatballs model: inhibition of thiobarbituric acid-reactive substances (TBARS) and hexanal formation**

These were measured at 100 and 200 g wet weight of spinach added to 1 kg meatballs. As a control, 100 or 200 g water/kg was added to the meat. Meat and spinach or water were mixed for approximately 1 min in a blender. Meatballs, made from pork meat (fat, 300 g/kg fresh weight; fatty acid composition was as follows: C14:0, 2.9%; C16:0, 51.4%; C16:1, 2.3%; C18:0, 18.7%; C18:1, 21.1%; C20:0, 1.1%; and C22:0, 0.8%) of 45 g weight were formed and heated in a microwave oven for 2 min. After cooling to room temperature, water loss was determined by weighing. Subsequently, the meatballs were stored in a plastic box for 48 h at 4°C. The antioxidant activity of the spinach extract was expressed as inhibition (%) of the formation of thiobarbituric acid-reactive substances (TBARS) and hexanal in the control sample. The meat sample (1 g) was homogenised (Ultra Turrax) with 5 mL trichloroacetic acid (35 g/100 mL) and 1 mL of the filtrate was mixed with 2 mL of TBARS reagent (20 mM 2-thiobarbituric acid) and heated

for 15 min in a water bath (100°C). The colour was measured at 532 nm. The blank consisted of 1 mL trichloroacetic acid and 2 mL thiobarbituric acid; samples were assayed in duplicate. The hexanal concentration was determined from 0.5 g meat mixed with 1 mL water in a headspace vial (NS20-vials, 10 mL). After incubating at 60°C for 15 min, 1 mL of the headspace was injected into the GC-Headspace (Carlo Erba 4130). The retention time of hexanal was approximately 4.5 min. The column was J &W DB1701 30 m x 0.32 mm with 1.0 μm film. The temperatures for the injector, oven and FID-detector were 180°C, 65°C, and 210°C, respectively. The gas flow was as follows: $N_2$, 140 kPa/7.5 mL/min; $H_2$, 50 kPa; and $O_2$, 100 kPa. A headspace autosampler (Carlo Erba HS 250) was used. The injection volume was 1000 μl and the temperatures of water bath and syringe were both 60°C. These samples were assayed in triplicate.

**Statistical analysis**

Values represent the mean of two (carotenoids, total phenolics, hydroperoxides, TBARS) or three (hexanal) replications. Data were analysed by analysis of variance (ANOVA) followed, when appropriate, by a Tukey or Dunnett test ($P < 0.05$). Pearson correlation coefficients were calculated ($P < 0.05$; two-tailed).

## RESULTS

Characteristics of the spinach products are given in Table 1.

**Table 1. Characteristics of differently processed spinach products (based on wet weight)**

| Type of spinach | Dry matter, *g/kg* | β-Carotene[a,c], *mg/kg* | Lutein[a,d], *mg/kg* | MEIS[b], *g/kg* | Polygalacturonic acid, *g/kg* | Total phenolics[a], *mg/kg* |
|---|---|---|---|---|---|---|
| Whole-leaf | 67 | 47 ± 6 | 101 ± 12 | 47.2 | 7.2 | 430 ± 35 |
| Minced | 59 | 40 ± 0.3 | 89 ± 1 | 39.4 | 5.9 | 470 ± 25 |
| Liquefied | 89 | 45 ± 7 | 79 ± 3 | 54.8 | 7.9 | 390 ± 10 |

[a] Mean ± SD, $n = 2$.
[b] MEIS = methanol-ether insoluble solids.
[c] β-Carotene/ total phenolics (*mg/mg*) is for whole-leaf, minced and liquefied spinach 0.109, 0.085 and 0.115, respectively.
[d] Lutein/ total phenolics (*mg/mg*) is for whole-leaf, minced and liquefied spinach 0.235, 0189 and 0.203, respectively.

## Antioxidant activity of spinach products

*Inhibition of formation of hydroperoxides*

At the level of 40 ppm, all samples completely inhibited the formation of hydroperoxides, which is comparable to an inhibition capacity of 20 ppm α-tocopherol. At the level of 10 ppm, the inhibition of hydroperoxide formation was highest for whole-leaf spinach (82 ± 3 %), followed by liquefied spinach (75 ± 2 %) and minced spinach (72 ± 2 %). In this test system, whole-leaf spinach had the highest antioxidant capacity.

*Inhibition of formation of TBARS*

Corrected for water loss measured in the control sample, whole-leaf and minced spinach increased the formation of TBARS at 100 and 200 g/kg meat (Table 2 and Figure 1). Thus, both the whole-leaf and minced spinach products were prooxidative at 100 and 200 g/kg, but with an increasing amount of spinach added to the meatballs, the whole-leaf spinach was less prooxidative ($P = 0.022$). The liquefied spinach showed antioxidant activity (inhibition of formation of TBARS) at 200 g/kg and was slightly prooxidative at 100 g/kg. The difference in antioxidant activity between the whole-leaf spinach and the liquefied spinach at 200 g/kg was significant ($P = 0.010$).

**Table 2. Inhibition (% ± SD) of formation of TBARS ($n$ = 2) and hexanal ($n$ = 3) with various spinach products added in amounts of 100 and 200 g/kg to cooked meat after 48 hours of storage at 4°C[a,b]**

| Type of spinach | Amount g/kg | Mean inhibition of formation | |
|---|---|---|---|
| | | TBARS | Hexanal |
| Whole-leaf | 100 | -75.4 ± 0.5[w] | -30.7 ± 5.9[d,x] |
| Minced | 100 | -47.1 ± 9.0 | 9.8 ± 5.6[d,y] |
| Liquefied | 100 | -15.6 ± 11.1 | 29.2 ± 3.0[d,z] |
| Whole-leaf | 200 | -22.4 ± 3.1[c,w] | -12.1 ± 3.7[e,x] |
| Minced | 200 | -12.3 ± 13.0 | 32.3 ± 5.1[e,y] |
| Liquefied | 200 | 32.3 ± 4.5[c] | 54.8 ± 1.8[e,z] |

[a] The control samples contained 100 or 200 g water/kg meat, respectively. The loss of water differed between the samples containing spinach and the controls and the data in the Table are corrected for the loss of water.

[b] Means within column with similar letter are significantly different ($P < 0.05$).

[c] Significant difference in inhibition of TBARS formation at 200 g spinach/kg meat between whole-leaf and liquefied spinach (Dunnett test after ANOVA: $P = 0.01$).

[d,e] Significant difference in hexanal inhibition (Tukey test after ANOVA, $P < 0.01$).

[w,x,y,z] Significant difference between inhibition at 100 and 200 g of each type of spinach tested/kg meat (independent samples $t$-test, $P < 0.001$).

*Inhibition of formation of hexanal*

With respect to the mean inhibition of hexanal formation, corrected for water loss in the control sample, the whole-leaf spinach was prooxidative at 100 g/kg and less prooxidative at 200 g spinach/kg meat ($P$ = 0.010). The minced and liquefied spinach were antioxidative at 100 g/kg and the activity increased at 200 g/kg ($P$ < 0.01). The liquefied spinach was more active than the minced spinach and both spinach products were more active than the whole-leaf spinach ($P$ < 0.01).

**Carotenoid content vs. antioxidant activity**

A correlation was observed between the inhibition of hydroperoxide formation and the calculated amount of lutein, but not of β-carotene, in the original spinach samples. Here we assume that the proportion of extracted carotenoids was similar for each sample. Whole-leaf spinach had the highest adjusted lutein concentration, followed by liquefied and minced spinach (Table 1). The Pearson correlation coefficient for adjusted lutein content was 0.999 ($P$ = 0.02, $n$ = 3). Correlations for β-carotene or total phenolic content or between carotenoid content of the spinach products and the inhibition of formation of TBARS and hexanal were not significant.

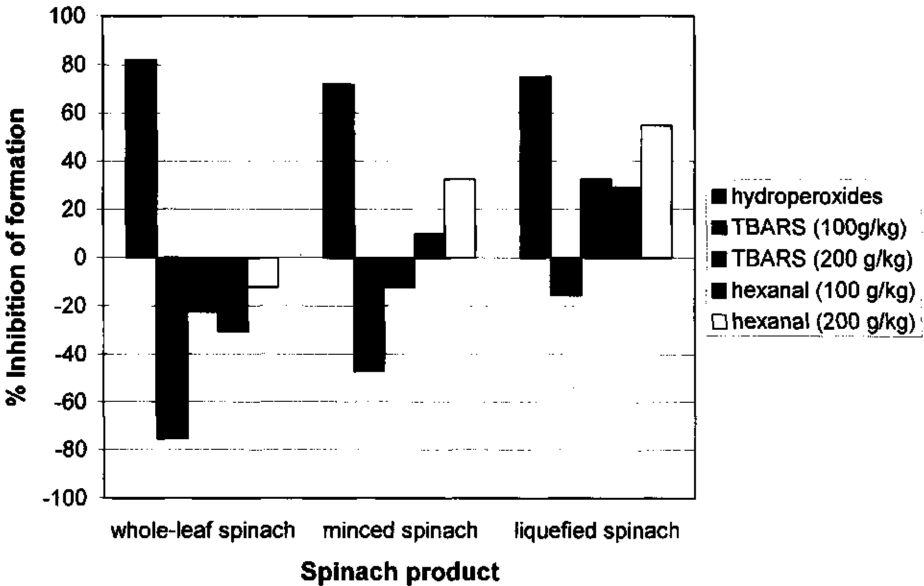


**Figure 1.** Prooxidant and antioxidant activity of spinach products. Inhibition (%) of formation of hydroperoxides (10 ppm), TBARS (100 and 200 g spinach/kg meat) and hexanal (100 and 200 g spinach/kg meat) by whole-leaf, minced and liquefied spinach.

**Amount of total phenolics, catechins, flavonols and flavones**
The amount of total phenolics ranged from 390 mg/kg in liquefied spinach to 470 mg/kg in minced spinach (see Table 1). The spinach products did not contain measurable amounts of catechins (detection limits): catechin (< 0.05 mg/kg); epicatechin (< 0.05 mg/kg); epicatechingallate (< 0.2 mg/kg); epigallocatechingallate (< 0.2 mg/kg); epigallocatechin (< 2 mg/kg). Also neither flavonols nor flavones were detectable: quercetin (< 1 mg/kg); myricetin (< 1 mg/kg); kaempferol (< 2 mg/kg); isorhamnetin (< 5 mg/kg); luteolin (< 1 mg/kg); apigenin (< 1 mg/kg). The amount of total phenolics was correlated with the MEIS on a wet weight basis of the different spinach products ($P = 0.005$, $r = -1.0$, $n = 3$).

## DISCUSSION

The present study showed that frozen spinach products demonstrated prooxidant and antioxidant activity depending on the extent of processing and the amount of spinach used. In a lipid methyl linoleate (MeLo) model, at a level of 40 ppm of total phenolics, all spinach extracts had an inhibitory activity on the formation of hydroperoxides comparable to 20 ppm α-tocopherol. At a level of 10 ppm of total phenolics the antioxidant activity in descending order was as follows: whole-leaf > liquefied > minced spinach. Higher concentrations of spinach in the meatballs (200 g/kg meat vs. 100 g/kg meat) significantly improved the antioxidant activity or decreased the prooxidant activity of all spinach products (inhibition of hexanal formation) and whole-leaf spinach (inhibition of formation of TBARS), respectively. Processing had a significant effect on the antioxidant activity of the spinach products. Differences in oxidant activity became significant for the difference between whole-leaf and liquefied spinach (inhibition of TBARS formation at 200 g/kg) and for all spinach products with respect to the inhibition of hexanal formation at all concentrations tested. An effect of processing has been reported before: strawberry extracts pre-treated with pectinase inhibited hexanal formation significantly less and blueberry extracts pre-treated with pectinase more than acetone extracts (Heinonen et al. 1998b).

None of the vegetables studied by Cao et al (1996), including spinach, showed prooxidant activity. Chopra et al. (1993) demonstrated that, at atmospheric oxygen concentrations, β-carotene slightly augmented the peroxidation of linoleic acid, whereas the xanthophyll carotenoids caused inhibition against peroxidation. However, experiments in the MeLo model with spontaneously autoxidised lipids, showed constantly increasing prooxidant activity (formation of hydroperoxides) of

β-carotene at concentrations of 5, 20, and 200 ppm, oxidised at 40°C in the dark and of lutein at concentrations of 5, 20, 30, and 40 ppm both in dark and light, at 40°C and 25°C, respectively (Haila et al. 1999). Minor amounts of tocopherol protected carotenoids from destruction and thus inhibited the prooxidant action of carotenoids (Haila et al. 1996). Tsuchihashi et al. (1995) used a MeLo model with a radical initiator and found that β-carotene suppressed methyl linoleate oxidation in benzene solution in a dose-dependent manner. The antioxidant activity in our MeLo model was positively related to the lutein content of the original spinach samples, adjusted for their total phenolics content. Carotenoids, if not in an α-tocopherol-free environment, reduce the rate of autoxidation of lipids by such processes as decomposing hydroperoxides to nonradical products.

The concentration of total phenolics and lutein per 100 g spinach was lowest in the liquefied spinach. Nevertheless, the liquefied spinach had the highest antioxidant activity in the meatball model. It may be speculated that when using a whole food instead of an extract the following factors may play a role: bioavailability of phenolics and carotenoids: liquefied spinach had the highest amounts of bioavailable β-carotene per 100 g spinach (Castenmiller et al. 1999); the polarity of the carotenoids; or other food components such as phenolic acids with a high antioxidant activity. The spinach samples did not contain detectable amounts of catechins, flavonols (quercetin, myricetin, kaempferol or isohamnetin) or flavones (luteolin and apigenin). This is in line with the analysis of Hertog et al. (1992) who also did not find measurable amounts of quercetin, kaempferol, luteolin, apigenin or myricetin in spinach. Nevertheless, the total amount of phenolics ranged from 390 to 470 mg/kg spinach. Possible other reducing compounds in spinach giving a response in the total phenolics analysis may include phenolic acids such as ferulic acid, caffeic acid and coumaric acid. A direct relationship exists between antioxidant capacity and the amount of phenolic compounds of wines and grapes (Abu-Amsha et al, 1996, Fogliano et al. 1999, Heinonen et al. 1998a, Kanner et al. 1994), different berries (Prior et al. 1998), medicinal plants (Pietta et al. 1998), and a number of fruit and vegetables and grain products (Velioglu et al. 1998). However, the relationship between phenolics and antioxidant activity was not significant for potato cultivars (Al-Saikhan et al. 1995) and anthocyanin-rich materials, including blueberry (Velioglu et al. 1998). The total phenolic content of berry and fruit wines and liquors did not correlate with the antioxidant activity in a MeLo system (Heinonen et al. 1998a). Our study showed that the total phenolic content, i.e., the reducing capacity of spinach, did not alone predict the antioxidant response. However, to explain the differences in antioxidant activity of various spinach products when tested under different

conditions, differences in the activities of the phenolic compounds and their antagonistic and synergistic reactions with other phenolics and other compounds present in spinach may be of importance.

Ascorbate was not a significant contributor to the antioxidant activity measured in a study of berries (Prior et al. 1998, Wang et al. 1996). Prior et al. (1998) observed that if the skin of the blueberry was broken, ascorbate may be oxidised and the concentration may be significantly reduced. We expect to find the lowest vitamin C content in the liquefied spinach due to its prolonged processing (matrix disruption and heat treatment) and thus conclude that vitamin C in spinach is indeed not a significant contributor to antioxidant activity. In calculations with other fruits, ascorbate has generally contributed less than 10% of the total antioxidant capacity (Wang et al. 1996). $\alpha$-Tocopherol, a phenolic compound, is the major liposoluble antioxidant found in leaves. Because bioactive compounds can act synergistically, antioxidative effects of fruits and vegetables may reflect a combined mechanism of antioxidant defence (Wise et al. 1996). Several studies have shown that the serum or blood lymphocyte antioxidant capacity increased following consumption of fruits and vegetables (Cao et al. 1998) or dried spinach powder and lutein (Pool-Zobel et al. 1997). These studies support the hypothesis that carotenoid containing plant products, but not synthetic carotenoids tested as single compounds, exert a cancer protective effect via a decrease in oxidative and other damage to DNA in humans (Pool-Zobel et al. 1997).

Apparently conflicting results have been obtained in our study under different testing conditions, using different models. In general, it is critical to establish the influence of the methodology used to evaluate natural antioxidants: the conditions of oxidation and the analytical method used to determine the extent and endpoint of oxidation must be taken into account. The degree of oxidation should be determined by more than one method and by measuring different types of products, including initial and decomposition products of lipid oxidation, such as hydroperoxides and volatiles by GC (Frankel 1993). Lipid peroxidation is a radical chain reaction that generates conjugated diene hydroperoxides as the initial main product (Abuja et al. 1998). Various types of antioxidants affect the formation of hydroperoxides and their decomposition quite differently. Antioxidant activity can be monitored by measuring the formation of conjugated diene hydroperoxides and the decomposition of hydroperoxides can be monitored by measuring hexanal, the major volatile product of linoleic acid hydroperoxides. The thiobarbituric acid (TBA) method is based on the colour reaction between TBA and oxidation products of polyunsaturated lipids. This simple test is sensitive and very precise, but the test is not specific because many secondary oxidation

products form TBARS. The test is more sensitive when used with polyunsaturated fatty acids containing three or more double bonds (Frankel 1993). It should be noted that linoleic acid is perhaps not the best substrate to evaluate natural antioxidants since food lipids are mainly triglyceride in nature (Frankel 1993). In conclusion, prooxidant and antioxidant properties of carotenoids, and other substances, depend on the conditions and the partners in the reaction (Buettner 1993, Gazzani et al. 1998), including partial oxygen pressure, carotenoid concentrations, interactions with other carotenoids, solvents, type of radical initiator, and type and polarity of the lipid system used (Britton 1995, Hopia et al. 1996b, Palozza, 1998, Tsuchihashi et al. 1995).

Our study may contribute to a better understanding of the antioxidant and prooxidant activity of spinach products and their carotenoid and total phenolics content. Of importance is the finding that spinach can have a prooxidative activity, which can become antioxidative by further processing of the spinach or by increasing the spinach concentrations. The results of this study are somewhat contradictory. Therefore in future studies the measurements made should be related to organoleptic and keeping qualities, which would be comparatively easy. They could also be related to health consequences once the food is eaten but this would be more difficult.

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## REFERENCES

Abu-Amsha R, Croft KD, Puddey IB, Proudfoot JM, Beilin LJ. Phenolic content of various beverages determines the extent of inhibition of human serum and low-density lipoprotein oxidation in vitro: identification and mechanism of action of some cinnamic acid derivatives from red wine. Clin Sci 1996;91:449-58.

Abuja PM, Murkovic M, Pfannhauser W. Antioxidant and prooxidant activities of elderberry (Sambucus nigra) extract in low-density lipoprotein oxidation. J Agric Food Chem 1998;46:4091-6.

Al-Saikhan MS, Howard LR, Miller JC Jr. Antioxidant activity and total phenolics in different genotypes of potato (Solanum tuberosum, L.). J Food Sci 1995;60:341-3.

Arts ICW, Hollman PCH. Optimization of a quantitative method for the determination of catechins in fruits and legumes. J Agric Food Chem 1998;46:5156-62.

Britton G. Structure and properties of carotenoids in relation to function. FASEB J 1995;9:1551-8.

Buettner GR. The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol, and ascorbate. Arch Biochem Biophys 1993;300:535-43.

Castenmiller JJM, West CE, Linssen JPH, van het Hof KH, Voragen AGJ. The food matrix of spinach is a limiting factor in determining the bioavailability of β-carotene and to a lesser extent of lutein in humans. J Nutr 1999;129:349-55.

Cao G, Russell RM, Lischner N, Prior RL. Serum antioxidant capacity is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. J Nutr 1998;128:2383-90.

Cao G, Sofic E, Prior RL. Antioxidant capacity of tea and common vegetables. J Agric Food Chem 1996;44:3426-31.

Chopra M, Wilson RL, Thurnham DI. Free radical scavenging of lutein in vitro. Ann NY Acad Sci 1993;691:246-9.

Craft NE, Wise SA, Soares JH Jr. Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids. J Chromatogr 1992;589:171-6.

Fogliano V, Verde V, Randazzo G, Ritieni, A. Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. J Agric Food Chem 1999;47:1035-40.

Frankel EN. In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. Trends Food Sci Technol 1993;41:220-5.

Gazzani G, Papetti A, Massolini G, Daglia M. Anti- and prooxidant activity of water soluble components of some common diet vegetables and the effect of thermal treatment. J Agric Food Chem 1998;46:4118-22.

Guo C, Cao G, Sofic E, Prior RL. High performance liquid chromatography coupled with coulometric array detection of electroactive components in fruits and vegetables: Relationship to oxygen radical absorbance capacity. J Agric Food Chem 1997;45:1787-96.

Haila K, Hopia A, Heinonen M. Effects of β-carotene and retinal on the formation and isomer distribution of methyl linoleate hydroperoxides. In: Haila K. Effects of carotenoids and carotenoid-tocopherol interaction on lipid oxidation in vitro. 1) Scavenging of free radicals. 2) Formation and decomposition of hydroperoxides. PhD thesis, EKT-series 1165. Helsinki, Finland: University of Helsinki, Department of Applied Chemistry and Microbiology, 1999.

Haila K, Lievonen S, Heinonen M. Effects of lutein, lycopene, annatto, and γ-tocopherol on autoxidation of triglycerides. J Agric Food Chem 1996;44:2096-2100.

Heinonen IM, Lehtonen PJ, Hopia AI. Antioxidant activity of berry and fruit wines and liquors. J Agric Food Chem 1998a;46:25-31.

Heinonen IM, Meyer AS, Frankel EN. Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. J Agric Food Chem 1998b;46:4107-12.

Hertog MGL, Hollman PCH, Katan MB. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. J Agric Food Chem 1992;40:2379-83.

Hopia A, Heinonen M. Antioxidant activity of flavonol aglycones and their glycosides in methyl linoleate. J Am Oil Chem Soc 1999;76:139-44.

Hopia AI, Huang S–W, Frankel EN. Effect of alpha-tocopherol and trolox C on the decomposition of methyl linoleate hydroperoxides. Lipids 1996a;31:357-65.

Hopia AI, Huang S-W, Schwarz K, German JB, Frankel EN. Effect of different lipid systems on antioxidant activity of rosemary constituents carnosol and carnosic acid with and without α-tocopherol. J Agric Food Chem 1996b;44:2030-6.

Hulshof PJM, Chao X, van de Bovenkamp P, Muhilal, West CE. Application of a validated method for the determination of provitamin A carotenoids in Indonesian foods of different maturity and origin. J Agric Food Chem 1997;45:1174-9.

Kanner J, Frankel E, Granit R, German B, Kinsella JE. Natural antioxidants in grapes and wines. J Agric Food Chem 1994;42:64-9.

Katan MB, van de Bovenkamp P. Determination of total dietary fiber by difference and of pectin by colorimetry or copper titration. In: James WPT, Theander O, Eds. The analysis of dietary fiber in foods, Ch 14, pp 217-39. New York, NY: Marcel Dekker Inc., 1981.

Kritchevsky SB. β-Carotene, carotenoids and the prevention of coronary heart disease. J Nutr 1999;129:5-8.

Mallet JF, Cerrati C, Ucciani E, Gamisans J, Gruber M. Antioxidant activity of plant leaves in relation to their alpha-tocopherol content. Food Chem1994;49:61-5.

Ness AR, Powles JW. Fruit and vegetables, and cardiovascular disease: a review. Int J Epidemiol 1997;26:1-13.

Palozza P. Prooxidant actions of carotenoids in biologic systems. Nutr Rev 1998;56:257-65.

Palozza P, Krinsky NI. β-Carotene and α-tocopherol are synergistic antioxidants. Arch Biochem Biophys 1992;297:184-7.

Pietta P, Simonetti P, Mauri P. Antioxidant activity of selected medicinal plants. J Agric Food Chem 1998;46:4487-90.

Pool-Zobel BL, Bub A, Muller H, Wollowski I, Rechkemmer G. Consumption of vegetables reduces genetic damage in humans: first results of a human intervention trial with carotenoid-rich foods. Carcinogenesis 1997;18:1847-50.

Prior RL, Cao G, Martin A, Sofic E, McEwen J, O'Brien C, Lischner N, Ehlenfeldt M, Kalt W, Krewer G, Mainland CM. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of Vaccinium species. J Agric Food Chem 1998;46:2686-93.

Satué MT, Huang S-W, Frankel EN. Effect of natural antioxidants in virgin olive oil on oxidative stability of refined, bleached, and deodorized olive oil. J Am Oil Chem Soc 1995;72:1131-7.

Singleton VL. Naturally occurring food toxicants: phenolic substances of plant origin common in foods. Adv Food Res 1981;27:149-242.

Tsuchihashi H, Kigoshi M, Iwatsuki M, Niki E. Action of β-carotene as an antioxidant against lipid peroxidation. Arch Biochem Biophys 1995;323:137-47.

Velioglu YS, Mazza G, Gao L, Oomah BD. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J Agric Food Chem 1998;46:4113-7.

Wang H, Cao G, Prior RL. Total antioxidant capacity of fruits. J Agric Food Chem 1996;44:701-5.

Wise JA, Morin RJ, Sanderson R, Blum K. Changes in plasma carotenoid, alpha-tocopherol, and lipid peroxide levels in response to supplementation with concentrated fruit and vegetable extracts: A pilot study. Current Therapeutic Res 1996;57:445-61.

World Cancer Research Fund/American Institute for Cancer Research. Food, nutrition and the prevention of cancer: a global perspective. Washington, DC: American Institute for Cancer Research, 1997.

# 7

# General discussion

In this thesis two aspects of spinach have been examined, namely the bioavailability of carotenoids and folate and the antioxidant activity of spinach products when consumed and in food products. This Chapter evaluates the contribution of the studies described in this thesis to present scientific knowledge. Conclusions and suggestions for future research are also presented.

## FACTORS AFFECTING BIOAVAILABILITY AND BIOCONVERSION OF CAROTENOIDS (SLAMENGHI FACTORS)

The SLAMENGHI factors were used to examine the knowledge and information available on the bioavailability and bioconversion of carotenoids (Chapter 2). Each carotenoid appears to have an individual pattern of absorption, plasma transport, and metabolism. Data on each SLAMENGHI factor is necessary to fill the gaps in our knowledge about carotenoids and their metabolism and actions, so that food-based strategies and interventions with specific foods to control vitamin A deficiency and to reduce the risk for chronic diseases can be properly devised. We conclude that the data available are not sufficiently comparable to allow a systematic comparison of results. However, a number of conclusions can be drawn. The bioavailability and bioconversion of various carotenoids and geometrical isomers of carotenoids differ; molecular linkage does not play a role; a positive relationship exists between intake and response of β-carotene; β-carotene supplementation increases serum α-carotene concentrations and decreases serum concentrations of lutein, lycopene and canthaxanthin; the bioavailability of β-carotene from foods is low, particularly from dark-green leafy vegetables; intake of dietary fat has a positive and intake of dietary fibre a negative effect on bioavailability; alcohol interferes with the bioconversion of β-carotene; and that nutrient status and genetic factors related to the host may explain some of the differences observed. There is a need for more carefully designed studies in order to define the individual SLAMENGHI factors (West & Castenmiller 1998).

## BIOAVAILABILITY OF CAROTENOIDS

The most commonly applied methods to measure carotenoid bioavailability include measuring the increase in serum or plasma concentrations following long-term administration of a controlled diet resulting in a new steady-state condition,

typically up to 17 days for β-carotene (Nierenberg et al. 1991), and use of postprandial chylomicron carotenoid response following a single dose of carotenoid (van den Berg & van Vliet 1998, van Vliet et al. 1995). In a three-week dietary controlled intervention study involving healthy subjects, the effect of the food matrix on the bioavailability of carotenoids was examined. Consumption of spinach (20 g/MJ), containing β-carotene and lutein, increased serum concentrations of all-*trans*-β-carotene, *cis*-β-carotene (and consequently total β-carotene), lutein, α-carotene and retinol and decreased the serum concentration of lycopene compared with the control group. Compared to a synthetic carotenoid supplement, the relative bioavailability of β-carotene was low, 5.1-9.5%, but much higher for lutein, 45-55%. This finding has been confirmed in a study by van het Hof et al. (1999a): they compared plasma responses of a mixed vegetable diet and found relative bioavailabilities for β-carotene and lutein of 14 and 67%, respectively. The low bioavailability of β-carotene from green vegetables (de Pee et al. 1995, Chapter 3) may partly be explained by the presence of 15-18% *cis*-β-carotene in the spinach products. All-*trans*-β-carotene is more readily absorbed than its *cis* isomers (Ben-Amotz & Levy 1996, Gaziano et al. 1995b, Jensen et al. 1987, Tamai et al. 1995, Chapter 2). However, also the relatively high lutein:β-carotene ratio in spinach may have negatively affected the bioavailability of β-carotene (van den Berg 1998). Plasma lutein concentrations were significantly reduced after single (Kostic et al. 1995, Martini et al. 1995, White et al. 1994) and multiple (Micozzi et al. 1992) doses of β-carotene, and lutein had an inhibitory effect on β-carotene plasma response (Kostic et al. 1995). Combined dosage with lutein decreased the area-under-the-curve (AUC) of β-carotene and retinyl palmitate in the triacylglycerol-rich lipoprotein (TRL) fraction adjusted for the triacylglycerol response by 66 and 74% respectively, compared with the response for β-carotene alone, but had no effect on β-carotene cleavage (van den Berg & van Vliet 1998, van den Berg 1998). A combined dosage with lycopene had no significant effect on β-carotene and retinyl palmitate response (van den Berg & van Vliet 1998). In the long-term Alpha-Tocopherol Beta-Carotene (ATBC) intervention study on β-carotene supplementation, a slight (approximately 11%) but significant decrease in serum lutein concentrations were observed as compared with a placebo (Albanes et al. 1997), but no effects on other serum carotenoids were observed in other long-term intervention studies (Fotouhi et al. 1996, Nierenberg et al. 1997). The increase in serum α-carotene levels after β-carotene supplementation (Micozzi et al. 1992, Pappalardo et al. 1997, de Pee et al. 1995, Wahlqvist et al. 1994, Chapter 3) might also be due to reduced cleavage

of α-carotene in the presence of excess β-carotene (van den Berg 1999). In conclusion, interactions or competition between carotenoids can occur during incorporation into mixed micelles in the intestinal lumen, during absorption into enterocytes, during β-carotene cleavage, or during chylomicron assemblage (van den Berg 1999). Van den Berg (1999) reviewed literature on carotenoid interactions and concluded that much of the evidence suggests an interaction between β-carotene and oxycarotenoids, such as canthaxanthin and lutein, and between the hydrocarbon carotenoids β-carotene and lycopene. These interactions needs further research because both of the effects reported, an interference of β-carotene on lutein uptake, and vice-versa, might have long-term health effects.

Granado et al. (1998) observed the occurrence of ester forms in subjects having a serum lutein concentration of over 1.05 μmol/L. This may indicate a ceiling effect on or saturation of the transport capacity for lutein, which may be re-esterified in vivo when it is supplied in excess of normal dietary intake. In our study, the mean serum lutein concentration per group was below 1 μmol/L (Chapter 3).

The relative bioavailability of β-carotene in dark-green leafy vegetables is extremely low (de Pee et al. 1995, de Pee et al. 1998, Chapter 3). Pure β-carotene is absorbed far more readily than that in foods. For example, the plasma response to pure β-carotene has been found to be about five times the response to a similar amount of β-carotene in carrots (Brown et al. 1989, Micozzi et al. 1992). The serum response of β-carotene from fruits was four times that from vegetables (de Pee et al. 1998) and the bioavailability of β-carotene and lutein varied substantially among different vegetables (van het Hof et al. 1999b). The bioavailability of carotenoids in dark-green leafy vegetables, root vegetables (e.g., carrots), and fruits partly depends on the matrix in which the carotenoid is located in the unprocessed product. Processing, cooking or fine grinding increased the bioavailability of carotenes possibly because of the softening or disruption of plant cell walls and the disruption of carotenoid-protein complexes (Congdon et al. 1981, Erdman et al. 1988, Hussein & El-Tohamy 1990, Törrönen et al. 1996, van Zeben & Hendriks 1948). Disruption of the vegetable matrix also enhanced the bioavailability of lycopene from tomatoes (Gärtner et al. 1997, Porrini et al. 1998, Stahl & Sies 1992). The effect of the food matrix on the lutein bioavailability has not been reported in the literature. Our study (Chapter 3) confirmed that disruption of the food matrix (cell wall structure) and loss of cellular structure had an effect on the bioavailability of β-carotene, whereas the bioavailability of lutein was not

affected. Serum β-carotene responses differed significantly between the whole-leaf and liquefied spinach groups and between the minced and liquefied spinach groups.

Addition of dietary fibre to the liquefied spinach to compensate for the fibre that was broken down during liquefaction had no effect on serum carotenoid responses. Dietary fibre has an effect on lipid metabolism by interacting with bile salts, resulting in a possible decreased absorption of carotenoids, and other fat-soluble substances. Rock and Swendseid (1992) reported that increases in plasma β-carotene in humans were significantly reduced by dietary pectin; in our study the proportion of dietary fibre added per mg β-carotene was much smaller.

Other experimental approaches are required to estimate the absolute bioavailability of carotenoids and to study the mechanisms involved. These approaches could include studies with isotopically labelled carotenoids.

## BIOAVAILABILITY OF FOLATE

Brouwer et al. (1999b) reviewed the literature and used the SLAMENGHI factors to describe the present state of knowledge on bioavailability and bioefficacy of folate and folic acid. They concluded that the food matrix and the amount of folic acid consumed are the major factors to influence folate bioavailability in healthy persons. These factors, in addition to the effectors of absorption, such as dietary fibre and the amount of fat, also play an important role in the bioavailability and bioconversion of carotenoids.

Studies examining the effect of single foods on the bioavailability of dietary folate showed that folate was absorbed, but the relative bioavailability, as compared to synthetic folic acid, differed greatly between different foods (Brouwer et al. 1999a, Cuskelly et al. 1996, Gregory et al. 1995, Keagy et al. 1988, Tamura & Stokstad 1973). Few studies have examined the bioavailability of folate from mixed diets (Sauberlich et al. 1987). The most commonly applied methods include measuring the increase in serum or plasma concentrations following long-term administration of a controlled diet resulting in a new steady-state condition. Plasma folate concentrations reached a steady state after four weeks of supplementation with 1 mg folic acid daily (Heseker & Schmitt 1987). In our controlled dietary human intervention study we examined the effect of consumption of spinach as part of a normal, mixed meal and destruction of the spinach food matrix (disruption of the cell wall and loss of dietary fibre) on plasma folate concentrations. Daily consumption for three weeks of 20 g/MJ of spinach

increased plasma folate concentrations significantly. Intake of minced and liquefied spinach resulted in greater plasma folate responses than consumption of whole-leaf spinach, which suggests that the food matrix of spinach plays a role with regard to bioavailability of folate (Chapter 4).

In an experiment with ten women, who were maintained in a metabolic unit for 92 d, dietary folates appeared to be no more than 50% available when compared to pteroylmonoglutamic acid added to the diets (Sauberlich et al. 1987). This was confirmed by Cuskelly and co-workers (1996) in a long-term study, who found that the bioavailability of added folic acid was higher than the bioavailability of food folate. Brouwer et al. (1999a) conducted a four-week controlled dietary intervention study and found that folate from vegetables and citrus fruits had a relative bioavailability, as compared to synthetic folic acid, of 60% based on plasma total homocysteine concentrations, 78% based on plasma folate concentrations, and 98% based on red blood cell folate concentrations. No studies have been published that have examined the effect of food processing on improving folate bioavailability. The consumption of the carotenoid supplement (see Chapter 3) had no effect on plasma folate concentrations compared with the control group. This implies that the study design could have been improved by supplying a synthetic folic acid supplement to a study group to estimate the relative bioavailability of folate from spinach.

Re-addition of dietary fibre to the liquefied spinach to compensate for the fibre broken down during liquefaction did not reduce the plasma folate response. The effect of dietary fibre on plasma folate concentrations is not conclusive. No effect on folate absorption was observed for cellulose or wheat bran, up to 8.2 g dietary fibre per meal (Russell et al. 1976), or 30 g wheat bran in a formula meal (Keagy et al. 1988). In contrast, 30 g wheat bran increased the folic acid absorption of a formula meal (Keagy et al. 1988) and 34 g wheat bran (19 g dietary fibre) reduced the plasma folate concentration of folates consumed in solution (Bailey et al. 1988). In our study, the total dietary fibre intake for the liquefied spinach and liquefied spinach plus added dietary fibre was 2.6 and 2.9 g/MJ, respectively. This difference was too small to become statistically significant.

The effect of food processing and heat treatment on the composition of folate forms in spinach and other foods is not clear and needs further research. Differences in chain length of food folates may explain differences in bioavailability of differently processed spinach products. An experimental approach involving isotopically labelled folates could be developed to answer these questions.

## ANTIOXIDANT ACTIVITY OF SPINACH IN MAN

Antioxidants inhibit or delay the oxidation of molecules by inhibiting the initiation or propagation of oxidising chain reactions (Halliwell 1996). Carotenoids have been found to inhibit free radical-induced lipid peroxidation (Krinsky 1993, Krinsky & Deneke 1982) and to quench singlet oxygen (Stahl et al. 1997).

The serum/plasma antioxidant concentrations, erythrocyte enzyme antioxidant activities, and concentrations of oxidatively damaged amino acids in plasma were measured in humans after consumption of spinach during a three-week period (Chapter 5). Consumption of spinach resulted in significantly greater erythrocyte glutathione reductase activity and lower erythrocyte catalase activity and serum α-tocopherol concentration compared with the control group. Consumption of the carotenoid supplement led to lower α-tocopherol responses and tended to increase the erythrocyte glutathione reductase activity. These changes may be related to increased serum lutein concentrations after carotenoid intake. Glutathione peroxidase catalyses the degradation of peroxides with concomitant oxidation of glutathione. In the presence of glutathione reductase and NADPH, the oxidised glutathione is immediately converted to the reduced form. Superoxide dismutase catalyses the dismutation of superoxide anion radicals and catalase catalyses the reduction of hydrogen peroxide to water. Thus, the removal of hydrogen peroxide ($H_2O_2$) is achieved by catalase, which is largely sequestered within the peroxisomal compartment of cells, and by the S-dependent enzyme glutathione peroxidase (Diplock 1994), whereby the affinity for hydrogen peroxide is higher for glutathione peroxidase than for catalase.

The increased glutathione reductase activity may be explained by decreased enzyme degradation due to a protective effect of carotenoids. Alternatively, carotenoids may induce the enzyme. The lower α-tocopherol concentrations in the spinach and carotenoid supplement groups may reflects its increased utilisation as an antioxidant, especially in combination with the lower ascorbic acid (vitamin C) contents of the diets in the spinach groups. In studies with synthetic β-carotene supplements, plasma α-tocopherol concentrations have been shown to decrease (Xu et al. 1992), increase (Goodman et al. 1994), or stay the same (Albanes et al. 1997, Nierenberg et al. 1994, Nierenberg et al. 1997). In our study a significant positive correlation was observed between serum responses of zeaxanthin and α-tocopherol and this finding may explain the inconclusive evidence for an effect of β-carotene supplementation on α-tocopherol concentrations.

A number of studies (Dugas et al. 1998, Romanchik et al. 1997) illustrate the

uncertainty that exists regarding the effects of β-carotene and other carotenoids on low-density lipoproteins (LDL) oxidative stability in vitro. Neither vegetable consumption (up to 490 g/d) nor a carotenoid supplement had effect on the resistance of LDL to oxidation ex vivo as compared to the control group (van het Hof et al. 1999a). *Administration of β-carotene did not reduce oxidation of LDL* particles, which actually carry β-carotene in the lipid phase of plasma, in vivo (Gaziano et al. 1995a, Reaven et al. 1994, Reaven et al. 1993). Recent studies (Bowen & Omaye 1998) even suggest that β-carotene participates as a prooxidant in the oxidative degradation of LDL (see Introduction for discussion of possible mechanisms) and that elevated LDL concentrations may counteract the protective effects of α-tocopherol. Following supplementation with concentrated fruit and vegetable extracts, decreases in lipid peroxide levels were coincident with increases in carotenoids and α-tocopherol (Wise et al. 1996). Hininger et al. (1997) found that an increased intake of carotenoid rich food had an inhibitory effect on the susceptibility of LDL to oxidation.

There are several methods to measure the prooxidant and antioxidant activity of β-carotene and other carotenoids, including measurements of cell damage, mainly as lipid peroxidation products, e.g., formation of thiobarbituric acid-reactive substances (TBARS) and hydroperoxides; non-enzymatic antioxidants concentrations and non-enzymatic antioxidant activity; and intermediate products of the reaction between free radical species and carotenoid molecules. Most studies on the ability of supplementary carotenoids to protect cells against oxidatively induced DNA damage have determined oxidatively induced DNA damage or protection against oxidation of LDL. However, by measuring activity of red blood cell antioxidative enzymes (Nielsen et al. 1998, Young et al. 1999), functional biomarkers for antioxidant activity could be developed. Measurements of erythrocyte enzyme antioxidant activities, serum or plasma non-enzymatic antioxidant concentrations and ferric reducing ability of plasma (FRAP) and uric acid concentrations can be considered biomarkers of defence in antioxidant research, whereas concentrations of oxidatively damaged amino acids in plasma is a biomarker for damage to proteins. Other biomarkers for damage include the determination of malondialdehyde (oxidation of fat) and the Comet assay (DNA damage).

Our data (Chapter 5) suggest that serum lutein concentration, or lutein and zeaxanthin intake, is positively related with erythrocyte glutathione reductase activity and negatively with serum α-tocopherol concentration. Moreover, the antioxidant responses in the pooled spinach group were not significantly different from those in the carotenoid supplement group, suggesting that the observed

changes in antioxidant activities or concentrations were not caused by the increased absorption of β-carotene, because the β-carotene response, but not that of lutein, was much higher in the carotenoid supplement group than in the spinach groups. In vivo oxidative metabolites have been described in human subjects after lutein supplementation (Khachik et al. 1995, Khachik et al. 1997, Olmedilla et al. 1997), indicating that lutein has biological activity with the potential of providing protection against cancer. The presence of the direct oxidation product of lutein and 3'-epilutein (metabolite of lutein and zeaxanthin) in human retina suggests that lutein and zeaxanthin may act as antioxidants to protect the macula against short-wavelength visible light and these carotenoids may therefore play an important role in prevention of age-related macular degeneration and cataracts (Khachik et al. 1997).

It is expected that in the near future analytical assays will be improved and more information on antioxidant enzyme activity will become available, thus enabling a better understanding of the kinetics and mechanisms of the complex antioxidant defence systems to be obtained.

## ANTIOXIDANT CAPACITY OF SPINACH IN FOOD

Generally, antioxidant and prooxidant properties of any substance depend on the conditions and the partners in the reaction (Buettner 1993). Antioxidant, autoxidation and even prooxidant processes are possible, and the relative efficiency of these processes in any system will determine whether a compound has an antioxidant action. Carotenoids are generally found in foods complexed with either proteins or fatty acids, which protect them from oxidation. The breakdown of these complexes allows the prooxidant action of carotenoids in lipids to take place and leads to degradation of carotenoids, resulting in blanching or discoloration of the product.

The effect of differently processed spinach products on inhibiting oxidation of food lipids, with special emphasis on the effect of total phenolics and carotenoids (β-carotene and lutein) present in spinach, was determined (Chapter 6). The antioxidant activity of spinach extracts and spinach products was tested using two oxidation systems: a methyl linoleate (MeLo) system and a cooked meat model, respectively. In the methyl linoleate system the inhibition of formation of hydroperoxides was measured after three days of autoxidation. The inhibition of thiobarbituric acid-reactive substances (TBARS) and hexanal concentrations was determined in cooked meatballs after two days of storage at 4°C. On the basis of

inhibition of hydroperoxide formation, the activity of oxidation, corrected for the content of phenolic compounds, decreased at 10 ppm in the descending order of minced (72% decrease) > liquefied (75% decrease) > whole-leaf spinach (82% decrease). Whole-leaf and minced spinach were more prooxidative and showed greater increased TBARS formation than the liquefied spinach. Minced and liquefied spinach were antioxidant active with respect to the mean inhibition of hexanal formation. Differences among the three spinach products (in descending order of inhibition of TBARS and hexanal formation: liquefied > minced > whole-leaf spinach) were significant. A strong correlation was observed between the calculated amount of lutein, but not for β-carotene, of the original spinach samples (amounts were adjusted for the content of phenolic compounds) and the inhibition of hydroperoxide formation. In contrast to this finding is the highest antioxidant activity of the liquefied spinach in the meatballs model: the liquefied spinach had the lowest content of total phenolics and lutein.

In fruits and vegetables, more than 80% of the total antioxidant capacity comes from ingredients other than ascorbic acid, indicating the presence of other potentially important antioxidants in these foods (Cao et al. 1996, Wang et al. 1996). There is evidence for a prooxidant activity of β-carotene in vitro (Haila et al. 1999) and in vivo (Lomnitsky et al. 1991, Reaven et al. 1993). Prooxidant means that the compound in question promotes oxidation. β-Carotene seems to act as a prooxidant when it is added to an α-tocopherol-deficient medium or diet (Haila et al. 1999, Lomnitsky et al. 1991). α-Tocopherol was the major liposoluble antioxidant found in leaves (Mallet et al. 1994). Even a minor amount of tocopherol protects carotenoid from destruction and thus inhibits the prooxidant action of carotenoids. It can be concluded that the naturally occurring combination of a carotenoid and a stabilising component such as tocopherol acts as a lipid antioxidant, whereas when tested as an isolated carotenoid, β-carotene, lutein and lycopene acted as lipid prooxidants (Haila et al. 1999, Haila et al. 1996, Palozza 1998). However, some of our spinach products, especially at low amounts, showed prooxidant activity. It must be assumed that the concentrations of carotenoids and co-antioxidants were too low in these cases to behave as antioxidants.

The differently processed spinach products behaved differently when tested for antioxidant activity (MeLo) or stability (meatballs). In the methyl linoleate model extracts were tested that contained at least phenolic compounds, whereas the whole spinach product was tested in the meatballs model. The thiobarbituric acid method has been widely used to evaluate the effectiveness of potentially antioxidative compounds to prevent lipid oxidation. The main drawback of this

method is that only an advanced oxidation status can be detected. A different approach is to measure the induction period before the rapid oxidation phase, which occurs in a lipid matrix exposed to conditions of accelerated oxidation. The methyl linoleate lipid model used in Chapter 6 is suitable for mechanistic studies, but may be less appropriate to evaluate the antioxidant capacity of most foods or biological systems (Hopia et al. 1996).

There is still much to be learned about the synergistic effects of potential antioxidants in foods and the effect of processing on the prooxidant or antioxidant activity of foods.

## HEALTH EFFECTS OF FRUITS AND VEGETABLES

Epidemiological studies strongly suggest that high intakes of fruits and vegetables, foods rich in β-carotene and other carotenoids and ascorbic acid, are associated with a reduced risk of heart disease and cancer. Whether this risk reduction is due to the action of one or several carotenoids, to other plant-based substances, or is secondary to confounding factors is at this point unclear. Evidence from clinical trials shows that supplemental β-carotene does not prevent coronary heart disease or cancer, though the benefits of other carotenoids has not been ruled out. The serum levels reported from the observational epidemiological studies, showing that β-carotene protects against cancer, were much lower than the levels achieved in the various intervention trials. Typical intakes of β-carotene in most populations are less than 5 mg/d (Russell 1998). The Finnish trial provided 20 mg, while the CARET trial supplemented men with 30 mg β-carotene and 8000 RE retinol each day. The β-carotene levels achieved were 5.6 μmol/L in the ATBC study, 3.9 μmol/L in the CARET study and 2.2 μmol/L in the Physicians Health Study. In the USA, the 95th percentile of serum carotenoid levels in the general population was only 0.9 μmol/L (Russell 1998). It is possible that beneficial effects of β-carotene might be seen only at physiological and not pharmacological levels. For example, there could be local accumulation of β-carotene and its metabolic products in lung tissue, or it could be that the high doses used in the intervention trials had a negative impact on one or more dietary protective factors (Russell 1998). The following working hypothesis has been introduced by Russell (1998): low doses of β-carotene can give rise to low levels of eccentric cleavage products, which can be converted to retinoic acid and thus have a beneficial effect in preventing cancer; high-dose β-

carotene supplements can give rise to high levels of eccentric cleavage products in oxidative conditions. This could especially be seen, for example, in the lung exposed to smoke. Such eccentric cleavage products in high concentrations could have biological activity per se, or compete with the biological functions of retinoic acid. Another explanation for the lack of beneficial effect of β-carotene could be the possibility that the prooxidant behaviour of carotenoids prevailed over the antioxidant behaviour because of carotenoid concentration, oxygen tension of tissues, and/or inadequate pre-existing antioxidant defences (Palozza 1998). In contrast, prooxidant activity could induce beneficial effects in already transformed cells, inhibiting tumour growth.

The results of the studies described in this thesis (Chapters 5 and 6) support the finding that β-carotene is not a good antioxidant in vivo or in vitro, but indicate that lutein may play a role as an antioxidant. Among the explanations for the disparity between epidemiological and intervention studies is the possibility that β-carotene may be a surrogate marker for other protective dietary factors. In addition, there are several other potentially beneficial micronutrients in fruits and vegetables. In many cases, it is not possible to separate the influence of these other dietary factors from that of carotenoids because data on these food components are not currently included in nutrient databases. Furthermore, the relation between carotenoid intake and disease risk may not be linear and carotenoid-related variables in disease outcomes may occur largely at the lower end of the intake spectrum, especially in women (Tribble 1998).

We are not aware of any other study that has determined effects of intake of fruits or vegetables on biomarkers of antioxidant activity in vivo and has measured antioxidant capacity in vitro. The studies described in Chapters 5 and 6 suggest that there is a direct relationship between in vitro and in vivo results of antioxidant activity from spinach. The antioxidative activity of spinach products in vitro could be confirmed in vivo as changes in certain biomarkers of antioxidative enzyme activity. It should be noted that spinach consumption decreased serum lycopene concentrations. Lycopene is an effective in vitro antioxidant that may have protected β-carotene from oxidation (Stahl et al. 1997). Clearly more research is needed to establish and validate good markers or biomarkers to measure antioxidant activity in human blood and in foods.

The finding that β-carotene as a single nutrient is not responsible for the health effects of fruits and vegetables supports the hypothesis that the synergistic effect of a number of nutrients and perhaps nonnutrients promotes health. Examples of synergistic effects include the formation of a complex of retinol and β-carotene with iron, keeping the iron soluble in the intestinal lumen and preventing the

inhibitory effect of phytates and polyphenols on iron absorption (Garcia-Casal et al. 1998) and the synergism between antioxidants. No controlled dietary trials have been conducted to study the effect of fruits and vegetables intake on endpoints for degenerative diseases. The DASH-trial assessed the effect of fruit and vegetable intake on blood pressure and found that a fruit and vegetable diet had a significant effect on systolic blood pressure. However, a diet of fruit and vegetables, low-fat diary foods and with reduced saturated total fat was much more effective in reducing both the systolic and diastolic blood pressure (Appel et al. 1997).

Gey (1995) concluded after reviewing complementary observational data, that these data consistently suggest that optimal, i.e., potentially protective plasma levels, should be > 0.4 μmol/L β-carotene (> 0.5 μmol/L total carotene). Russell (1998) suggests that it may well be that once serum levels exceed 1.5 mg/L (ca. 2.8 μmol/L), harmful effects ensue. Lin et al. (1998) measured carbonyl production by $CuSO_4$-challenged LDL from healthy women on a controlled diet plus a mixed carotenoid supplement and concluded that LDL seemed fully protected with 2.3 μmol β-carotene/L plasma.

In conclusion, optimal health and protection against oxidative damage and related diseases is best served by the variety of bioactive substances found in fruits and vegetables, which act together, instead of a single component. There is insufficient or inconclusive evidence regarding carotenoids, although β-carotene per se is unlikely to be a causative factor. The combination of α-tocopherol, β-carotene and ascorbic acid may be effective in inhibiting oxidative damage, especially in vivo where oxygen concentration is low (Niki et al. 1995).

## FUTURE STUDIES

A number of suggestions for further studies are set out below.

- Future studies on nutrient bioavailability and bioconversion should be designed in such manner that the results between studies can be compared and that they provide information to contribute to the quantification of one or more SLAMENGHI factors influencing bioavailability and bioconversion of carotenoids and the bioavailability of folate. Food composition databases used for observational studies should provide information on the bioavailability of carotenoids and of folate.
- New methods should be developed to study the bioavailability and bioconversion of nutrients. Until now, most studies have measured β-carotene,

retinol or folate concentrations in serum or plasma. One complicating factor in comparative studies that include provitamin A carotenoids and use the serum or plasma response as a measure of absorption is that provitamin A carotenoid cleavage is not accounted for and, thus, absorption of these carotenoids is underestimated. Another complication of postprandial plasma responses is the presence of endogenous carotenoids and resecretion of absorbed carotenoids by the liver (van den Berg 1999). For example, by feeding labelled β-carotene and retinol with 10 $^{13}C$-atoms at predetermined positions to participants the degree of labelling of these compounds can be measured in serum using liquid chromatography/mass spectrometry (LC-MS) (van Breemen et al. 1998, van Lieshout et al. 1999). This enables the bioavailability of β-carotene and retinol to be estimated. Alternatively, measuring carotenoid responses in the triacylglycerol-rich lipoprotein or chylomicron plasma fractions can be advantageous because the carotenoid content in these fractions reflects newly absorbed carotenoids and accounts for effects of intestinal β-carotene cleavage.

- There is a need for validated and good biomarkers to measure antioxidant activity in humans. Methodologies to measure biomarkers of impact of fruit and vegetable consumption should be improved. This includes the development and provision of certified reference materials for the analytical methods used.
- Studies should be directed at obtaining a better understanding of the mechanisms underlying the effect of individual fruit and vegetable components on antioxidant activity and stability in foods. The synergistic effects of potential antioxidants in foods and the effect of processing on the prooxidant or antioxidant activity of foods should be further explored.

## CONCLUSIONS

In order to better understand the potential role of carotenoids and folate in foods, their relative bioavailability is a critical factor. This thesis has evaluated a number of studies on factors affecting the bioavailability and bioconversion of carotenoids. It is not yet possible to accurately quantify these factors due to a lack of comparable studies, but factors affecting bioavailability and bioconversion have been identified. A carefully designed study was carried out to examine the effect of the food matrix on carotenoid and folate bioavailability. The consumption of spinach (*Spinacea oleracea* L.), a green, leafy vegetable containing carotenoids

(β-carotene and lutein) and folate, increased after three weeks the serum or plasma concentrations of β-carotene, lutein, α-carotene, retinol, and folate, and decreased the serum concentration of lycopene. The bioavailability of lutein was higher than that of β-carotene and the disruption of the food matrix (cell wall structure) enhanced the bioavailability of β-carotene from whole-leaf and minced spinach, but had no effect on lutein bioavailability. The processing method of spinach also had an effect on the bioavailability of folate. Processing methods, such as mincing or liquefaction may increase the plasma folate response. The effect of disruption of the food matrix on carotenoid and folate bioavailability could not be reversed by adding dietary fibre in an amount comparable to that lost in the cell wall structure.

Blood samples were collected to determine enzyme and non-enzyme antioxidant activities. Spinach consumption significantly increased responses of erythrocyte glutathione reductase activity and decreased responses of erythrocyte catalase activity and serum α-tocopherol concentrations. β-Carotene was unlikely to be a causative factor for changes in erythrocyte glutathione reductase activity or serum α-tocopherol concentrations in vivo. Our data suggest that these changes can be attributed to an increased intake of lutein (and zeaxanthin). This finding is further supported by in vitro measurements of antioxidant capacity of the spinach products. Frozen spinach products possessed prooxidant or antioxidant activity, depending on the extent of processing and the amount of spinach used. In a lipid methyl linoleate model, at a level of 10 ppm total phenolics the antioxidant activity was descending as follows: whole-leaf > liquefied > minced spinach. Differences among the spinach products tested in the meatball model were significant: the inhibition of the formation of thiobarbituric acid-reactive substances (TBARS) and hexanal was in the following descending order: liquefied > minced > whole-leaf spinach. Of importance is the finding that spinach can have prooxidative activity, which can become antioxidative by further processing of the spinach or by increasing the amount of spinach present.

We used the same dietary intervention trial to study a number of aspects of spinach consumption. The major advantage of this approach is that the results of measurements of various parameters can be related to each other. A complication is that it is difficult to design a study with multiple aims. For example, subjects could not be stratified according to their baseline concentrations of serum or plasma antioxidant vitamins or enzyme antioxidant activities. This complicated the statistical analysis.

Antioxidants and antioxidant enzyme defence systems are very complex and changes in one antioxidant may result in changes in serum/plasma

concentrations of other antioxidants or erythrocyte enzyme activities. It is concluded that a severe lack of knowledge on the bioavailability and antioxidant activity of carotenoids hampers adequate suggestions for human supplementation. We support the statement of Vainio et al. (1998) that, pending further research, supplemental β-carotene, α-carotene, lutein, and other carotenoids should not be recommended as an approach to prevent cancer in the general population.

## REFERENCES


Albanes D, Virtamo J, Taylor PR, Rautalahti M, Pietinen P, Heinonen OP. 1997. Effects of supplemental β-carotene, cigarette smoking, and alcohol consumption on serum carotenoids in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. Am J Clin Nutr 1997;66:366-72.

Appel LJ, Moore TJ, Obarzanek E, Vollmer WM, Svetkey LP, Sacks FM, Bray GA, Vogt TM, Cutler JA, Windhauser MM, Lin P-H, Karanja N for the DASH Collaborative Research Group. A clinical trial of the effects of dietary patterns on blood pressure. New Engl J Med 1997;336:1117-24.

Bailey LB, Barton LE, Hillier SE, Cerda JJ. Bioavailability of mono and polyglutamyl folate in human subjects. Nutr Rep Int 1988;38:509-18.

Ben-Amotz A, Levy Y. Bioavailability of a natural isomer mixture compared with synthetic all-*trans* β-carotene in human serum. Am J Clin Nutr 1996; 63:729-34.

Berg H van den, van Vliet T. Effect of simultaneous, single oral doses of β-carotene with lutein or lycopene on the β-carotene and retinyl ester responses in the triacylglycerol-rich lipoprotein fraction of men. Am J Clin Nutr 1998;68:82-9.

Berg H van den. Effect of lutein on beta-carotene absorption and cleavage. Internat J Vit Nutr Res 1998;68:360-5.

Berg H van den. Carotenoid interactions. Nutr Rev 1999;57:1-10.

Bowen HT, Omaye ST. Oxidative changes associated with β-carotene and α-tocopherol enrichment of human low-density lipoproteins. J Am Coll Nutr 1998;17:171-9.

Breemen RB van, Nikolic D, Xu X, Xiong Y, van Lieshout M, West CE, Schilling AB. Development of a method for quantitation of retinol and retinyl palmitate in human serum using high-performance liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry. J Chromatogr A 1998;794:245-51.

Brouwer IA, van Dusseldorp M, West CE, Meyboom S, Thomas CMG, Duran M, van het Hof KH, Eskes TKAB, Hautvast JGAJ, Steegers-Theunissen RPM. Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J Nutr 1999a;129:1135-9.

Brouwer IA, van Dusseldorp M, West CE, Steegers-Theunissen RPM. Bioavailability of folate and folic acid: Review of the literature. In: Brouwer IA. Folic acid, folate and homocysteine: Human intervention studies. PhD thesis. Wageningen, The Netherlands: Wageningen Agricultural University, 1999b.

Brown ED, Micozzi MS, Craft NE, Bieri JG, Beecher G, Edwards BK, Rose A, Taylor PR, Smith JC. Plasma carotenoids in normal men after a single ingestion of vegetables or purified β-carotene. Am J Clin Nutr 1989;49:1258-65.

Buettner GR. The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol,

and ascorbate. Arch Biochem Biophys 1993;300:535-43.

Cao G, Sofic E, Prior RL. Antioxidant capacity of tea and common vegetables. J Agric Food Chem 1996;44:3426-31.

Congdon PJ, Kelleher J, Edwards P, Littlewood JM. Benign carotenaemia in children. Arch Dis Child 1981;56:292-4.

Cuskelly GJ, McNulty H, Scott JM. Effect of increasing dietary folate on red-cell folate: implications for prevention of neural tube defects. Lancet 1996;347:657-9.

Diplock AT. Antioxidants and disease prevention. Molec Aspects Med 1994;15:293-376.

Dugas TR, Morel DW, Harrison EH. Impact of LDL carotenoid and alpha-tocopherol content on LDL oxidation by endothelial cells in culture. J Lipid Res 1998;39:999-1007.

Erdman JW, Poor CL, Dietz JM. Factors affecting the bioavailability of vitamin A, carotenoids and vitamin E. The bioavailability of fat-soluble vitamins is affected not only by food processing procedures but also by the individual's nutritional status. J Agric Food Chem 1988;42:214-21.

Fotouhi N, Meydani M, Santos MS, Meydani SN, Hennekens CH, Gaziano JM. Carotenoid and tocopherol concentrations in plasma, peripheral blood mononuclear cells, and red blood cells after long-term β-carotene supplementation in men. Am J Clin Nutr 1996;63:553-8.

Gärtner C, Stahl W, Sies H. Lycopene is more bioavailable from tomato paste than from fresh tomatoes. Am J Clin Nutr 1997;66:116-22.

Garcia-Casal MN, Layrisse M, Solano L, Baron MA, Arguello F, Llovera D, Ramirez J, Leets I, Tropper E. Vitamin A and beta-carotene can improve nonheme iron absorption from rice, wheat and corn by humans. J Nutr 1998;128:646-50.

Gaziano JM, Hatta A, Flynn M, Johnson EJ, Krinsky NI, Ridker PM, Hennekens CH, Frei B. Supplementation with beta-carotene in vivo and in vitro does not inhibit low density lipoprotein oxidation. Atherosclerosis 1995a;112:187-95.

Gaziano JM, Johnson EJ, Russell RM, Manson JE, Stampfer MJ, Ridker PM, Frei B, Hennekens CH, Krinsky NI. Discrimination in absorption or transport of β-carotene isomers after oral supplementation with either all-*trans*- or 9-*cis*-β-carotene. Am J Clin Nutr 1995b;61:1248-52.

Gey KF. Ten-year retrospective on the antioxidant hypothesis of arteriosclerosis: Threshold plasma levels of antioxidant micronutrients related to minimum cardiovascular risk. J Nutr Biochem 1995;6:206-36.

Goodman GE, Metch BJ, Omenn GS. The effect of long-term beta-carotene and vitamin A administration on serum concentrations of alpha-tocopherol. Cancer Epidemiol Biomark Prev 1994;3:429-32.

Granado F, Olmedilla B, Gil-Martinez E, Blanco I. Lutein ester in serum after lutein supplementation in human subjects. Br J Nutr 1998;80:445-9.

Gregory JF. The bioavailability of folate. In: Bailey LB, ed. Folate in health and disease. New York, NY: Marcel Dekker, Inc. 1995:195-235.

Haila K, Hopia A, Heinonen M. Effects of β-carotene and retinal on the formation and isomer distribution of methyl linoleate hydroperoxides. In: Haila K. Effects of carotenoids and carotenoid-tocopherol interaction on lipid oxidation *in vitro*. 1) Scavenging of free radicals. 2) Formation and decomposition of hydroperoxides. PhD thesis, EKT-series 1165. Helsinki, Finland: University of Helsinki, Department of Applied Chemistry and Microbiology, 1999.

Haila K, Lievonen S, Heinonen, M. Effects of lutein, lycopene, annatto, and γ-tocopherol on autoxidation of triglycerides. J Agric Food Chem 1996;44:2096-2100.

Halliwell B. Antioxidants in human health and disease. Annu Rev Nutr 1996;16:33-50.

Heseker H, Schmitt G. Effect of long-term supplementation of folate on folate status in plasma and erythrocytes. J Nutr Sci Vitaminol 1987;33:163-8.

Hininger I, Chopra M, Thurnham DI, Laporte F, Richard MJ, Favier A, Roussel AM. Effect of increased

fruit and vegetable intake on the susceptibility of lipoprotein to oxidation in smokers. Eur J Clin Nutr 1997;51:601-6.

Hof KH van het, Brouwer IA, West CE, Haddeman E, Steegers-Theunissen RPM, van Dusseldorp M, Weststrate JA, Eskens TKAB, Hautvast JGAJ. Bioavailability of lutein from vegetables is 5 times higher than that of β-carotene. Am J Clin Nutr 1999a;70:261-8.

Hof KH van het, Tijburg LBM, Pietrzik K, Weststrate JA. Influence of feeding different vegetables on plasma levels of carotenoids, folate and vitamin C. Effect of disruption of the vegetable matrix. Br J Nutr 1999b, in press.

Hopia AI, Huang SW, Schwarz K, German JB, Frankel EN. Effect of different lipid systems on antioxidant activity of rosemary constituents carnosol and carnosic acid with and without α-tocopherol. J Agric Food Chem 1996;44:2030-6.

Hussein L, El-Tohamy M. Vitamin A potency of carrot and spinach carotenes in human metabolic studies. Internat J Vit Nutr Res 1990;60:229-35.

Jensen CD, Howes TW, Spiller GA, Pattison TS, Whittam JH, Scala J. Observations on the effects of ingesting *cis* and *trans*-beta-carotene isomers on human serum concentrations. Nutr Rep Int 1987;35:413-22.

Keagy PM, Shane B, Oace SM. Folate bioavailability in humans: effect of wheat bran and beans. Am J Clin Nutr 1988;47:80-8.

Khachik F, Beecher GR, Smith JC. Lutein, lycopene, and their oxidative metabolites in chemoprevention of cancer. J Cellular Biochem 1995;22(suppl.):236-46.

Khachik F, Bernstein PS, Garland DL. Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. Invest Ophthalmol Vis Sci 1997;38:1802-11.

Kostic D, White WS, Olson JA. Intestinal absorption, serum clearance, and interactions between lutein and β-carotene when administered to human adults in separate or combined oral doses. Am J Clin Nutr 1995;62:604-10.

Krinsky NI, Deneke SM. Interaction of oxygen and oxy-radicals with carotenoids. J Natl Cancer Inst 1982;69:205-10.

Krinsky NI. Actions of carotenoids in biological systems. Annu Rev Nutr 1993;13:561-87.

Lieshout M van, West CE, Lugtenburg J, van Breemen RB, Muhilal. Quantification of bioavailability and bioconversion of β-carotene using [$^{13}C_{10}$]-β-carotene and [$^{13}C_{10}$]-retinyl palmitate. XIX IVACG Meeting Report. Washington, DCV: IVACG, 1999:87 (Abstr).

Lin YM, Burri BJ, Neidlinger TR, Muller HG, Dueker SR, Clifford AJ. Estimating the concentration of beta-carotene required for maximal protection of low-density lipoproteins in women. Am J Clin Nutr 1998;67:837-45.

Lomnitsky S, Bergman M, Schon I, Grossman S. The effects of dietary vitamin E and β-carotene on oxidation processes in rat testis. Biochim Biophys Acta 1991;1082:101-7.

Mallet JF, Cerrati C, Ucciani E, Gamisans J, Gruber, M. Antioxidant activity of plant leaves in relation to their alpha-tocopherol content. Food Chem 1994;49:61-5.

Martini MC, Campbell DR, Gross MD, Grandits GA, Potter JD, Slavin JL. Plasma carotenoids as biomarkers of vegetable intake: the University of Minnesota Cancer Prevention Research Unit Feeding Studies. Cancer Epidemiol Biomarkers Prev 1995;4:491-6.

Micozzi MS, Brown ED, Edwards BK, Bieri JG, Taylor PR, Khachik F, Beecher GR, Smith JC Jr. Plasma carotenoid response to chronic intake of selected foods and β-carotene supplements in men. Am J Clin Nutr 1992;55:1120-5.

Nielsen SE, Young JF, Daneshvar B, Lauridsen ST, Knuthsen P, Sandström B, Dragsted LO. Effect of parsley intake on urinary apigenin excretion, blood antioxidant enzymes and on biomarkers for oxidative stress in humans. In: Metabolism and biomarker studies of dietary flavonoids. PhD thesis. Copenhagen, Denmark: Institute of Analytical and Pharmaceutical Chemistry, The Royal

Danish School of Pharmacy, 1998.

Nierenberg DW, Dain BJ, Mott LA, Baron JA, Greenberg ER. Effects of 4 y of oral supplementation with β-carotene on serum concentrations of retinol, tocopherol, and five carotenoids. Am J Clin Nutr 1997;66:315-9.

Nierenberg DW, Stukel TA, Baron JA, Dain BJ, Greenberg ER, Skin Cancer Prevention Study. Determinants of increase in plasma concentration of beta-carotene after chronic oral supplementation. Am J Clin Nutr 1991;53:1443-9.

Nierenberg DW, Stukel TA, Mott LA, Greenberg ER. Steady-state serum concentration of alpha-tocopherol not altered by supplementation with oral β-carotene. J Nat Cancer Inst 1994;86:117-20.

Niki E, Noguchi N, Tsuchihashi H, Gotoh N. Interaction among vitamin C, vitamin E, and β-carotene. Am J Clin Nutr 1995;62(suppl.):1322S-6S.

Olmedilla B, Granado F, Gil-Martínez E, Blanco I. Supplementation with lutein (4 months) and α-tocopherol (2 months), in separate or combined oral doses, in control men. Cancer Lett 1997;114:179-81.

Palozza P. Prooxidant actions of carotenoids in biologic systems. Nutr Rev 1998;56:257-65.

Pappalardo G, Maiani G, Mobarhan S, Guadalaxara A, Azzini E, Raguzzini A, Salucci M, Serafini M, Trifero M, Illomei G, FerroLuzzi A. Plasma (carotenoids, retinol, α-tocopherol) and tissue (carotenoids) levels after supplementation with β-carotene in subjects with precancerous and cancerous lesions of the sigmoid colon. Eur J Clin Nutr 1997;51:661-6.

Pee S de, West CE, Muhilal, Karyadi D, Hautvast JGAJ. Lack of improvement in vitamin A status with increased consumption of dark-green leafy vegetables. Lancet 1995;346:75-81.

Pee S de, West CE, Permaesih D, Martuti S, Muhilal, Hautvast JGAJ. Orange fruit is more effective than are dark-green, leafy vegetables in increasing serum concentrations of retinol and β-carotene in schoolchildren in Indonesia. Am J Clin Nutr 1998;68:1058-67.

Porrini M, Riso P, Testolin G. Absorption of lycopene from single or daily portions of raw and processed tomato. Br J Nutr 1998;80:353-61.

Reaven PD, Ferguson E, Navab M, Powell FL. Susceptibility of human LDL to oxidative modification. Effects of variations in beta-carotene concentration and oxygen tension. Arterioscler Thromb 1994;14:1162-9.

Reaven PD, Khouw A, Beltz WF, Parthasarathy S, Witztum JL. Effect of dietary antioxidant combinations in humans. Protection of LDL by vitamin E but not by beta-carotene. Arterioscler Thromb 1993;13:590-600.

Rock CL, Swendseid ME. Plasma β-carotene response in humans after meals supplemented with dietary pectin. Am J Clin Nutr 1992;55:96-9.

Romanchik JE, Harrison EH, Morel DW. Addition of lutein, lycopene, or β-carotene to LDL or serum in vitro: Effects on carotenoid distribution, LDL composition, and LDL oxidation. Nutr Biochem 1997;8:681-8.

Russell RM. Physiological and clinical significance of carotenoids. Internat J Vit Nutr Res 1998;68:349-53.

Russell RM, Ismail-Beigi F, Reinhold JG. Folate content of Iranian breads and the effect of their fiber content on the intestinal absorption of folic acid. Am J Clin Nutr 1976;29:799-802.

Sauberlich HE, Kretch MJ, Skala JH, Johnson HL, Taylor PC. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr 1987;46:1016-28.

Stahl W, Sies H. Uptake of lycopene and its geometrical isomers is greater from heat-processed than from unprocessed tomato juice in humans. J Nutr 1992;122:2161-6.

Stahl W, Nicolai S, Briviba K, Hanusch M, Broszeit G, Peters M, Martin H-D, Sies H. Biological activities of natural and synthetic carotenoids: induction of gap junctional communication and singlet oxygen quenching. Carcinogenesis 1997;18:89-92.

Tamai H, Morinobu T, Murata T, Manago M, Mino M. 9-*cis* β-carotene in human plasma and blood cells after ingestion of β-carotene. Lipids 1995;30:493-8.

Tamura T, Stokstad EL. The availability of food folate in man. Br J Haematol 1973;25:513-32.

Törrönen R, Lehmusaho M, Häkkinen S, Hänninen O, Mykkänen H. Serum β-carotene response to supplementation with raw carrots, carrot juice or purified β-carotene in healthy non-smoking women. Nutr Res 1996;16:565-75.

Tribble DL. Further evidence of the cardiovascular benefits of diets enriched in carotenoids (editorial). Am J Clin Nutr 1998;68:521-2.

Vainio H, Rautalahti M. An international evaluation of the cancer preventive potential of carotenoids. Cancer Epidemiol Biomarkers Prev 1998;7:725-8.

Vliet T van, Schreurs WHP, van den Berg H. Intestinal β-carotene absorption and cleavage in man: response of β-carotene and retinyl esters in the triglyceride-rich lipoprotein fraction after a single oral dose of β-carotene. Am J Clin Nutr 1995;62:110-6.

Wahlqvist ML, Wattanapenpaiboon N, Macrae FA, Lambert JR, MacLennan R, Hsu-Hage BHH (Aus Polyp Prev Proj Invest). Changes in serum carotenoids in subjects with colorectal adenomas after 24 mo of β-carotene supplementation. Am J Clin Nutr 1994;60:936-43.

Wang H, Cao G, Prior RL. Total antioxidant capacity of fruits. J Agric Food Chem 1996;44:701-5.

West CE, Castenmiller JJM. Quantification of the "SLAMENGHI" factors for carotenoid bioavailability and bioconversion. Internat J Vit Nutr Res 1998;68:371-7.

White WS, Stacewicz-Sapuntzakis M, Erdman JW, Bowen PE. Pharmokinetics of β-carotene and canthaxanthin after ingestion of individual and combined doses by human subjects. J Am Coll Nutr 1994;13:665-71.

Wise JA, Morin RJ, Sanderson R, Blum K. Changes in plasma carotenoid, alpha-tocopherol, and lipid peroxide levels in response to supplementation with concentrated fruit and vegetable extracts: A pilot study. Current Therapeutic Res 1996;57:445-61.

Xu MJ, Plezia PM, Alberts DS, Emerson SS, Peng YM. Reduction in plasma or skin alpha-tocopherol concentration with long-term oral administration of beta-carotene in humans and mice. J Natl Cancer Inst 1992;84:1559-65.

Young JF, Nielsen SE, Haraldsdóttir J, Daneshvar B, Lauridsen ST, Knuthsen P, Crozier A, Sandström B, Dragsted LO. Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. Am J Clin Nutr 1999;69:87-94.

Zeben W van, Hendriks TF. The absorption of carotene from cooked carrots. Zschr Vitamin Forsch 1948;19:265-6.

# SUMMARY

Fruits and vegetables are generally considered important contributors to a healthy diet and, for some years now, health professionals have been advising the general public to increase their intake of fruits and vegetables to reduce the risk of specific cancers, cardiovascular disease, neural tube defects, and cataracts. Although the mechanisms are not fully understood, carotenoids, folic acid and dietary fibre appear to play important roles in the prevention of these diseases. The hypothesis that β-carotene plays a role in cancer prevention is based on studies suggesting that carotenoids function as antioxidants, as enhancers of immune function, as precursors of retinoids and/or by stimulation of cell-cell communication. Epidemiological studies reveal associations but cause and effect have not been demonstrated. In order to determine whether β-carotene is the active phytochemical in the prevention of cancer and cardiovascular disease, four major intervention trials in humans have been completed, all of which included β-carotene supplementation with or without other nutrients. Two studies, provided evidence suggesting that β-carotene increased the risk of lung cancer in high-risk groups: one study was in male Finnish smokers and the other was in smokers or workers who had been occupationally exposed to asbestos. Another study showed no effect on the incidence of cancer in a healthy population supplemented for 12 years with β-carotene. A further study, which involved combined supplementation of β-carotene with vitamin E and selenium to marginally malnourished people, showed lower mortality from gastric and total cancers in the supplemented group. With respect to cardiovascular disease, the clinical trials, in which β-carotene was given without other antioxidants, no evidence was found that supplementation had a protective effect. Thus, with respect to the chemopreventive effect of fruits and vegetables, these intervention trials indicated that β-carotene alone is not the active phytochemical in the prevention of cancer and cardiovascular disease. Another candidate contributing to the positive effects of fruit and vegetable intake on degenerative diseases is folate. Folates are abundant in many of the same fruits and vegetables that are rich in carotenoids. A low intake of folate is associated with increased plasma homocysteine levels, cardiovascular disease, colon cancer and neural tube defects. No intervention studies have yet been conducted to examine the effect of fruit and vegetable intake on endpoints of degenerative diseases.

In this thesis two aspects of spinach, a dark-green, leafy vegetable are examined. The first aspect is the bioavailability of the carotenoids and folate

present in spinach. The second aspect is the antioxidant activity of spinach consumption in humans and the antioxidant capacity of spinach products. Bioavailability is defined as the fraction of an ingested nutrient that is available for utilisation in normal physiological functions or for storage. Accurate information on the bioavailability of nutrients and nonnutrients is needed in order to devise food-based strategies for long-term alleviation of deficiency and/or to devise food or supplement-based interventions to reduce risk for chronic diseases, which appear linked to these food constituents. The term bioconversion is used to refer to the conversion to the active form of a nutrient. The overall process from ingestion to the formation of an active form of a nutrient is termed bioefficacy although this is often referred to as bioconversion.

We refer to the factors that affect nutrient and nonnutrient bioavailability, and bioconversion, as SLAMENGHI factors derived from the capitalised letters in the following description: Species of the constituent; molecular Linkage; Amount of constituent consumed in a meal; Matrix in which the constituent is incorporated; Effectors of absorption and bioconversion; Nutrient status of the host; Genetic factors; Host related factors; and Interactions. Current knowledge of each of the SLAMENGHI factors for the bioavailability and bioconversion of carotenoids is discussed in Chapter 2. From the literature review, we concluded that each carotenoid appears to have an individual pattern of absorption, plasma/serum transport, and metabolism. We further concluded that although data are at present not sufficiently comparable to allow a systematic comparison of results, a number of conclusions can be drawn. The bioavailability and bioconversion of various carotenoids and geometrical isomers of carotenoids differ; molecular linkage does not play a role; a positive relationship exists between intake and response of β-carotene; β-carotene supplementation increases serum α-carotene concentrations and decreases serum concentrations of lutein, lycopene and canthaxanthin; the bioavailability of β-carotene from foods is low, particularly from dark-green leafy vegetables; intake of dietary fat has a positive and intake of dietary fibre a negative effect on bioavailability; alcohol interferes with the bioconversion of β-carotene. Nutrient status and genetic factors related to the host may explain some of the differences observed.

We conducted a dietary intervention study with 70 healthy human subjects, 42 women and 28 men, to examine the effect of the food matrix on the bioavailability of carotenoids and folate and to evaluate the effect of spinach intake on biomarkers of antioxidant activity. The study started with a three-week run-in period, in which subjects chose their own diets but were instructed to avoid foods rich in carotenoids, vitamin A and folate. The run-in period was followed by a

three-week dietary intervention period. During the intervention period, subjects were supplied with total diets, except for a limited choice of free products, and divided over six treatment groups. Four groups received a basic diet plus a spinach product (whole-leaf, minced, enzymatically liquefied, and liquefied spinach plus added dietary fibre), one group received the basic diet plus a carotenoid supplement of β-carotene, lutein and a small amount of zeaxanthin dissolved in oil, and one group received the basic diet only. Consumption of spinach (20 g/MJ), containing β-carotene and lutein, increased serum concentrations of all-*trans*-β-carotene, *cis*-β-carotene (and consequently total β-carotene), lutein, α-carotene and retinol and decreased the serum concentration of lycopene compared with the control group. Compared with the synthetic carotenoid supplement, the relative bioavailability of β-carotene was low, 5.1-9.5%, but much higher for lutein, 45-55% (Chapter 3). The low bioavailability of β-carotene from spinach products can partly be explained by the presence of 15-18% *cis*-β-carotene, which is less readily absorbed than all-*trans*-β-carotene. Furthermore, the relatively high lutein:β-carotene ratio in spinach may have negatively affected the bioavailability of β-carotene. Combined intake of lutein and β-carotene has been shown to have an inhibitory effect on serum β-carotene concentrations. Another factor that contributed to the low bioavailability of β-carotene is the food matrix, because β-carotene dissolved in oil is absorbed far more readily than that in plant foods. Our study (Chapter 3) showed that disruption of the food matrix (cell wall structure) and loss of cellular structure had an effect on the bioavailability of β-carotene, whereas the bioavailability of lutein was not affected. Serum β-carotene responses differed significantly between the whole-leaf and liquefied spinach groups and between the minced and liquefied spinach groups. Addition of dietary fibre to the liquefied spinach to compensate for the fibre that was broken down during liquefaction had no effect on serum carotenoid responses. The plasma folate response was significantly greater in the spinach groups compared with the control group. Intake of minced and liquefied spinach resulted in greater plasma folate responses than consumption of whole-leaf spinach, which suggests that the food matrix of spinach plays a role with regard to bioavailability of folate (Chapter 4). Also in the folate study the amount of dietary fibre added to the liquefied spinach was too small to have a significant effect on the plasma folate response compared with that of the liquefied spinach group. The folate response in the carotenoid supplement group was similar to that in the control group, suggesting that there is no interaction between the absorption of carotenoids and folate.

Foods and the human body are under constant oxidative stress from free radicals, reactive oxygen and nitrogen species and other prooxidants. Cells have intact prooxidant/antioxidant systems that function continuously to generate and to detoxify oxidants during normal aerobic metabolism. The first line of defence is largely enzymatic and prevents the generation of radical species derived from molecular oxygen. The enzymes superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase are free radical control biomolecules that are present in most tissues. Superoxide dismutase prevents the accumulation of superoxide anion ($O_2^{\bullet -}$) by converting it to hydrogen peroxide ($H_2O_2$). Hydrogen peroxide is metabolised by catalase or glutathione peroxidase to water. Although these endogenous antioxidant enzymes are very effective, they do not provide (sufficient) defence against some reactive oxygen species such as singlet oxygen or hydroxyl radicals. Food-derived derived antioxidants, such as vitamins C and E, uric acid, glutathione, carotenoids and flavonoids, form the second line of defence. There is no doubt that antioxidants are of great importance in the aetiology of a number of different human diseases. However, it is critical to provide evidence that a diet containing a high antioxidant activity from fruits and vegetables can either increase the overall antioxidant capacity or change the relative balance between individual antioxidant components in the human body; otherwise, an antioxidant hypothesis related to the protection of fruits and vegetables against diseases cannot be sustained.

Thus, we examined the effect of consumption of spinach after three weeks on several enzymatic antioxidant activities in erythrocytes as well as antioxidants concentrations in serum/plasma and concentrations of oxidatively damaged amino acids in plasma (Chapter 5). Consumption of spinach resulted in significantly greater erythrocyte glutathione reductase activity and lower erythrocyte catalase activity and serum α-tocopherol concentration compared with the control group. Consumption of the carotenoid supplement led to lower α-tocopherol responses and tended to increase the erythrocyte glutathione reductase activity. Serum lutein concentration was positively related with erythrocyte glutathione reductase activity and negatively with serum α-tocopherol concentration. Moreover, the antioxidant responses in the pooled spinach group were not significantly different from those in the carotenoid supplement group, suggesting that the observed changes in antioxidant activities or concentrations were not caused by the increased absorption of β-carotene, because the β-carotene response, but not that of lutein, was much higher in the carotenoid supplement group than in the spinach groups (Chapter 3). The antioxidant capacity of the differently processed spinach products was determined (Chapter

6). The antioxidant activity was tested using two oxidation systems: a methyl linoleate system and a cooked meat model. In the methyl linoleate system the inhibition of formation of hydroperoxides by spinach extracts was measured after three days of oxidation. The inhibition of thiobarbituric acid-reactive substances (TBARS) and hexanal concentrations by spinach samples was determined in cooked meat after two days of storage at 4°C. On the basis of inhibition of hydroperoxide formation, the activity of oxidation, corrected for the total phenolics content of the spinach, decreased at 10 ppm in the descending order of minced (72% inhibition) > liquefied (75% inhibition) > whole-leaf spinach (82% inhibition). Whole-leaf and minced spinach were more prooxidative and showed greater increased TBARS formation than the liquefied spinach. Minced and liquefied spinach were antioxidatively active with respect to the mean inhibition of hexanal formation, whereas whole-leaf spinach showed prooxidant activity. Differences among the three spinach products (in descending order: liquefied > minced > whole-leaf spinach) were significant. A correlation was observed between the calculated amount of lutein, but not for $\beta$-carotene, in the original spinach samples (amounts were adjusted for the total phenolics content of spinach) and the inhibition of hydroperoxide formation. The results of the studies described in this thesis (Chapters 5 and 6) support the finding that $\beta$-carotene is not a good antioxidant in vivo or in vitro, but indicate that lutein may play a role as antioxidant.

Among the explanations for the disparity between observational epidemiological and dietary intervention studies is the possibility that $\beta$-carotene may be a surrogate marker for other protective nutrients and nonnutrients in fruits and vegetables. In many cases, it is not possible to decipher the influence of these other dietary factors because they are not currently included in databases. The finding that $\beta$-carotene as a single nutrient is not responsible for the health effects of fruits and vegetables supports the hypothesis that the synergistic effect of a number of nutrients and perhaps nonnutrients promotes health. Furthermore, the relation between carotenoid intake and disease risk may not be linear: beneficial effects of $\beta$-carotene might only be seen at physiological and not at pharmacological levels.

Controlled dietary trials should be conducted to study the effect of fruit and vegetable intake on endpoints for degenerative diseases. More research is needed to establish and validate good markers or biomarkers to measure antioxidant activity in human blood and in foods. It should be taken into account that the most effective antioxidant in oxidative stress depends on the specific molecules causing the stress and the cellular or extracellular location of the

source of these molecules.

In conclusion, the bioavailability of β-carotene from differently processed spinach products is low and can be improved by mincing or liquefaction of whole-leaf spinach. The food matrix also has an effect on the bioavailability of folate from spinach. Optimal health and protection against oxidative damage and related diseases is best served by the variety of bioactive substances found in fruits and vegetables, which act together, instead of a single component. Consumption of spinach or a carotenoid supplement changed erythrocyte enzyme activities and non-enzymatic serum/plasma antioxidant concentrations, but β-carotene is unlikely to be a causative factor. Lutein may play a role in the antioxidant defence of human blood.

# SAMENVATTING

**Spinazie als bron van carotenoïden, foliumzuur en antioxidantactiviteit**
Groenten en fruit leveren een belangrijke bijdrage aan een gezonde voeding. Nationale campagnes worden gericht op de gehele Nederlandse bevolking om de inneming van groenten en fruit te verhogen. Een hoge inneming van groenten en fruit wordt geassocieerd met een verlaagde kans op kanker, hart- en vaatziekten en andere negatieve gezondheidseffecten. Carotenoïden, foliumzuur en voedingsvezel lijken een belangrijke rol te spelen in het voorkomen van deze ziekten, alhoewel de hierbij behorende reactiemechanismen niet volledig bekend zijn. De hypothese dat β-caroteen, een van de carotenoïden die in het lichaam kunnen worden omgezet in vitamine A, een rol speelt bij de preventie van kanker, is gebaseerd op onderzoeken waarin de werking en functie van carotenoïden zijn beschreven en op epidemiologisch onderzoek. Carotenoïden werken als antioxidanten, versterken de immuunfunctie, kunnen worden omgezet in retinol (vitamine A), en stimuleren de communicatie tussen cellen. Epidemiologische onderzoeken laten verbanden zien maar geen oorzaak en gevolg. Om te kunnen vaststellen of β-caroteen inderdaad een actieve verbinding is in de preventie van kanker en hart- en vaatziekten, zijn een viertal interventie-onderzoeken uitgevoerd met vrijwilligers. Tijdens deze onderzoeken kregen de deelnemers synthetisch β-caroteen zonder of in combinatie met andere voedingsstoffen. Twee onderzoeken toonden aan dat het toedienen van β-caroteen de kans op longkanker verhoogde bij mensen met een hoog risico, namelijk Finse rokers en werknemers, die blootgesteld waren geweest aan asbest. Een derde onderzoek liet geen effect zien op de incidentie van kanker bij een gezonde populatie, die werd gesuppleerd met β-caroteen gedurende 12 jaren. Een vierde onderzoek omvatte het toedienen van β-caroteen in combinatie met vitamine E en selenium aan marginaal gevoede mensen in China. Dit leidde tot een verlaagde sterfte aan maagkanker en kanker in het algemeen. Met betrekking tot hart- en vaatziekten, werd in de klinische onderzoeken waarin alleen β-caroteen of β-caroteen in combinatie met andere antioxidanten werd gegeven, geen bewijs gevonden voor een beschermend effect van β-caroteen. Uit deze onderzoeken kan worden geconcludeerd dat β-caroteen in groenten en fruit niet de actieve component is, die een beschermend effect heeft op het ontstaan van kanker en hart- en vaatziekten. Een mogelijke andere kandidaat voor een positieve bijdrage van groenten en fruit aan ziekten, is foliumzuur. Foliumzuur is aanwezig in veelal dezelfde groenten- en fruitsoorten die ook veel carotenoïden bevatten. Een lage

foliumzuurinneming wordt geassocieerd met het optreden van verhoogde homocysteïneconcentraties in plasma, hart- en vaatziekten, darmkanker en neurale buis-defecten. Tot heden zijn er nog geen interventie-onderzoeken uitgevoerd om het effect van de inneming van groenten en fruit op harde eindpunten van chronische ziekten te bestuderen.

In dit proefschrift zijn twee aspecten van spinazie, een donkergroene bladgroente, onderzocht. Het eerste aspect is de biobeschikbaarheid van carotenoïden en foliumzuur in spinazie. Het tweede aspect is tweeledig, namelijk het effect van de consumptie van spinazie op de antioxidantactiviteit in het bloed en de antioxidantcapaciteit van spinazieproducten.

Biobeschikbaarheid wordt gedefinieerd als het gedeelte van een ingenomen voedingsstof, dat beschikbaar is voor normale fysiologische functies of voor opslag in het lichaam. Accurate informatie over de biobeschikbaarheid van voedingsstoffen en andere stoffen in voedingsmiddelen is nodig om voedselgerichte strategieën te kunnen ontwerpen ter voorkoming van voedingstekorten en/of om strategieën te ontwerpen gebaseerd op voedsel- of suppletie-interventies om het risico van chronische ziekten, gerelateerd aan deze voedingsstoffen, te verminderen. De term bioconversie wordt gebruikt om de omzetting naar een actieve vorm van een voedingsstof aan te geven. Het gehele proces van inneming tot de formatie van een actieve vorm van een voedingsstof wordt 'bioefficacy' (biodoeltreffendheid) genoemd, maar wordt vaak verward met bioconversie.

De factoren, die van invloed kunnen zijn op de biobeschikbaarheid en bioconversie van stoffen, zijn de zogenaamde SLAMENGHI factoren, waarbij elke letter staat voor een bepaalde factor. De factoren zijn de volgende: soort stof (Species); moleculaire verbinding (Linkage); hoeveelheid van een stof aanwezig in een maaltijd (Amount); Matrix waarin de stof zich bevindt; verbindingen die de absorptie of bioconversie bewerkstelligen of bevorderen (Effectors); Nutriëntenstatus van de gastheer; Genetische factoren; individuele (Host-related) factoren; en Interacties. De hedendaagse kennis over elk van de SLAMENGHI factoren voor de biobeschikbaarheid en bioconversie van carotenoïden wordt besproken in hoofdstuk 2. Uit het literatuuronderzoek kon worden geconcludeerd dat elk van de carotenoïden een individueel patroon van absorptie, plasma of serum transport, en metabolisme lijkt te volgen. Alhoewel de gegevens niet voldoende vergelijkbaar waren om tot een systematisch vergelijk van resultaten te komen, konden toch een aantal conclusies worden getrokken uit de resultaten van gepubliceerde onderzoeken. De biobeschikbaarheid en bioconversie van de verschillende carotenoïden en geometrische isomeren van carotenoïden zijn

verschillend; moleculaire verbindingen spelen geen rol; er bestaat een positieve relatie tussen de inneming en de β-caroteenconcentratie in serum of plasma; β-caroteensuppletie verhoogt α-caroteenconcentraties in serum en verlaagt serumconcentraties van luteïne, lycopeen and canthaxantine; de biobeschikbaarheid van β-caroteen uit voedingsmiddelen, met name uit donkergroene bladgroenten, is laag; vetinname heeft een positief effect terwijl inname van voedingsvezel een negatief effect heeft op de biobeschikbaarheid; alcohol beïnvloedt de bioconversie van β-caroteen. De voedingsstatus en genetische factoren van een individu kunnen een gedeelte van de waargenomen verschillen in biobeschikbaarheid verklaren.

Om de invloed van de voedselmatrix op de biobeschikbaarheid van carotenoïden en foliumzuur te onderzoeken en om het effect van consumptie van spinazie op biomerkers van antioxidantactiviteit te evalueren, werd een voedingsinterventie-onderzoek uitgevoerd. Aan dit onderzoek namen 70 gezonde vrijwilligers deel (42 vrouwen en 28 mannen). Het onderzoek begon met een drie weken durende inloopperiode, waarin alle deelnemers zelf hun voeding samenstelden, maar instructies ontvingen om voedingsmiddelen rijk aan carotenoïden, vitamine A en foliumzuur te vermijden. De inloopperiode werd gevolgd door een voedingsinterventieperiode van drie weken, waarin de deelnemers werden verdeeld over zes onderzoeksgroepen. Gedurende de interventieperiode werden alle deelnemers voorzien van een volledige dagvoeding met een beperkte keuze aan vrije producten. Vier onderzoeksgroepen kregen naast de basisvoeding een van de volgende spinazieproducten: heel-blad, gehakte, enzymatisch vervloeide, of enzymatisch vervloeide spinazie waaraan voedingsvezel was toegevoegd. De vijfde groep kreeg naast de basisvoeding een supplement van β-caroteen, luteïne en een kleine hoeveelheid zeaxantine, opgelost in olie. De laatste groep (controlegroep) kreeg alleen de basisvoeding. De spinazie bevatte de carotenoïden β-caroteen en luteïne. Spinazieconsumptie (20 g/MJ) verhoogde de serumconcentraties van all-*trans*-β-caroteen, *cis*-β-caroteen (en dus van totaal β-caroteen), luteïne, α-caroteen en retinol en verlaagde de serumconcentratie van lycopeen vergeleken met de controlegroep. Vergeleken met het synthetische carotenoïdensupplement, was de relatieve biobeschikbaarheid van β-caroteen laag (5,1-9,5%), maar was veel hoger voor luteïne (45-55%) (hoofdstuk 3). De lage biobeschikbaarheid van β-caroteen uit spinazieproducten kan gedeeltelijk worden verklaard door de aanwezigheid van 15-18% *cis*-β-caroteen in de spinazieproducten, dat minder makkelijk wordt geabsorbeerd dan het all-*trans*-β-caroteen waaruit het supplement bestond. Voorts kan de relatief hoge verhouding tussen luteïne en β-

caroteen in spinazie de biobeschikbaarheid van β-caroteen negatief hebben beïnvloed. Een gecombineerde inneming van luteïne en β-caroteen verlaagt de serum β-caroteenconcentraties. Een andere factor, die bijgedragen heeft aan de lage biobeschikbaarheid van β-caroteen is de voedselmatrix. β-Caroteen opgelost in olie wordt veel beter geabsorbeerd dan β-caroteen uit plantaardige voedingsmiddelen. Het onderzoek beschreven in hoofdstuk 3 laat zien dat het verbreken van de voedselmatrix (de celwandstructuur) en het verlies van de celstructuur een invloed hadden op de biobeschikbaarheid van β-caroteen, terwijl de biobeschikbaarheid van luteïne hierdoor nauwelijks werd beïnvloed. De verschillen in β-caroteenconcentraties in serum tussen het begin en eind van de proefperiode waren statistisch significant voor het verschil tussen de heel-blad en vervloeide spinaziegroepen en tussen de gehakte en vervloeide spinaziegroepen. Het toevoegen van voedingsvezel aan de vervloeide spinazie om de voedingsvezel, die verloren is gegaan tijdens het vervloeien, te compenseren, had geen invloed op de serumcarotenoïdenconcentraties. Na drie weken spinazie te hebben gegeten, was de foliumzuurconcentratie in plasma statistisch significant hoger in de spinaziegroepen dan in de controlegroep. De inneming van gehakte en vervloeide spinazie resulteerde in een groter verschil in foliumzuurconcentraties in plasma dan de inneming van heel-blad spinazie. Dit suggereert dat de voedselmatrix ook een rol speelt bij de biobeschikbaarheid van foliumzuur (hoofdstuk 4). Ook in het foliumzuuronderzoek bleek de hoeveelheid voedingsvezel, die was toegevoegd aan de vervloeide spinazie, een te klein verschil in foliumzuurconcentraties te veroorzaken om een statistisch significant verschil aan te tonen tussen de groepen die vervloeide spinazie en vervloeide spinazie met toegevoegd voedingsvezel ontvingen. De foliumzuurconcentraties in plasma van de controle- en supplement-groepen reageerden hetzelfde, hetgeen suggereert dat er geen interactie is tussen de absorptie van carotenoïden en foliumzuur.

Het menselijk lichaam en voedingsmiddelen ondervinden een continue oxidatieve druk van vrije radicalen, reactieve zuurstof- en stikstofverbindingen en andere pro-oxidanten. Cellen bezitten intacte pro-oxidant/antioxidant-systemen, die tijdens het normale aërobe metabolisme continu functioneren om oxidanten te genereren of te ontgiften. Een eerste afweermechanisme is voornamelijk enzymatisch en voorkomt de vorming van radicalen afgeleid van moleculair zuurstof. De enzymen superoxidedismutase, catalase, glutathionreductase en glutathionperoxidase zijn aanwezig in bijna alle weefsels en deze biomoleculen reageren met vrije radicalen. Superoxidedismutase voorkomt de accumulatie van het superoxide-anion ($O_2^{\bullet-}$) door het om te zetten in waterstofperoxide ($H_2O_2$).

Waterstofperoxide wordt gemetaboliseerd door catalase of glutathionperoxidase tot water ($H_2O$). Alhoewel deze endogene antioxidantenzymen effectief zijn, voorzien ze niet in een (voldoende) afweer tegen sommige reactieve zuurstofsoorten, zoals $^1O_2$ of hydroxylradicalen. Antioxidanten afkomstig uit voedingsmiddelen, zoals bijvoorbeeld de vitamines C en E, urinezuur, glutathion, carotenoïden en flavonoïden vormen een tweede afweermechanisme. Er bestaat geen twijfel over dat antioxidanten een belangrijke rol spelen in het ontstaan van een aantal ziekten. Echter, het is van essentieel belang om het bewijs te leveren dat een voeding rijk aan groenten en fruit, en dus met een veronderstelde hoge antioxidantactiviteit, inderdaad de algehele antioxidantactiviteit kan verhogen of een verandering teweeg kan in de relatieve balans tussen individuele antioxidantcomponenten in het lichaam. Als deze relatie niet gestaafd kan worden met bewijs, kan de antioxidanthypothese met betrekking tot het beschermende effect van groenten en fruit niet worden gehandhaafd.

De invloed van inneming van spinazie gedurende drie weken op verschillende enzymatische antioxidantactiviteiten in erytrocyten (rode bloedcellen), antioxidantconcentraties in serum/plasma en concentraties van oxidatief beschadigde aminozuren in plasma is onderzocht (hoofdstuk 5). De consumptie van spinazie resulteerde in een statistisch significant grotere glutathionreductase-activiteit en lagere catalase-activiteit in de erytrocyten. Ook werden lagere α-tocoferolconcentraties in serum gemeten vergeleken met de controlegroep. De inneming van het carotenoïdensupplement leidde tot lagere α-tocoferolconcentraties en vertoonde een net niet statistisch significante toename in de glutathionreductase-activiteit in de erytrocyten. De luteïneconcentraties in serum waren positief gecorreleerd met de erytrocyt-glutathionreductase-activiteit en negatief met de α-tocoferolconcentratie in serum. Bovendien waren de gemeten antioxidantactiviteiten en -concentraties in de samengevoegde spinaziegroepen niet statistisch significant verschillend van die in de carotenoïdensupplementgroep. Dit geeft aan dat de waargenomen veranderingen niet werden veroorzaakt door een verhoogde absorptie van β-caroteen, omdat de absorptie van β-caroteen in tegenstelling tot de absorptie van luteïne, veel hoger was in de carotenoïdensupplementgroep dan in de spinaziegroepen (hoofdstuk 3). Vervolgens is de antioxidantactiviteit van de verschillend bewerkte spinazieproducten bepaald (hoofdstuk 6). De antioxidantactiviteit werd getest in twee oxidatiesystemen: in een methyl-linoleaatsysteem en in een model van gekookte gehaktballen. In het methyl-linoleaatsysteem werd de remming van de formatie van hydroperoxiden door spinazie-extracten gemeten na drie dagen van oxidatie. De remming van de vorming van thiobarbituurzuur-reactieve substanties

(TBARS) en hexanal door de spinaziemonsters werd bepaald in gekookt gehakt na opslag van twee dagen bij 4°C. Op basis van de remmende werking op de formatie van hydroperoxiden, nam de oxidatie-activiteit, gecorrigeerd voor de totale hoeveelheid fenolische verbindingen in de spinazieproducten, bij 10 ppm het sterkst af voor gehakte spinazie (72% remming), gevolgd door vervloeide spinazie (75% remming) en heel-blad spinazie (82% remming). In het gekookte vlees-model, waren de heel-blad en gehakte spinazieproducten pro-oxidatiever (de vorming van TBARS werd minder geremd) dan de vervloeide spinazie. Wat betreft de gemiddelde remming van de hexanalvorming, bleken de gehakte en vervloeide spinazieproducten een antioxidatieve activiteit te hebben, terwijl de heel-blad spinazie een pro-oxidatieve activiteit vertoonde. De verschillen tussen de drie spinazieproducten (afnemend naar antioxidantactiviteit: vervloeid > gehakt > heel-blad spinazie) waren statistisch significant. De berekende hoeveelheid luteïne in de originele spinaziemonsters, gecorrigeerd voor de totale hoeveelheid fenolische verbindingen, was gecorreleerd met de remming van de hydroperoxidevorming. De resultaten van de onderzoeken die beschreven zijn in dit proefschrift (hoofdstukken 5 en 6) steunen de bevinding dat β-caroteen geen goede antioxidant is in de mens of in voedingsmiddelen, maar geven aan dat luteïne een rol zou kunnen spelen als antioxidant.

Een van de verklaringen voor de uiteenlopende bevindingen van observationeel epidemiologisch onderzoek en voedingsinterventie-onderzoek is de mogelijkheid dat β-caroteen het effect van andere voedingsstoffen en andere stoffen in groenten en fruit maskeert. In vele gevallen is het niet mogelijk om de invloed van een groot aantal (voedings)stoffen die voorkomen in groenten en fruit te bestuderen, omdat informatie over hun hoeveelheden (nog) niet zijn opgenomen in voedingsmiddelenbestanden. De bevinding dat β-caroteen als enkelvoudige voedingsstof niet alleen verantwoordelijk is voor de gezondheidseffecten, die worden toegeschreven aan een hoge inneming van groenten en fruit, ondersteunt de hypothese dat een synergistisch effect van een aantal (voedings)stoffen gezondheidsbevorderend werkt. Ook kan het zo zijn dat de relatie tussen de inneming van carotenoïden en het ziekterisico wellicht niet lineair is: het zou kunnen zijn dat een heilzaam effect van β-caroteen alleen optreedt bij fysiologische en niet bij farmacologische hoeveelheden.

Gecontroleerde voedingsproeven dienen uitgevoerd te worden om de invloed van inneming van groenten en fruit op harde eindpunten voor chronische ziekten te bestuderen. Meer onderzoek is nodig naar het vaststellen en valideren van goede (bio)merkers om de oxidantactiviteit te bepalen in bloed en in voedingsmiddelen. Hierbij dient rekening gehouden te worden met het feit dat de

meest effectieve antioxidant in een bepaalde situatie afhankelijk is van de specifieke moleculen, die de balans van de oxidanten verstoren, en de cellulaire of extracellulaire herkomst van deze moleculen.

Concluderend kan gesteld worden dat de biobeschikbaarheid van β-caroteen uit verschillend verwerkte spinazieproducten laag is maar kan worden verbeterd door het hakken of vervloeien van heel-blad spinazie. De voedselmatrix heeft ook een invloed op de biobeschikbaarheid van foliumzuur uit spinazie. Een optimale gezondheid en bescherming tegen oxidatieve schade en gerelateerde ziekten kan worden bereikt door het eten van groenten en fruit met een verscheidenheid aan bioactieve stoffen in plaats van een enkel bestanddeel.

De inneming van spinazie of een carotenoïdensupplement leidde tot veranderingen in de gemeten enzymactiviteiten in de erytrocyten en in antioxidantconcentraties in serum en plasma. Echter, het is onwaarschijnlijk dat deze veranderingen werden veroorzaakt door β-caroteen; wel zou het kunnen zijn dat luteïne een rol speelt in de antioxidatieve afweer van het bloed.

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# ABOUT THE AUTHOR

Jacqueline J.M. Castenmiller was born on 17 September 1959 in Geleen, The Netherlands. In 1977, she passed the gymnasium-B examination at the Scholengemeenschap Sint Michiel in Geleen. In the same year she started her study of Human Nutrition at Wageningen Agricultural University. She obtained her MSc degree in 1984 specialising in human nutrition and epidemiology. Immediately after obtaining her degree she moved to Somalia and worked as a nutritionist on short-term contracts for WFP/WHO and OXFAM in Somalia. In 1986, she accepted a two-year contract with FAO and started work in the Caribbean as a Marketing and Nutrition Development Officer. This project was mainly concerned with reducing post-harvest losses of fruits and vegetables.

Upon returning to The Netherlands, she was employed by the FLAIR Eurofoods-Enfant Foundation in Wageningen as a project officer responsible for all daily activities of an EU-Concerted Action concerned with improvement of the quality and compatibility of food consumption and food composition data in Europe. When the Project ended in January 1994, she remained under contract with the Foundation and wrote several research proposals on micronutrients and a follow-up to the FLAIR-project (COST99), conducted a pilot-study to examine the iron status of Dutch girls, organised seminars on micronutrients, organised and participated in a 'Postgraduate course on production and use of food composition data in nutrition' in 1994, and worked on the 'Constructing a nutritional database for use in Europe' project. In 1995, work was carried out for the Netherlands Food and Nutrition Council on preparing advice and organising meetings to discuss and evaluate recommended allowances, addition of amino acids to foods, food consumption monitoring, and natural anticarcinogens and carcinogens in the diet.

From January 1996, she participated in the EU-sponsored project 'Natural antioxidants in foods' (project leader: Prof. B. Sandström, Denmark). The research activities conducted for this project are described in this thesis. The project received funding for three years and during 1999 writing up the results from the project was combined with work as a secretary for the working group on Vitamins of the Netherlands Health Council (the former Food and Nutrition Council), which has prepared draft recommendations for the daily intake of B vitamins. Since August 1999, she is employed with the Centre for Micronutrient Research TNO-WU. She is married to Kees Schuur and has two children, Freek (1989) and Michiel (1992).