Dietary factors that affect carotenoid bioavailability

Karin H. van het Hof

Promotoren: Dr. J.G.A.J. Hautvast
Hoogleraar in de leer van de voeding en gezondheid,
Landbouwuniversiteit Wageningen

Dr. C.E. West
Universitair hoofddocent aan de afdeling Humane Voeding en Epidemiologie, Departement Levensmiddelentechnologie en Voedingswetenschappen, Landbouwuniversiteit Wageningen
Visiting Professor of International Nutrition, Rollins School of Public Health, Emory University, Atlanta GA, USA

Co-promotor: Dr. Ir. J.A. Weststrate
Unilever Research Vlaardingen

Stellingen

behorend bij het proefschrift 'Dietary factors that affect carotenoid bioavailability' van Karin H. van het Hof. Wageningen, 4 juni 1999.

1. "Natuurlijk" wordt vaak ten onrechte verward met gezond; wat betreft de biobeschikbaarheid van carotenoïden zijn juist de "onnatuurlijke" en technologisch bewerkte bronnen beter *(dit proefschrift)*.

2. Optimalisatie van de biobeschikbaarheid van carotenoïden is geen geldig excuus voor een vetrijke maaltijd *(dit proefschrift)*.

3. Homogenisatie verbetert de biobeschikbaarheid van carotenoïden uit groenten. De Hollandse stamppot is daarom zo slecht nog niet *(dit proefschrift)*.

4. Een beperking in de aanwezigheid van gevalideerde biomarkers en gevoelige analysemethoden is een limiterende factor voor de vooruitgang in de voedingswetenschap.

5. Het gebruik van functional foods noopt tot een verdergaande individualisering van het eetgedrag.

6. Zolang een meerderheid van de vrouwen valt op mannen die ouder en minstens even intelligent zijn als zijzelf, vindt emancipatie aan de top van organisaties geen doorgang.

7. Voor sommige mensen veroorzaakt de stiptheid van de Nederlandse Spoorwegen meer stress dan zijn vertragingen.

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Katharina Henriëtte van het Hof

Proefschrift

ter verkrijging van de graad van doctor
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Abstract

Dietary factors that affect carotenoid bioavailability

PhD thesis by Karin H. van het Hof, Department of Human Nutrition and Epidemiology, Wageningen Agricultural University, the Netherlands, June 4 1999.

Carotenoids are thought to contribute to the beneficial effects of increased vegetable consumption. To better understand the potential benefits of carotenoids, we investigated the bioavailability of carotenoids from vegetables and dietary factors which might influence carotenoid bioavailability.

In a four weeks intervention trial, we found that the increases in plasma concentrations of β-carotene and lutein after consumption of a high vegetable diet were 14% and 67%, respectively, of those after consumption of the same amount of carotenoids, supplied in their purified form. In another study, it appeared that the bioavailability of β-carotene was particularly low from spinach. Broccoli and green peas were more effective in enhancing plasma concentrations of β-carotene after four days consumption (relative bioavailability ca. 3%, 74% and 96% for spinach, broccoli and green peas, respectively). Disruption of the vegetable matrix by mechanical homogenisation significantly improved the bioavailability of lutein from spinach by 14% and of lycopene from tomatoes by 20 to 60%. One hour additional heating of the tomatoes (100°C) also enhanced the bioavailability of lycopene but this effect lacked significance.

Carotenoids are absorbed in association with dietary fat and therefore the presence of dietary fat is thought crucial for carotenoid absorption. Four weeks consumption of a full-fat margarine (80% fat), supplemented with α-carotene and β-carotene, effectively enhanced blood concentrations of these carotenoids. In a further study, we found that in healthy adult volunteers, only a small amount of fat (i.e. 3-5 g per meal) was sufficient to ensure uptake of α-carotene and β-carotene. For lutein supplied as lutein esters, however, the amount of fat required for optimal uptake was greater. Daily consumption of an unabsorbable fat replacer, sucrose polyester, with the main meal for four weeks, significantly reduced the bioavailability of carotenoids. Plasma concentrations of β-carotene and lycopene were reduced by 20% and 38% if 3 g/d sucrose polyester was consumed.

Interaction among carotenoids appeared to interfere with carotenoid bioavailability in some but not all cases. Simultaneous ingestion of α-carotene and β-carotene did not affect the bioavailability of β-carotene whereas four weeks supplementation with β-carotene and lutein significantly reduced the plasma concentration of lycopene by 39%.

In conclusion, the type of food matrix in which carotenoids are located largely determines their bioavailability. Processing, such as mechanical homogenisation or heat treatment, has the potential to enhance the bioavailability of carotenoids from vegetables. The amount of dietary fat needed to ensure carotenoid absorption seems low, although it depends on the physico-chemical characteristics of the carotenoids ingested. Unabsorbable, fat-soluble compounds reduce carotenoid absorption and interaction among carotenoids may also result in a reduced carotenoid bioavailability.

Research into the functional benefits of carotenoids should consider the fact that the bioavailability of β-carotene in particular is one order of magnitude higher when provided as pure compound added to foods than when naturally present in foods.

Contents

1

General introduction

INTRODUCTION

The importance of fruit and vegetable consumption has been linked in the past 50-100 years to the prevention of micronutrient deficiencies. More recently, epidemiological studies have indicated that a high intake of fruits and vegetables is also associated with a reduced risk of chronic diseases, such as some types of cancer and cardiovascular disease (Steinmetz & Potter, 1991; Block et al, 1992; Ness & Powles, 1997; Willet & Trichopoulos, 1997; Law & Morris, 1998). These beneficial effects of fruits and vegetables cannot fully be explained by their established role as a source of micronutrients. It has been suggested that other plant compounds, such as carotenoids, which are abundantly present in fruits and vegetables, contribute to the beneficial effects of fruit and vegetable consumption (Peto et al, 1981; Gey, 1995; Kohlmeier & Hastings, 1995; Van Poppel, 1996).

Carotenoids belong to a large class of fat-soluble, yellowish-red pigments which are synthesised by bacteria, algae, fungi and plants, but not by animals, including humans. More than 600 carotenoids have been identified in nature. In contrast, only seven carotenoids are found in appreciable amounts in humans. These include α-carotene, β-carotene, lutein, zeaxanthin, lycopene, α-cryptoxanthin and β-cryptoxanthin (Figure 1). These carotenoids are found in a wide range of foods, among which fruits and vegetables are the richest dietary sources (Mangels et al, 1993; Heinonen et al, 1989; Ollilainen et al, 1989). Carrots are particularly rich in α-carotene and β-carotene, green vegetables are highest in lutein, maize in zeaxanthin and tomatoes and tomato products are the major dietary sources of lycopene. Cryptoxanthin is mainly provided by oranges and tangerines (Chug-Ahuja et al, 1993; Mangels et al, 1993; Scott et al, 1996; Heinonen et al, 1989). Average dietary intake of carotenoids in the US and European countries is about 6-8 mg/d of total carotenoids and 0.5-3 mg/d of individual carotenoids, among which the intake of β-carotene, lycopene and lutein is highest (Chug-Ahuja et al, 1993; Järvinen et al, 1995; Nebeling et al, 1996; Scott et al, 1996; Goldbohm et al, 1998).

The function of carotenoids in plants is related to light energy collection and photoprotection in photosynthetic membranes of the leaves (Cogdell & Gardiner, 1993). In humans, some of the carotenoids, such as α-carotene, β-carotene and β-cryptoxanthin, can be converted into vitamin A. Other functions of carotenoids may be related to their antioxidant properties (Burton and Ingold, 1984; Sies and Stahl, 1995), ability to interfere with inter-cellular communication (Zhang et al, 1991) and/or immunomodulation (Santos et al, 1996).

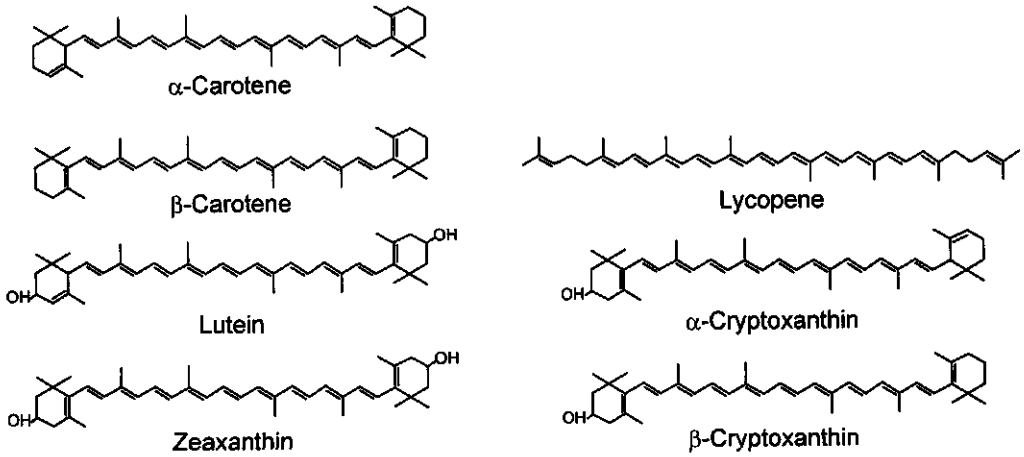

Figure 1 Structures of major carotenoids found in human plasma.

Currently, the role of carotenoids in the prevention of chronic diseases is still a hypothesis and is not yet confirmed by intervention trials on the effects of carotenoid supplementation at dietary intake levels (IARC, 1998; Kritchevsky, 1999). To increase our understanding of the possible beneficial effects of carotenoids, it is important to obtain more knowledge of the bioavailability of these compounds. In addition, it may be of interest to identify options to enhance the contents or bioavailability of carotenoids present in fruits and vegetables, in order to optimise their health potential. In particular increasing the bioavailability seems relevant for carotenoids. De Pee et al (1995) showed that the bioavailability of β-carotene from green leafy vegetables was lower than previously thought (Hume & Krebs, 1949). They therefore questioned the effectiveness of programmes stimulating home gardening of green leafy vegetables as a way to reduce the prevalence of vitamin A deficiency in developing countries. Consumption of foods enriched or fortified with carotenoids may be an easier approach than increasing total fruit and vegetable consumption to improve the health status of the population. To ensure the effectiveness, the choice of the food carrier is very important and requires knowledge of the factors that may interfere with the bioavailability and bioefficacy of carotenoids.

The term "bioavailability" is used above as a descriptor of the processes that occur after consumption of a nutrient, which include absorption at the intestinal level, and subsequent distribution and metabolism or storage of the nutrient in the body. A working definition of bioavailability that is often used originates from pharmacology, i.e.: "the proportion of a nutrient ingested that becomes available for usage or storage in a target tissue". This introductory chapter describes the current knowledge of the processes

underlying carotenoid bioavailability, i.e. carotenoid absorption, distribution, metabolism and excretion. At the end of this introduction, the outline of this thesis and the research questions that will be addressed, are presented.

CAROTENOID ABSORPTION

Absorption process

Carotenoids are fat-soluble compounds which are absorbed along with dietary fat. Therefore, their absorption process resembles that of fat absorption. Figure 2 shows a schematic overview of carotenoid absorption. The first step after ingestion includes disruption of the food matrix, mechanically and by digestive enzymes, and the subsequent release of the carotenoids from this matrix and from protein complexes (Britton, 1995). After solubilisation in lipid droplets, the carotenoids released are incorporated into mixed micelles which are formed from triglycerides, phospholipids and cholesteryl esters and their hydrolysis products together with conjugated and unconjugated bile salts. Subsequently, carotenoids are taken up, most likely by passive diffusion, into the enterocytes of the intestinal wall. Within the enterocytes, a proportion of the provitamin A carotenoids is enzymatically cleaved and converted to retinol and eventually to retinyl esters. These cleavage products and intact carotenoids are then incorporated into chylomicrons and transported via the lymph into the blood. Chylomicrons contain triacylglycerol, phopholipids, cholesteryl esters, carotenoids, retinyl esters, and other fat-soluble compounds. Triacylglycerols in chylomicrons are lipolysed, mediated by lipoprotein lipase in extrahepatic tissues, resulting in the uptake of lipolysis products by these tissues and formation of chylomicron remnants which still contain the carotenoids absorbed. The majority of these chylomicron remnants are cleared from the plasma by the liver, and a small part is directly taken up by other tissues (Erdman et al, 1993; Olson, 1994; Parker, 1996; Furr & Clark, 1997).

Quantification of carotenoid absorption

Only few data are available on the extent of β-carotene absorption in humans and no data are available on carotenoids other than β-carotene. Using β-carotene (0.04 - 40 mg) labelled with radioactive or stable isotopes and dissolved in oil, it was shown that 13-23% is absorbed after ingestion of a single dose (Goodman et al, 1966; Blomstrand & Werner, 1967; Novotny et al, 1995). There are certain limitations to these studies, as only a small number of volunteers (n=1-4) was included, some of whom were patients, and because it was necessary to make assumptions with respect to kinetics of absorption and distribution. In comparison with the extent to which fat is absorbed, i.e. 95-99% (Small,

1991), the findings suggest that the uptake of β-carotene is rather low. The reason for this difference is not clear and it may be related to the poor solubility of β-carotene (Borel et al, 1996).

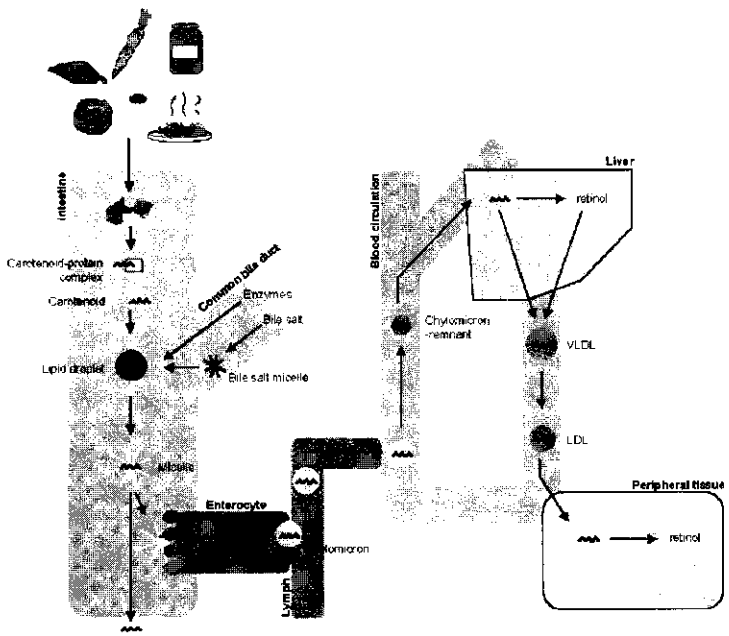

Figure 2 Schematic overview of carotenoid absorption and distribution in humans

The extent to which carotenoids from natural foods are absorbed may be even lower as the matrix in which the carotenoids naturally occur has been shown to be a limiting factor. β-Carotene located in a complex matrix, such as vegetables, is less bioavailable than β-carotene dissolved in oil or added to a fat-rich matrix (Micozzi et al, 1992; De Pee et al, 1995). In addition, disruption of the vegetable matrix improves the bioavailability of carotenoids, as the uptake of lycopene from tomato paste was superior to that of lycopene from fresh tomatoes (Gärtner et al, 1997). Furthermore, the physical form of carotenoids may be an important factor. Crystalline β-carotene has been shown to be less available than dissolved β-carotene (Bierer et al, 1995; Zhou et al, 1996). In carrots, β-carotene is present as crystals and this may also explain the low bioavailability of β-carotene from carrots as compared to β-carotene dissolved in oil (Micozzi et al, 1992; Zhou et al, 1996).

There are some indications that the extent of absorption may also vary among different carotenoids. Serum responses to a single ingestion of lutein or canthaxanthin were larger than those found after ingestion of a similar amount of β-carotene (2.3-fold larger area under the serum concentration curve for lutein; 72% higher maximum increase in serum concentration of canthaxanthin) (Kostic et al, 1995; Paetau et al, 1997). The observed differences may however be affected by differences in serum clearance and rates of tissue uptake between the carotenoids investigated and thus not reflect true differences in absorption. On the other hand, Paetau et al (1997) found the same difference in carotenoid responses in the triglyceride-rich lipoprotein fraction of serum.

In addition to the variation in extent of carotenoid absorption, caused by the type of carotenoid and/or dietary factors interfering with carotenoid absorption, a substantial interindividual variation may be present also. De Pee & West (1996) have summarised factors related to genetics and other characteristics of the "host", such as gastrointestinal infections and parasites, which may influence carotenoid uptake from the diet. In healthy, well-nourished populations, a large variability of in particular responses of plasma or serum β-carotene concentrations to supplementation has been reported (Brown et al, 1989; Nierenberg et al, 1991). Some volunteers have even been identified as so-called non-responders; people who show no or a very small increase in β-carotene concentration in chylomicrons or plasma following β-carotene supplementation (Johnson & Russell, 1992; Stahl et al, 1995). The mechanism underlying this variation may not only be related to differences in carotenoid absorption but also to differences in lipoprotein metabolism among people (Borel et al, 1998).

In summary, although the pathway of carotenoid absorption is largely known, the extent to which carotenoids are absorbed is less clear and may depend on their nature as well as on external factors in the diet. In addition, the extent of carotenoid absorption varies among individuals.

CAROTENOID DISTRIBUTION

After absorption and incorporation in chylomicrons within the enterocytes and subsequent uptake in the blood stream via the lymph, carotenoids are released from chylomicron remnants into the liver and other tissues. In the liver, provitamin A carotenoids can be converted to retinoids as the cleavage enzyme is not only present in enterocytes but also in liver cells. Alternatively, carotenoids as such can be stored in the liver or incorporated in very low density lipoprotein (VLDL) and be resecreted into the blood. VLDL is subsequently transformed into low density lipoprotein (LDL) and carotenoids located in LDL are transported to tissues. This is analogous to the role of LDL

in delivering cholesterol to peripheral tissues (Figure 2). High density lipoproteins (HDL) on the other hand, transport cholesterol from peripheral tissues to the liver and carotenoids located in HDL are thus transported along with this reverse cholesterol transport. Johnson & Russell (1992) confirmed these processes as they showed that after consumption of a β-carotene supplemented meal (120 mg), the β-carotene concentration initially increased in chylomicrons, with maximum concentrations 3 h post ingestion, followed by an increase in VLDL carotenoid concentration, reaching peak concentrations after 9 h. After an initial reduction, a significant increase in concentrations of β-carotene in LDL and HDL was observed from 12 h to 3 days following β-carotene ingestion.

During fasting, LDL and HDL are the major carriers of carotenoids in plasma as chylomicrons and VLDL are formed only after consumption of dietary fat and cleared rapidly within 12 hours. Generally, 60-70% of the carotenoids is found in LDL and 10-30% in HDL when subjects have been fasting for at least 12 hours (Johnson & Russell, 1992; Manago et al, 1992; Romanchik et al, 1995; Traber et al, 1994). This distribution is however dependent on the type of carotenoid as lutein, which is less lipophilic than β-carotene and lycopene, was shown to be equally distributed over LDL and HDL in fasting subjects (44% and 38% respectively) (Romanchik et al, 1995). Due to this direct association of carotenoids to lipoproteins, plasma or serum concentrations of carotenoids are positively related to total cholesterol concentrations (Brady et al, 1996; Vogel et al, 1997).

Carotenoids are found in various organs. Examples of tissues which specifically accumulate lutein (and zeaxanthin) are the corpus luteum and the yellow spot in the retina (*macula lutea*). The mechanism and importance of this accumulation is not clear, although lutein and zeaxanthin may accumulate in the macula to filter blue light and to prevent photo-oxidative stress and thus reduce the risk of age-related macular degeneration (Eye Disease Case-Control Study Group, 1993; Seddon et al, 1994). The concentrations of carotenoids reported to be present in various tissues are shown in Table 1. Adipose tissue, liver and the adrenals seem to be the major sites of deposition, although tissue distribution varies among the different types of carotenoids.

As indicated above, LDLs transport carotenoids from the liver to peripheral tissues, concurrent with cholesterol transport. Therefore, the number of LDL receptors on tissue cells has been suggested to be related to the tissue content of carotenoids because tissue uptake of carotenoids may be mediated by these receptors (Parker, 1989). This is in line with the relatively high carotenoid concentrations in the liver and adrenals. However, the mechanisms underlying the accumulation of specific carotenoids in certain tissues has not yet been revealed.

TABLE 1 Tissue concentrations of carotenoids, in humans.

			Mean carotenoid concentration (nmol/g tissue wet weight)					
Tissue	Reference	Subjects, country	α-Carotene	β-Carotene	Lutein	Lycopene	β-Cryptoxanthin	Total carotenoids
Liver	Kaplan et al, 1990	n=13 m + f, USA	0.21	1.82	(zeaxanthin: 0.53)	2.44	0.06	4.91
	Schmitz et al, 1991	n=19 m + f, USA	1.7	4.2	2.7	5.4	1.9	16.2
	Stahl et al, 1992	n=9 m + f, Germany	0.51	3.02		1.28	0.32	5.1
	Tanumihardjo et al, 1990	n=5, sex unspecified, France	4.2	15.1	2.9	25.5		47.7
Kidney	Kaplan et al, 1990	n=12 m + f, USA	0.06	0.31	0.16	0.39	0.03	0.96
	Schmitz et al, 1991	n=13 m + f, USA	0.3	0.6	1.2	0.6	0.4	3.1
	Stahl et al, 1992	n=9 m + f, Germany	0.07	0.55		0.15	0.09	0.9
Adrenal	Kaplan et al, 1990	n=11 m + f, USA	1.15	9.40	1.60	21.57	0.29	33.3
	Stahl et al, 1992	n=9 m + f, Germany	1.22	5.60		1.90	0.66	9.4
Spleen	Kaplan et al, 1990	n=11 m + f, USA	0.09	0.44	0.17	0.19	0.05	0.95
Colon	Nierenberg & Nann, 1992	n=16 m + f, USA	0.03	0.17	0.15	0.31	0.09	
Lung	Nierenberg & Nann, 1992	n=16 m + f, USA, benign and malignant tissue	0.004	0.11	0.17	0.22	0.04	
	Schmitz et al, 1991	n=13 m + f, USA	0.2	0.4	0.5	0.6	0.4	2.1
Adipose tissue	Kaplan et al, 1990	n=12 m + f, USA	0.09	0.38	1.39	1.30	0.05	3.27
	Parker et al, 1988	n=19 m + f, USA		0.62		0.58		
	Stahl et al, 1992	n=5 m + f, Germany	0.13	0.38		0.20	0.08	0.8

TABLE 1 - *continued*

Tissue	Reference	Subjects, country	α-Carotene	β-Carotene	Lutein	Lycopene	β-Cryptoxanthin	Total carotenoids
Skin	Nierenberg & Nann, 1992	n=14 m + f, USA, benign and malignant tissue	0.03	0.27	0.05	0.43	0.09	
	Ribaya-Mercano et al, 1995	n=16 f, USA		1.4		1.6		
Cataractous lens nuclei	Bates et al, 1996	n=9 m + f, UK	<0.05	<0.05	0.028	<0.05	<0.05	
Testes	Kaplan et al, 1990	n=5 m, USA	0.48	4.37	0.37	21.32	0.12	26.0
	Stahl et al, 1992	n=4 m, Germany	0.37	2.68		4.34	0.16	7.6
Prostate	Clinton et al, 1996	n=25 m, USA	0.35	0.86	0.26; (zeaxanthin: 0.19)	0.63	0.14 (α-cryptoxanthin: 0.22)	
Ovary	Kaplan et al, 1990	n=3 f, USA	0.31	0.99	0.75	0.28	0.14	2.55
	Stahl et al, 1992	n=3 f, Germany	0.08	0.45		0.25	0.08	0.9
Breast	Nierenberg & Nann, 1992	n=16 f, USA, benign and malignant tissue	0.10	0.71	0.43	0.78	0.24	

CAROTENOID METABOLISM

The metabolism of β-carotene and other provitamin A carotenoids (i.e., α-carotene and β-cryptoxanthin) to retinoids has been studied most extensively. Estimates of the conversion of labelled β-carotene to retinoids at the intestinal level in vitamin A sufficient humans vary widely among different studies and among subjects, ranging from 10% to 98% (Goodman et al, 1966; Blomstrand & Werner, 1967; Parker et al, 1993; Novotny et al, 1995). As indicated before, these studies have some limitations with respect to the number of patients included, the use of hospitalised patients and calculations based on assumptions related to kinetics of absorption and tissue distribution of β-carotene and retinoids. However, more recent studies, in which the postprandial response of β-carotene and retinyl esters in triglyceride-rich lipoproteins was monitored in healthy subjects, also reported that the proportion of β-carotene converted varies substantially among individuals. The proportion of β-carotene converted was estimated to range between 28% and 80% (Van Vliet et al, 1995; O'Neill & Thurnham, 1998).

Very few data are available on the conversion of (non-)provitamin A carotenoids to compounds other than thôse related to retinoids. Metabolites of lutein, zeaxanthin and lycopene have been found in plasma (Khachik et al, 1992, Khachik et al, 1995). It was speculated that these metabolites resulted from oxidation of the parent carotenoids.

In summary, the conversion of provitamin A carotenoids to retinoids is well documented whereas, in contrast, little is known about the formation of other metabolites and the metabolism of non-provitamin A carotenoids.

CAROTENOID EXCRETION

Excretion of carotenoids or their metabolites may occur via different pathways although the nature of excretion of absorbed carotenoids has not yet been revealed and data are scarce. Certainly a considerable proportion of the ingested carotenoids is not absorbed and excreted in the faeces, either as intact carotenoids or after metabolism by microflora in the colon. As discussed above, the proportion excreted is expected to generally exceed 77%. Absorbed carotenoids are metabolised and they are thought to, either after conversion to vitamin A or as intact carotenoids, be oxidised and/or conjugated in the liver, secreted into the bile and excreted via the faeces. Another pathway is utilisation in tissues and ultimately excretion in the urine or as carbon dioxide in respired air (Olson, 1994).

OUTLINE OF THE THESIS

The objective of this thesis is to obtain insight in the bioavailability of carotenoids from different food matrices and to identify dietary factors that may interfere with carotenoid bioavailability. The mnemonic "SLAMENGHI" has been introduced by De Pee and West (1996) to describe the factors that may interfere with the bioavailability of carotenoids and their provitamin A value. The factors include: Species of carotenoids, Linkages at molecular level, Amount of carotenoid, Matrix, Effectors, Nutrient status, Genetics, Host-related factors and Interactions among these factors (De Pee & West, 1996; Castenmiller & West, 1998). In this thesis, we primarily determine the effect on carotenoid bioavailability of the type and intactness of the food matrix (Matrix), of the digestibility and amount of dietary fat (Effectors) and of the interaction among carotenoids (Effectors).

Disruption of the food matrix is the first step in the process of carotenoid absorption, as shown in Figure 2. Therefore, incomplete disruption of the food matrix and thus the type or intactness of the matrix in which carotenoids are located upon ingestion, may be a limiting factor of carotenoid bioavailability. As discussed above, the presence of dietary fat is thought crucial for carotenoid absorption, as carotenoids need to be incorporated in mixed micelles and subsequently absorbed along with dietary fat. The amount and type of fat present may thus be another factor affecting the bioavailability of carotenoids. The step(s) of carotenoid absorption, during which interaction among carotenoids may take place and affect their bioavailability, may include solubilisation in lipid droplets and uptake in micelles or in the enterocytes. In addition, carotenoids may compete for incorporation in chylomicrons, after being absorbed. Such competition between carotenoids can be regarded as the influence of the Amount of carotenoids. However, as also a positive effect may occur if carotenoids spare each other, e.g. by protection against oxidation, we here evaluate the possible interaction among carotenoids as Effectors of carotenoid bioavailability.

We choose to study the effect of the factors mentioned above in healthy human volunteers. Although various *in vitro* and animal models have been proposed as valuable models to study carotenoid bioavailability (Poor et al, 1987; Wilson, 1990; Scita et al, 1992; Wang et al, 1992; Bierer et al, 1995; Moore et al, 1996; Snodderly et al, 1997; Clark et al, 1998; Lee et al, 1998) and most of our knowledge about the mechanism of carotenoid absorption actually originates from *in vitro* studies (El-Gorab et al, 1975; Hollander et al, 1978), the uncertainty about the value of extrapolation of the results obtained to the human *in vivo* situation remains. However, studies in humans involve practical problems which have to be considered also. One important limitation is the

access to body tissues. We used changes in plasma carotenoid concentrations after four days to four weeks intervention as a measure of carotenoid bioavailability. In the following chapters of this thesis, bioavailability is thus determined as the availability of carotenoids ingested to the blood stream. It may be assumed that an increase in blood carotenoid concentrations relates to an increase in availability of the carotenoid to tissues. Another limitation is the control of free living individuals. Approximately three to four weeks are needed to induce a new steady state of plasma carotenoid concentrations (Micozzi et al, 1992). Such a long period requires a major effort of the volunteers to comply with the instructions of the study as well as of the investigators to organise such a longer term intervention. In the present thesis, we tried to find alternatives for long term studies. In addition to four weeks interventions (Chapters 2, 5, 7), we used four to seven days protocols during which volunteers received instructions which limited the variation in their background diet, thus increasing the contrasts among groups or treatments (Chapters 3, 4, 6, 8). We determined the value of these short term protocols by comparing the changes in fasting plasma carotenoid concentrations observed after four days intervention with the postprandial response of carotenoids in triglyceride-rich lipoproteins following single consumption of the test products (Chapter 4). This fraction of blood contains newly absorbed carotenoids and comparison of the carotenoid content of this fraction following different sources of carotenoids is thus an indicator of differences in carotenoid absorption.

The following research questions will be addressed:

Does the bioavailability of carotenoids vary among different food matrices and does disruption of the matrix enhance the bioavailability of carotenoids from vegetables?

Previous studies have indicated that the bioavailability of β-carotene from carrots and green leafy vegetables is low as compared to purified β-carotene ingested as supplement or added to a wafer (Brown et al, 1989; Micozzi et al, 1992; De Pee et al, 1995). Furthermore, yellow-orange fruits are more effective sources of β-carotene than green leafy vegetables (De Pee et al, 1998). Chapter 2 describes a study in which we determined the relative bioavailability of β-carotene and lutein from a mixed vegetable diet. In another study, described in Chapter 3, we compared the effectiveness of broccoli, green peas and spinach in increasing β-carotene and lutein concentrations in plasma.

The first step of carotenoid absorption includes disruption of the food matrix in which they are located (Figure 2). It has been suggested that the intactness of the vegetable matrix is an important limiting factor in the bioavailability of carotenoids. For example,

Gärtner et al (1997) found that consumption of tomato paste induced a larger lycopene response in triglyceride-rich lipoproteins than fresh tomatoes. The effects of mechanical homogenisation and/or heat treatment of spinach and tomatoes on the bioavailability of β-carotene, lutein and/or lycopene were addressed in two studies, described in Chapters 3 and 4.

Does the amount and digestibility of dietary fat affect the bioavailability of carotenoids?

Previous studies have shown that the bioavailability of carotenoids ingested without any dietary fat or any foods is significantly reduced (Dimitrov et al, 1988; Henderson et al, 1989; Prince & Frisoli, 1993; Shiau et al, 1994). However, the minimum amount of dietary fat required is not clear and has been investigated in vitamin A deficient children only (Jayarajan et al, 1980). Therefore, we determined the impact of a full-fat margarine, enriched with palm oil carotenoids on plasma carotenoid levels (Chapter 5) and we compared the bioavailability of carotenoids added to a low fat meal (3 g fat) with those added to a high fat meal (35 g fat) in healthy adults (Chapter 6).

Sucrose polyester is an effective fat replacer which mimics the organoleptic and satiating properties of fat, but does not provide the energy (Hulshof et al, 1995; De Graaf et al, 1996; Peters et al, 1997). It is a large molecule which is indigestible and is thus excreted unmodified. Carotenoids are absorbed along with dietary fat and sucrose polyester may interfere with their uptake into mixed micelles from absorbable dietary fat and thereby reduce their bioavailability. This has been investigated in the studies described in Chapter 7.

Do individual carotenoids affect the bioavailability of other carotenoids?

Various studies have investigated whether individual carotenoids affect the absorption of other carotenoids. There are indications, for example, that lutein reduces the appearance of β-carotene in triglyceride-rich lipoproteins (Van den Berg & Van Vliet, 1998). Such an interaction may also occur between other carotenoids. We compared the bioavailability of β-carotene from a palm oil carotenoids supplement, containing both α-carotene and β-carotene, with that of synthetic β-carotene (Chapter 8). In addition, we assessed the effect of supplementation with purified β-carotene and lutein on plasma concentrations of other carotenoids (Chapter 2).

The results described in this thesis are discussed in a review of the current knowledge on dietary factors that affect carotenoid bioavailability (Chapter 9). The answers to the research questions mentioned above and the overall conclusion of this thesis are also

presented in this chapter. Although other compounds and research topics have been investigated in the research described in Chapters 2-8, the discussion and conclusions of this thesis focusses on the dietary factors that affect carotenoid bioavailability.

Seven of the nine chapters were written by the author of this thesis and two chapters (Chapters 6 and 7) were co-authored by her because she provided an essential contribution to the design, execution and reporting of the studies. As the research described closely relates to the subject of this thesis, it was decided to also include these chapters.

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2

Bioavailability of lutein from vegetables is five times higher than that of β-carotene

Karin H. van het Hof, Ingeborg A. Brouwer, Clive E. West, Edward Haddeman, Régine P.M. Steegers-Theunissen, Marijke van Dusseldorp, Jan A. Weststrate, Tom K.A.B. Eskes, Joseph G.A.J. Hautvast

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ABSTRACT

Background: Carotenoids may contribute to the inverse association between vegetable consumption and the risk of chronic diseases. To obtain more insight into this relationship, it is important to determine the bioavailability of carotenoids from vegetables and the impact of vegetable consumption on suggested biomarkers of chronic diseases.

Objectives: To assess the bioavailability of β-carotene and lutein from vegetables and the effect of increased vegetable consumption on the *ex vivo* oxidizability of low-density lipoproteins (LDL).

Design: During four weeks, 22 healthy adult volunteers consumed a high vegetable diet (490 g/d), 22 volunteers consumed a low vegetable diet (130 g/d) and 10 volunteers consumed a low vegetable diet supplemented with pure β-carotene (6 mg/d) and lutein (9 mg/d).

Results: Plasma levels of vitamin C and carotenoids (i.e., α-carotene, β-carotene, lutein, zeaxanthin and β-cryptoxanthin) were significantly increased following the high vegetable diet as compared to the low vegetable diet. In addition to an increase in plasma levels of β-carotene and lutein, the pure carotenoid supplemented diet induced a significant decrease in plasma concentration of lycopene (mean (95%CI): -0.11 μmol/L (-0.21, -0.0061)). The plasma β-carotene and lutein responses to the high vegetable diet were 14% and 67%, respectively, of those to the pure carotenoid supplemented diet. Conversion of β-carotene into retinol may have attenuated its plasma response as compared to lutein. There was no significant effect on the resistance of LDL to oxidation *ex vivo*.

Conclusion: Increased vegetable consumption enhances plasma levels of vitamin C and carotenoids substantially, but not resistance of LDL to oxidation. The relative bioavailability of lutein from vegetables is five times higher than that of β-carotene.

INTRODUCTION

Epidemiological studies have shown that a high vegetable intake is associated with reduced risk of free radical-mediated degenerative diseases, such as epithelial cancers (Willet & Trichopoulos, 1997), cardiovascular disease (Ness & Powles, 1997) and age-related eye diseases (Jacques & Chylack, 1991; Hankinson et al, 1992; Seddon et al, 1994). Vegetables are a major source of carotenoids and mainly due to their antioxidant properties, carotenoids are thought to contribute to the beneficial effects of vegetable consumption (Seddon et al, 1994; Sie & Stahl, 1995; Van Poppel & Goldbohm, 1995).

Various studies have shown a significant correlation between habitual vegetable intake and plasma concentration of carotenoids (Campbell et al, 1994; Drewnowski et al, 1997; Polsinelli et al, 1998). In addition, increased vegetable consumption results in increased levels of carotenoids in blood (Yeum et al, 1996; Rock et al, 1997; Zino et al, 1997). However, the effectiveness of vegetables as a source of carotenoids has been questioned because studies have shown that the bioavailability of β-carotene from vegetables is less than previously thought (De Pee & West, 1996; Castenmiller & West, 1998). This has been shown most conclusively for green leafy vegetables (De Pee et al, 1995). The bioavailability of β-carotene may however vary among different types of vegetables, as a difference was also found between fruits and green leafy vegetables (De Pee et al, 1998). Green leafy vegetables are not the sole type of vegetables in many diets and it is therefore of interest to determine the relative bioavailability of β-carotene from a mixed vegetable diet.

Furthermore, it is necessary to obtain more information on the bioavailability of carotenoids other than β-carotene, because evidence is accumulating that these may also have important health benefits (Seddon et al, 1994; Giovannucci et al, 1995; Ziegler et al, 1996). Lutein is a major carotenoid in vegetables and it has been implicated in the etiology of age-related macular disease (Seddon et al, 1994). No information is however available on the relative bioavailability of lutein from vegetables.

Therefore, we performed a 4-weeks dietary controlled intervention study in which we investigated the relative bioavailability of β-carotene and lutein from mixed vegetables as compared to purified β-carotene and lutein. In addition, we determined the impact of increased vegetable consumption on the resistance of low-density lipoproteins (LDL) to oxidation *ex vivo*. Witztum and Steinberg (1991) have suggested that oxidative modification of LDL is an important step in the etiology of atherosclerosis. Although the impact of β-carotene supplementation on the susceptibility of LDL to oxidation *ex vivo* was shown to be limited (Princen et al, 1992; Reaven et al, 1994; Meraji et al, 1997),

increased vegetable consumption may be more effective as a range of antioxidants is supplied.

SUBJECTS AND METHODS

Volunteers

Fifty-five apparently healthy, non-smoking men and women, aged between 18 and 45 y, were selected for participation in the present study. They did not use dietary supplements containing vitamins or minerals, malaria prophylactics or anti-convulsants in the three months prior to selection and they reported no gastro-intestinal problems which could interfere with nutrient uptake. None of the women were pregnant or lactating. The volunteers were recruited among inhabitants of Wageningen and surrounding areas. The study protocol was explained to the volunteers before they gave their written informed consent.

Study design

In a four week strictly controlled dietary intervention study, 22 volunteers received a low vegetable diet (130 g/d vegetables; control group), 23 volunteers received a high vegetable diet (490 g/d; vegetable group) and 10 volunteers received a low vegetable diet (130 g/d vegetables) supplemented with pure β-carotene and lutein (carotenoid supplemented group). As this experiment was designed to also investigate the bioavailability of folate from fruits and vegetables, an additional group of 22 volunteers was included to receive supplemental folic acid. This part of the study will be reported separately. The treatment groups were stratified for total energy intake, sex and number of vegetarians. Fasting blood samples were taken before the start and at the end of the study for analysis of plasma concentrations of retinol, carotenoids and other antioxidants (i.e. vitamins C and E), total antioxidant activity and resistance of LDL to oxidation *ex vivo*. For practical reasons, we limited the number of volunteers in the carotenoids supplemented group. Power calculations based on data of previous studies, showed that n=10 would be sufficient to show a 33-50% difference in plasma responses of β-carotene and lutein and a 15% difference in LDL oxidizability (lag phase) (α=0.05; β=0.20). The study was executed at the Department of Human Nutrition and Epidemiology of Wageningen Agricultural University from November - December 1996. The study protocol was approved by the medical ethical committee of the Department.

Diets

During the intervention period, the major part of the diet was supplied to the volunteers. Diets were individually tailored to meet each volunteer's energy requirement (±0.5 MJ/d), which was estimated by questionnaire before the start of the study (Feunekes et al, 1993). Body weight was measured twice per week and, if necessary, energy intake was adjusted to prevent further changes in body weight. About 90% of total energy intake was supplied to the volunteers. They were allowed to choose a limited number of additional food items, low in carotenoids, vitamin C, vitamin E and fat. From the food diaries that were kept during the study, it was calculated that these foods provided on average (SD) 11% (1.3) of the total energy intake (Stichting NEVO, 1993).

The diets were provided as a six-day menu cycle and comprised conventional foods and drinks. All volunteers received the same basic diet, which was supplemented with a fixed amount of additional vegetables and fruits, independent of the volunteers' total energy requirements. The control and carotenoid supplemented groups were provided with the same additional products (depending on the day of the menu cycle: a rice or pasta salad, a soup containing little or no vegetables, a pear or apple and apple juice or grape juice). For the carotenoids supplemented group, purified β-carotene (all-*trans* β-carotene 30% FS (E160a), 30% suspension in vegetable oil, Hoffmann-La Roche, Basel, Switzerland) and lutein (Flora GLO™ all-*trans* lutein, 20% suspension in safflower oil, Kemin Foods LC, Des Moines, Iowa, USA) were added to the salad dressing. The vegetable group received, in addition to the basic diet, 185 g/d of cooked vegetables (depending of the day of the menu cycle: green beans, broccoli, spinach, green peas, Brussels sprouts or a vegetable mix) and a salad and soup based on vegetables. Instead of an apple or pear they received an orange or two tangerines, and instead of the apple or grape juice they were supplied with orange juice. The "low" vegetable diet provided on average 130 g/d of vegetables, whereas the total daily amount of vegetables in the high vegetable diet was 490 g. The amount of vegetables provided by the "low" vegetable diet (130 g/d) is comparable with the average vegetable intake in the Dutch population (Voorlichtingsbureau voor de Voeding, 1993), whereas the amount of vegetable in the high vegetable diet (490 g/d) was chosen as a high but acceptable amount for consumption during a four week period. The additional vegetables provided to the vegetable group were frozen vegetables, obtained from Birds Eye Walls (United Kingdom) Langnese-Iglo (Germany), Frudesa (Spain) and Sagit (Italy). The fruit juices were from Albert Heijn (Zaandam, The Netherlands) and fresh vegetables and fruits were obtained from a local supermarket.

Hot meals (including the additional cooked vegetables) were consumed under supervision at lunch-time at the university from Monday to Friday. Foods for the rest of the

day (including additional salad, soup, fruit juice and fruit) and for the weekend were taken home by the volunteers. Volunteers were carefully instructed how to prepare these foods. Compliance was checked by diaries.

Analysis of diets

Duplicate portions of the diets were taken on each day of the six-day menu cycle. One pooled sample was prepared and stored at -20°C for analysis of fat, protein, carbohydrate and dietary fiber. To assess the amount of carotenoids and vitamin C in the diets, one sample from each day of the m- u cycle was analyzed and results were averaged per treatment. For these analyses, samples were stored at -80°C. For vitamin C, 5% metaphosphoric acid was added for stabilization. Carotenoid content was determined by reversed phase HPLC. Samples were extracted with methanol/tetrahydrofuran (1:1, v/v). An aliquot of the filtrate was saponified in boiling ethanolic 2 mol/L KOH after addition of a sodium ascorbate (10%)/sodiumdisulfide-glycerol mixture (2:1, v/v). After cooling, the saponification mixture was extracted with di-isopropylether. The extract was washed three times with water. The solvent was evaporated and the residue was dissolved in di-isopropylether. Carotenoids and α-tocopherol were separated on a Hypochrome column filled with nucleosil 120-3C_{18} (Sandon Southern Products, UK) with acetonitrile/methanol/methylene chloride/ammonium acetate (900/50/40/10, v/v) as mobile phase at a flow rate of 1 mL/min and room temperature. Calibration was performed using external standards. Recovery tests of this method showed 86-103% recovery of the carotenoids and the coefficient of variation ranged between 5.4% and 15.3%, depending on the type of carotenoid. For analysis of the vitamin C content, the samples were extracted with metaphosphoric acid/acetic acid (60/80, w/v). Vitamin C content was subsequently determined fluorimetrically as ascorbic acid plus dehydro-ascorbic acid (Vuilleumier & Keck, 1989). The composition of the diets is shown in Table 1. As we planned to replace the vegetables from the high vegetable diet by other fiber-rich foods (e.g. rice or pasta), the difference in fiber content between the high and low vegetables diets was only 0.7 g/MJ. Differences in carotenoid and vitamin C levels among the diets were generally as expected. However, the lycopene content of the low vegetables and carotenoids supplemented diets was higher than that of the high vegetable diet. Two of the ready-to-eat soups that were provided in the six days menu cycle to the low vegetable and carotenoid supplemented groups but not to the high vegetable group apparently contained more lycopene than we expected. The diets were calculated to provide ca. 500-600 µg/d of preformed vitamin A (Stichting NEVO, 1993). The β-carotene, α-carotene and β-cryptoxanthin in the control, high vegetable and carotenoid supplemented diets hypothetically provided an additional 300, 1000 and 1219

µg retinol equivalents/d, respectively (based on data of Table 1 and the assumption that 6 µg β-carotene or 12 µg α-carotene or β-cryptoxanthin equals 1 µg retinol equivalents).

TABLE 1 Composition of the diets[1,2].

	Intervention group		
	Low vegetable diet	High vegetable diet	Carotenoid supplement
Fat (energy%)	30.5	31.7	30.7
Protein (energy%)	13.6	14.1	13.7
Carbohydrate (energy%)	55.9	53.0	55.7
Fiber (g/MJ)	4.1	4.8	4.1
Vitamin C (mg/d)	27.5 (6.3)	169 (70)	28.4 (5.4)
α-Carotene (mg/d)	0.31 (0.25)	0.99 (1.2)	0.23 (0.07)
β-Carotene (mg/d)	1.5 (1.4)	5.1 (3.2)	7.2 (1.5)
Lutein (mg/d)	2.7 (3.3)	10.7 (9.4)	12.0 (3.1)
Zeaxanthin (mg/d)	nd[3]	nd[3]	nd[3]
β-Cryptoxanthin (mg/d)	0.21 (0.04)	0.84 (0.55)	nd[3]
Lycopene (mg/d)	2.1 (2.1)	1.1 (0.99)	2.2 (2.1)
α-Tocopherol (mg/d)	20.9 (2.8)	27.4 (2.9)	23.6 (2.9)

[1] Values are based on analysis of duplicate portions of complete daily menus plus the calculated contribution from the free choice items (see methods section). As the amount of additional vegetables or carotenoids was the same for all volunteers, irrespective of their total energy intake (see methods section), differences in vitamin C, carotenoid and α-tocopherol content among the groups are the same for all volunteers.

[2] Values are presented as mean (SD)

[3] Not detectable: <0.2 mg/d

Analysis of blood samples

Venous blood samples were obtained while subjects were fasting before the start and at the end of the study. Blood samples were collected into heparinized tubes for analysis of vitamin C and into sodium EDTA coated tubes for the other analyses. Plasma was prepared by centrifugation at 3000 x g for 10 minutes (4°C). Before storage, 5% metaphosphoric acid was added for the analysis of vitamin C (9/1, v/v). Plasma samples for LDL isolation were stabilized with 6 g sucrose/L. It has been reported that freezing LDL in this way does not influence oxidation variables (Ramos et al, 1995). Samples were stored at -80°C until analysis.

Vitamin C concentration in trichloroacetic acid treated plasma was determined fluorimetrically as ascorbic acid plus dehydro-ascorbic acid (Vuilleumier & Keck, 1989). Plasma levels of carotenoids, retinol and α-tocopherol were assessed by reversed phase HPLC on a 201TP54 Vydac column (Separations Group, Hesperia CA, USA) with retinyl

acetate as internal standard. After extraction with n-heptane/diethyl ether (1:1, v/v) and evaporation of the solvents, the residue was dissolved in eluent and compounds were separated at a flow rate of 0.8 mL/min and a column temperature of 20 °C by using a step gradient: 0-80 min methanol/amonium acetate (950/50, v/v), 80-85 min methanol/tetrahydrofurane (950/50, v/v). Peak areas were measured spectrophotometrically at 292 nm for α-tocopherol, at 325 nm for retinol, at 450 nm for α-carotene, β-carotene, lutein, zeaxanthin and β-cryptoxanthin and at 470 nm for lycopene. The percent recovery of the internal standards varied between 86% and 99.9% for the different compounds and the detection limit was ≤ 0.02 µmol/L for carotenoids, 0.03 µmol/L for retinol and 0.65 µmol/L for α-tocopherol. Intra assay variation ranged from 0.6% to 5.1%.

Total cholesterol and triacylglycerol concentrations were measured in plasma using enzymatic colorimetric methods (Boehringer Mannheim, Germany). The antioxidant activity of plasma was assessed as its ferric reducing ability (Benzie & Strain, 1996). LDL was isolated from thawed plasma by discontinuous density gradient ultracentrifugation for 24 h at 4°C (Redgrave et al, 1975). EDTA was removed as described by Puhl et al (1994). Immediately thereafter, LDL protein content was determined using bovine serum albumin (Fraction V, Sigma, St Louis, Mo., USA) as the standard (Markwell et al, 1978). Subsequently, resistance to copper-mediated oxidation of the EDTA-free LDL-fraction was determined as described by Princen et al (1992). Intra assay variations were 10% for the lag phase and 4% for the maximum rate of oxidation.

Statistical evaluation

Differences in changes during the experiment among the three groups were compared by one-way analysis of variance. Significance of the differences was assessed by Tukey (α=0.05). For plasma concentrations and changes in plasma concentrations of β-carotene, data were log-transformed to minimize correlation between mean values and standard error. Data for β-carotene are therefore presented as geometric mean with the standard error as percentage of the geometric mean. Other data are shown as mean with their standard error, or as mean and standard deviation in case of descriptive parameters.

RESULTS

Volunteers

One male withdrew from participation in the vegetable group for personal reasons and 54 volunteers completed the study. Table 2 shows the descriptive characteristics of the participants.

TABLE 2 Characteristics of the volunteers[1].

	Intervention group		
	Low vegetable diet	High vegetable diet	Carotenoid supplement
Males/Females (n)	7/15	6/16	4/6
Vegetarians (n)	5	5	2
Age (y)	22.2 (7.5)	22.4 (5.2)	20.9 (2.5)
Body Mass Index (kg/m^2)	22.6 (1.7)	22.1 (2.3)	23.0 (2.9)
Energy intake (MJ/d)	9.9 (2.6)	9.9 (2.5)	10.6 (2.5)

[1] Values are expressed as mean (SD). Baseline age and Body Mass Index are shown, whereas energy intake is based on the average intake over the four weeks intervention period.

Blood parameters

Four weeks of increased vegetable consumption resulted in significantly increased plasma levels of vitamin C and five of the six carotenoids measured (Table 3). Plasma concentration of lycopene was significantly reduced during consumption of the high vegetable diet, reflecting the 50% lower lycopene content of this diet compared with the low vegetable diet (Table 1).

As anticipated, consumption of the purified β-carotene and lutein supplemented diet significantly enhanced plasma levels of these carotenoids, as compared to the changes found in the low vegetable or control group. Surprisingly, however, the plasma concentration of lycopene was significantly decreased in the carotenoid supplemented group, while the lycopene intake was similar to that of the control group (Tables 1 and 3).

In comparison to the changes in the carotenoid supplemented group, consumption of the high vegetable diet induced significantly smaller increases in plasma concentrations of β-carotene and lutein. The high vegetable and carotenoid supplemented diets contained slightly different amounts of these carotenoids. We therefore calculated the relative plasma carotenoid responses for the high vegetable and carotenoid supplemented groups by dividing the changes in plasma carotenoid levels by the carotenoid intake (mg/d), both corrected for those observed in the low vegetable group. This revealed that the relative plasma β-carotene response to the high vegetable diet was substantially less than that to the carotenoid supplement (Table 4). From the ratio of the two responses, a measure of relative bioavailability can be obtained by dividing the relative plasma response to vegetable β-carotene by that to synthetic β-carotene. This gave a figure of 14% for the relative bioavailability of β-carotene from vegetables (Table 4). For lutein, the difference in the relative plasma carotenoid responses between the high vegetable and the carotenoid supplement group was not as large as for β-carotene and the relative bioavailability of lutein from vegetables was calculated to be 67% (Table 4).

TABLE 3 Baseline plasma concentrations of antioxidants and oxidizability of LDL and changes after four weeks consumption of diets containing low or high amounts of vegetables or a low vegetable diet supplemented with β-carotene and lutein[1].

		Intervention group		
		Low vegetable diet (n=22)	High vegetable diet (n=22)	Carotenoid supplement (n=10)
Vitamin C (µmol/L)	Baseline	63.6 (3.7)	69.1 (3.6)	71.4 (4.3)
	Change	-7.5 (3.1)[a]	19.0 (3.1)[b]	-14.0 (4.5)[a]
α-Carotene (µmol/L)	Baseline	0.087 (0.017)	0.083 (0.010)	0.065 (0.008)
	Change	-0.029 (0.010)[a]	0.053 (0.008)[b]	-0.017 (0.004)[a]
β-Carotene (µmol/L)	Baseline	0.34 (9.6%)	0.37 (11%)	0.32 (18%)
	Change	-0.017 (4.2%)[a]	0.18 (6.5%)[b]	2.23 (5.6%)[c]
Lutein (µmol/L)	Baseline	0.19 (0.016)	0.21 (0.021)	0.18 (0.016)
	Change	0.068 (0.008)[a]	0.40 (0.027)[b]	0.64 (0.048)[c]
Zeaxanthin (µmol/L)	Baseline	0.048 (0.005)	0.056 (0.005)	0.049 (0.005)
	Change	0.018 (0.003)[a]	0.13 (0.015)[b]	0.022 (0.005)[a]
β-Cryptoxanthin (µmol/L)	Baseline	0.32 (0.043)	0.37 (0.046)	0.24 (0.028)
	Change	-0.17 (0.031)[a]	0.096 (0.029)[b]	-0.077 (0.018)[a]
Lycopene (µmol/L)	Baseline	0.27 (0.027)	0.28 (0.024)	0.28 (0.059)
	Change	0.047 (0.020)[a]	-0.10 (0.023)[b]	-0.062 (0.047)[b]
Retinol (µmol/L)	Baseline	1.70 (0.071)	1.67 (0.082)	1.59 (0.13)
	Change	-0.056 (0.036)	-0.008 (0.053)	-0.016 (0.058)
α-Tocopherol (mmol/L)	Baseline	8.63 (0.38)	8.42 (0.41)	8.30 (0.59)
	Change	0.99 (0.22)	0.71 (0.26)	0.61 (0.41)
FRAP (mmol/L)	Baseline	1.04 (0.03)	1.08 (0.04)	1.10 (0.05)
	Change	0.03 (0.2)	0.06 (0.2)	0.04 (0.06)
Lag time of LDL oxidation[2] (min)	Baseline	89.2 (4.4)	83.1 (3.8)	105 (5.7)
	Change	3.5 (5.2)	3.8 (5.8)	-4.0 (2.4)
Maximum rate of LDL oxidation[2] (nmol dienes/min/mg LDL protein)	Baseline	10.1 (0.3)	9.5 (0.2)	9.8 (1.1)
	Change	1.2 (0.3)	1.6 (0.4)	0.9 (0.2)

[1] Values are presented as mean (SE), except for β-carotene, which was log-transformed and thus the geometric mean with the coefficient of variation from the mean (CVM%) is shown

[2] Low vegetable group: n=15; High vegetable group: n=8; Carotenoid group: n=5

[a,b,c] Mean changes within the same row with different superscripts are significantly different ($P<0.05$)

Note: There were no significant differences among the groups at baseline

Plasma concentrations of retinol and α-tocopherol remained unchanged in all of the three groups and no significant differences were found among the groups (Table 3).

No significant differences in changes among the groups were found in plasma levels of total cholesterol and triacylglycerol (results not shown).

Despite the significant increases in plasma levels of vitamin C and/or carotenoids in the vegetable and carotenoid supplemented group, the total antioxidant activity of plasma, measured as ferric reducing ability (FRAP), remained unchanged (Table 3). Furthermore, consumption of the diets supplemented with vegetables or carotenoids did not enhance protection of LDL against copper-induced oxidation *ex vivo*. Neither the changes in lag time before onset of oxidation, nor the maximum rate of oxidation were significantly different from those found in the control group (Table 3).

TABLE 4 Relative bioavailability of β-carotene and lutein calculated from the relative plasma responses to carotenoids ingested from vegetables or from a preparation of pure carotenoids suspended in oil[1,2].

	Relative plasma carotenoid response (nmol/L/mg)		Relative bioavailability from vegetables (%)
	High vegetable diet	Carotenoid supplement	
β-Carotene	55 (3.3)	394 (2.2)	14 (1.1)
Lutein	41 (3.5)	62 (5.2)	67 (8)

[1] Values are presented as mean (SE)

[2] *See methods section for details of calculations*

DISCUSSION

The present study shows that four weeks of increased vegetable consumption significantly improves plasma levels of carotenoids and vitamin C. There is a striking difference in the bioavailability of β-carotene and lutein from mixed vegetables, as calculated from the plasma carotenoid response relative to that following consumption of pure carotenoids in oil. The relative bioavailability of β-carotene was 14% and of lutein 67%. The larger amount of all the carotenoids in the high vegetable diet compared with the low vegetable diet was reflected in the increased plasma concentrations of carotenoids. Consumption of pure β-carotene and lutein induced a significant reduction in plasma concentration of lycopene.

Bioavailability of β-carotene and lutein

*The relatively low bioavailability of β-carotene from vegetables as compared to pure β-*carotene has been reported previously. Our results confirm the assumption that the matrix in which β-carotene is located is a major limiting factor for its bioavailability. The relative bioavailability of 14% for β-carotene from the mixed vegetables diet lies within the range of relative bioavailabilities from different vegetable types that have been reported by

others. It is higher than the 7% availability from green leafy vegetables (De Pee et al, 1995) but lower than the 19-30% from carrots and 22-24% from broccoli (Brown et al, 1989; Micozzi et al, 1992; Törrönen et al, 1996). In the present study, spinach was the only green leafy vegetable in the menu cycle and it contributed about 43% of the total β-carotene intake (Mangels et al, 1993). It seems that the low bioavailability of β-carotene from spinach has been compensated by the higher bioavailability of β-carotene from other types of vegetables in the menu cycle.

Until recently, major focus has been on the health benefits of β-carotene. However, it is now being recognized that other carotenoids present in vegetables may also be crucial for optimal health (Seddon et al, Giovannucci et al, 1995; Ziegler et al, 1996). Therefore, the carotenoid supplemented group received not only β-carotene but also lutein so that we could calculate the relative bioavailability of this carotenoid. For lutein in vegetables, the bioavailability was found to be 67%. This suggests that the bioavailability from vegetables of the more hydrophilic lutein is five times greater than that of β-carotene. On the other hand, the difference in relative plasma response between β-carotene and lutein may not reflect the true differences in absorbability. Part of the absorbed β-carotene is cleaved and converted to retinyl esters before entering the blood stream. In the vegetable group, a relatively larger percentage of the absorbed β-carotene may have been converted than in the β-carotene supplemented group, because of the large difference in absorbed β-carotene between these two groups. As this phenomenon does not occur for lutein, which has no provitamin A activity, this may have resulted in an underestimation of the relative bioavailability of β-carotene from vegetables as compared to lutein.

Interestingly, the plasma response of lutein following supplementation with pure lutein and β-carotene was substantially smaller than that of β-carotene. There are a number of explanations for this finding. Firstly, less lutein may have been absorbed per mg ingested. The solubility of lutein in oil is lower than that of the more lipophilic β-carotene (Borel et al, 1996) and a larger part of lutein may have been present as crystals in the salad dressing that contained the carotenoids. It has been suggested that the crystalline form of carotenoids is less bioavailable (Zhou et al, 1996). In addition, a relatively smaller uptake of lutein may have resulted from competition for absorption. As more β-carotene was released from the food matrix following ingestion of the purified carotenoids, the ratio of released β-carotene to lutein was larger than in the case of the vegetables supplemented diet, in which part of the β-carotene was still locked in the cellular compartments of the vegetables. Kostic et al (1995) showed that simultaneous ingestion of purified lutein and β-carotene decreases the bioavailability of lutein. Secondly, a difference in plasma response between different carotenoids may not reflect a difference in true absorption, as

the rate and extent of tissue uptake and subsequent metabolism may vary among carotenoids. A faster serum clearance of lutein than β-carotene has indeed been observed in preruminant calves (Bierer et al, 1995). On the other hand, β-carotene has provitamin A activity and the rate and extent of β-carotene metabolism may thus be higher. This would however imply, in contrast to our findings, a lower β-carotene response as compared to lutein.

Interaction between β-carotene, lutein and lycopene

The increases in plasma concentrations of other carotenoids and vitamin C, and the decrease in plasma concentration of lycopene in the vegetable group were anticipated, based on the composition of the diets (Table 1). The decrease in plasma lycopene concentration during consumption of the β-carotene and lutein supplemented diet was however surprising. The lycopene content of the control and carotenoid supplemented diets was similar (Table 1). Other studies have been equivocal with respect to the effect of β-carotene supplementation on plasma or LDL levels of lycopene, whereas no information is available on the effect of lutein. Some studies also showed a reduction of lycopene levels during supplementation with β-carotene (Prince et al, 1991; Gaziano et al, 1995) whereas others found no effect (Fotouhi et al, 196; Albanes et al, 1997) or even an enhancing effect (Wahlqvist et al, 1994; Johnson et al, 1997). The enhancing effect in the single dose study of Johnson et al (1997) was attributed to an increased solubility of lycopene in the suspension which contained β-carotene, whereas during the long-term study, reported by Wahlqvist et al (1994), a sparing effect of lycopene as antioxidant may have occurred. However, the results of the present study and those of others (Prince et al, 1991; Gaziano et al, 1995) suggest that the supplemented carotenoids compete with lycopene for absorption and/or transport in plasma. This phenomenon may be particularly important with respect to the risk of prostate cancer as an inverse association with lycopene intake has been reported (Giovannucci et al, 1995).

Effects on antioxidant capacity

Consumption of the high vegetable diet increased plasma β-carotene levels by about 50% to 0.55 µmol/L, whereas the increases in the carotenoid supplemented group were even higher (Table 3). These levels and the plasma levels of total carotenoids are beyond the threshold levels that were suggested in relation to reducing risk of cardiovascular disease (Gey, 1995). Despite these substantial increases in plasma antioxidant levels in the vegetable and carotenoids supplemented groups, we found however no significant effect on the total antioxidant activity of plasma or on the susceptibility of LDL to oxidation *ex*

vivo. Oxidative modification of LDL has been proposed as an important step in the etiology of atherogenesis (Witztum & Steinberg, 1991). Recently, Hininger et al (1997) reported that increased fruit and vegetable consumption significantly enhanced the resistance of LDL to oxidation, both in smokers and non-smokers. However, the effect reported may have been due to external factors as they did not include a control group which received a lower level of fruits and vegetables. The decrease in plasma levels of lycopene we observed in the high vegetable and carotenoid supplemented groups may also have outweighed a possible protective effect of the other carotenoids, thus explaining the lack of effect of vegetable or carotenoid supplementation in the present study. On the other hand, previous studies have shown that in particular vitamin E supplementation is effective in increasing the resistance of LDL to oxidation (Jalal et al, 1992; Princen et al, 1992), whereas studies on the benefits of synthetic β-carotene only have been equivocal (Princen et al, 1992; Reaven et al, 1994; Levy et al, 1995; Lin et al, 1998). The increased antioxidant levels may have had an impact on other oxidative stress-related parameters that were not assessed in this study, such as for example isoprostanes (Patrono & FitzGerald, 1997). This should perhaps be addressed in future research.

Conclusion

When designing future studies on health benefits of carotenoids or formulating recommendations on carotenoid intake, the variation in the bioavailability of β-carotene in particular should be taken into account. The present study clearly shows that vegetable consumption induces a more moderate increase of β-carotene in plasma than purified β-carotene (Table 4). Five mg β-carotene from vegetables would equal only ca. 0.7 mg β-carotene from a supplement. This aspect may be less crucial for lutein because the differences in plasma responses between vegetables and purified lutein were less pronounced.

In conclusion, the present study shows that increased vegetable consumption (i.e., an additional 360 g/day) enhances the plasma levels of vitamin C and carotenoids substantially, but not the resistance of LDL to oxidation. The relative bioavailability of β-carotene and lutein from mixed vegetables as compared to purified carotenoids is 14% and 67% respectively.

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3

Influence of feeding different vegetables on plasma levels of carotenoids, folate and vitamin C. Effect of disruption of the vegetable matrix

Karin H. van het Hof, Lilian B.M. Tijburg, K. Pietrzik, Jan A. Weststrate

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ABSTRACT

Carotenoids, folate and vitamin C may contribute to the observed beneficial effects of increased vegetable intake. Currently, knowledge on the bioavailability of these compounds from vegetables is limited. We compared the efficacy of different vegetables, at the same level of intake (i.e. 300 g/d), in increasing plasma levels of carotenoids, folate and vitamin C and we investigated if disruption of the vegetable matrix would enhance the bioavailability of these micronutrients. In an incomplete block design, 69 volunteers consumed a control meal without vegetables and three out of four vegetable meals (i.e. broccoli, green peas, whole leaf spinach, chopped spinach; containing between 1.7 and 24.6 mg β-carotene, 3.8 and 26 mg lutein, 0.22 and 0.60 mg folate and 26 and 93 mg vitamin C) or a meal supplemented with synthetic β-carotene (33.3 mg). Meals were consumed during four days and fasting blood samples were taken at the end of each period. Consumption of the spinach supplemented meal did not affect plasma levels of β-carotene, although the β-carotene content was 10-fold that of broccoli and green peas, which induced significant increases in plasma β-carotene levels (28% (95% CI: 6.4, 55) and 26% (95% CI: 2.6, 54), respectively). The β-carotene supplemented meal increased plasma concentrations of β-carotene effectively (517%, 95% CI: 409, 648). All vegetable meals increased the plasma concentration of lutein and vitamin C significantly. Broccoli and green peas were, when expressed per mg carotenoid consumed, also more effective sources of lutein than spinach. A significant increase in plasma folate concentration was found only after consumption of the spinach supplemented meal, which was most abundant in folate. Disruption of the spinach matrix increased the plasma response of both lutein (14%, 95% CI: 3.7, 25) and folate (10%, 95% CI: 2.2, 18), whereas it did not affect the response of β-carotene. We conclude that the bioavailability of β-carotene and lutein varies substantially among different vegetables and that the bioavailability of lutein and folate from spinach can be improved by disruption of the vegetable matrix.

INTRODUCTION

Many epidemiological studies have indicated that an increased intake of vegetables is associated with a decreased risk of certain cancers (Willet & Trichoupoulos, 1997), cardiovascular disease (Ness & Powles, 1997) and age-related eye diseases (Jacques & Chylack, 1991; Hankinson et al, 1992; Seddon et al, 1994). This has raised interest in determining the nature and bioavailability of active compounds present in vegetables. Antioxidants, such as carotenoids and vitamin C, may contribute to the beneficial effects of vegetable consumption (Seddon et al, 1994; Van Poppel & Goldbohm, 1995; Gey, 1995; Weber et al, 1996). In addition, vegetables are a major dietary source of folate (De Bree et al, 1997). High intake of folate may be associated with a reduced risk of cancer (Glynn & Albanes, 1994) and there is increasing evidence that folate may reduce the risk of cardiovascular disease by lowering homocysteine levels in plasma (Boushey et al, 1995; Verhoef et al, 1996).

There are indications that the bioavailability of carotenoids and folate from vegetables is limited as compared to supplements (Micozzi et al, 1992; Gregory, 1995; De Pee et al, 1995). It is plausible that the vegetable matrix plays a major role in determining the bioavailability of these micronutrients. For instance, disruption of the vegetable matrix enhanced the bioavailability of β-carotene from carrots and lycopene from tomatoes (Van Zeben & Hendriks, 1948; Gärtner et al, 1997). It is of interest to investigate whether disruption of the matrix also affects the bioavailability of β-carotene from green leafy vegetables, which was found to be low (De Pee et al, 1995). In addition, there are indications that there may be significant differences between different vegetables as to the bioavailability of carotenoids and folate (Tamura & Stokstad, 1973; Babu & Srikantia, 1976; Micozzi et al, 1992). The present study was set up to compare three types of vegetables, i.e. broccoli, green peas and spinach, at the same level of intake (300 g/day), in their effectiveness to increase plasma levels of carotenoids, folate and vitamin C. These vegetables are similar in colour, but clearly distinct with respect to the part of the plants they represent (i.e. flowers, seeds and leaves). In addition, we investigated whether mechanical homogenisation of whole leaf spinach would enhance the bioavailability of carotenoids, folate and vitamin C.

SUBJECTS AND METHODS

Volunteers

Seventy-two volunteers were selected for participation in the study. They were recruited among inhabitants of Vlaardingen and surroundings and employees of Unilever

Research Laboratorium in Vlaardingen, The Netherlands and gave their written informed consent before participation.

Volunteers were eligible if they had a Quetelet Index between 19 and 30 kg/m^2 and if they were apparently healthy, as assessed by questionnaire (i.e. they regarded themselves as being healthy, they did not receive medical treatment or used medicines and they reported no chronic gastro-intestinal problems). They did not use dietary supplements (e.g. vitamins, minerals, carotenoids) in the month preceding the study and they were not on a slimming diet, did not use excessive amounts of alcohol (less than 21 units/week for females or 28 units/week for males) and smoked maximally 15 cigarettes/day. Volunteers who adhered to a vegetarian, macrobiotic or other alternative diet were excluded from participation. Also pregnant or lactating women were excluded.

Study design

The study had an incomplete block design with four experimental periods and with persons as blocks. All volunteers would consume a control meal during one of the experimental periods and they would consume meals supplemented with vegetables or synthetic β-carotene during the other three periods. The vegetable meals contained 300 g of one of the following vegetables: broccoli or green peas or whole leaf spinach or chopped spinach. The control and the β-carotene supplemented meals contained no vegetables. The test meals were consumed for four consecutive days, followed by 10 days of wash-out. The duration of the wash-out period was chosen as approximately the *in vivo* half-life of plasma β-carotene (Rock et al, 1992). Fasting blood samples were taken at the end of each four day experimental period to assess plasma levels of folate (only after control and vegetable supplemented meals), carotenoids and vitamin C. To determine the effect of the experimental regime without any vegetables and fruits, a sub-group of 26 randomly chosen volunteers supplied an additional fasting blood sample before the start of the experimental period in which they would consume the control meal. The protocol was approved by the Medical-Ethical Committee of Unilever Nederland BV.

Test meals and background diet

The control meal consisted of a basic pasta meal with ham and a white sauce and custard for dessert. The vegetables and β-carotene were added to this basic meal. Energy and fibre content and macronutrient composition was equal for all test meals and similar to that of an average Dutch main meal (Voorlichtingsbureau voor de Voeding, 1993). Differences in fibre content was corrected for by addition of beet fibre (Fibrex, Tefco Food Ingredients BV, Bodegraven, the Netherlands) to the sauce. Synthetic β-carotene (30% microcrystalline suspension in oil, 30% FS (E160a), Hoffmann-La Roche,

Basel, Switzerland) was added to the sauce. The vegetables (300 g/meal: broccoli, Iglo/Mora, The Netherlands; garden peas, Birds Eye Wall's, UK; whole leaf spinach, Sagit, Italy) were served simultaneously with the basic meal. Broccoli and green peas were cooked conventionally in boiling water during 6 min and 3-4 min respectively. Whole leaf spinach was microwaved (3200 Watt) for 16 min with a stir after 8 min. For preparation of chopped spinach, whole leaf spinach was minced after 8 min in the microwave (3200 Watt) and subsequently microwaved for another 8 min.

The hot meals were served at lunch time and volunteers were instructed not to consume any vegetables or vegetable containing products, fruits, fruit juices or red sauces (e.g. tomato ketchup) during the rest of the days of the experimental periods. During the wash-out periods the volunteers returned to their habitual diet.

Composition of test meals

Duplicate portions (n=5) of the complete test meals (as consumed by the volunteers) were analysed and found to provide on average (SD) 20 (1.5) g fat, 80 (4.7) g carbohydrates, 32 (1.8) g protein and 16 (1.8) g fibre. Carotenoid, folate and vitamin C concentrations were determined in duplicate portions (n=4-8) of the vegetables or sauces (as consumed by the volunteers). After extensive extraction of the vegetables or sauces with n-heptane/ether (1:1, v/v), ethyl-carotenoate was added as internal standard. (α+β)-Carotene, lutein and lycopene were separated by straight phase HPLC using a nucleosil 5CN column (Machery & Nagel, Duren, Germany) and n-heptane/iso-propanol (1000:25, v/v) as mobile phase at a flow rate of 1.0 mL/min and a column temperature of 20°C. The eluent was monitored by UV-Vis detection at 450 nm for (α+β)-carotene and lutein and at 470 nm for lycopene. In this system, α-carotene coelutes with β-carotene. As the vegetables and β-carotene supplement contained virtually no α-carotene (i.e. <0.04 mg/serving, Mangels et al, 1993a), the response of (α+β)-carotene is considered as β-carotene. The vegetables and control sauce were extracted with 0.1 mol/L phosphate buffer (pH 6.1, +0.2% sodium ascorbate) and the filtrate was used for analysis of the folate concentration by microbiological assay with Lactobacillus rhamnosus (NCIB 10463), using a commercially obtained assay medium (Merck Folic acid medium, Difco Laboratories, Detroit, USA). For total folate content, an aliquot of the extract was incubated (3 h, pH 4.5, 37°C) with human plasma deconjugase (Sigma Chem Co.) (Finglas et al, 1993). This step was omitted for analysis of free folate. After precipitation of the proteins and stabilization with 5% metaphosphoric acid, the vitamin C content of the vegetables or sauces was determined fluorimetrically as ascorbic acid plus dehydroascorbic acid, as described by Vuilleumier and Keck (1989).

TABLE 1 Carotenoid, folate and vitamin C content of the control sauce, vegetables and synthetic β-carotene supplemented sauce (mean (SD)).

Compound	Type of test meal					
	Control	Broccoli	Green peas	Whole leaf spinach	Chopped spinach	Synthetic β-carotene
	(mg/serving)[1]					
Total folate	nd	0.35 (0.029)	0.22 (0.015)	0.60 (0.049)	0.59 (0.030)	--
Monoglutamyl folate	--	--	--	0.17 (0.015)	0.18 (0.0)	--
β-Carotene	nd	2.43 (0.2)	1.72 (0.07)	24.6 (1.8)	23.8 (2.4)	33.3 (1.2)
Lutein	nd	3.78 (0.4)	4.22 (0.2)	25.0 (2.7)	26.0 (2.8)	nd
Vitamin C	nd	84.6 (14.9)	25.7 (2.1)	91.6 (10.3)	92.6 (9.1)	nd

[1] Based on analysis of duplicate portions of the prepared vegetables or sauces, as consumed by the volunteers; N=4 for folate and vitamin C analysis, N=8 for carotenoid analysis

nd = not detectable: < 0.03 mg/serving for folate; < 0.3 mg/serving for carotenoids; < 5 mg/serving for vitamin C

-- = not determined

Note: the amount of lycopene was below detection level in all of the meals (< 0.3 mg/serving)

Plasma and serum analyses

Fasting blood samples were taken by vena puncture. Plasma samples were stored at -70°C for analysis of carotenoids and trichloroacetic acid-treated plasma was stored at -70°C for analysis of vitamin C. For the other analyses, plasma and serum samples were stored at -20°C. All analyses were executed within six months after the study. Plasma concentrations of β-carotene, lutein and lycopene were determined by straight phase HPLC as described previously (Weststrate & Van het Hof, 1995). β-Carotene and lycopene were separated on a Nucleosil 5-N $(CH_3)_2$ column with n-heptane as mobile phase at a flow rate of 1 ml/min and ethyl-β-apo 8'-carotenoate as internal standard. Lutein was separated on a Nucleosil 5CN column with n-heptane/dichloromethane/ isopropanol (900/100/5, v/v) as mobile phase at a flow rate of 1 ml/min and β-apo-8'-carotenol as internal standard (intra-assay variation: <5.7%, as determined in control plasma samples with the following average carotenoid concentrations: β-carotene 0.2 µmol/L; lycopene 0.4 µmol/L; lutein 0.15 µmol/L). Plasma folate concentration was assessed by using a chemiluminescence competitive protein binding test (Magic Lite, Ciba Corning Diagnostics GmbH, Fernwald, Germany) (intra-assay variation: 4.7%, as determined in plasma samples varying in folate concentration between 10 and 48 nmol/L). Vitamin C was analysed fluorimetrically in trichloroacetic acid-treated plasma as the concentration of ascorbic acid plus dehydroascorbic acid (intra-assay variation: 1.9%, as determined in control plasma samples with 58 and 283 µmol/L vitamin C) (Vuilleumier & Keck, 1989). Total cholesterol and triacylglycerol concentration in serum was assessed by using commercially available colorimetric test kits (respectively CHOD-PAP, Boehringer,

Mannheim, Germany and GPO-PAP (Roche, Basel, Switzerland)/GPO-Trinder (Sigma, St. Louis, USA)).

Statistical evaluation

The significance of the changes in plasma carotenoid, folate and vitamin C concentration during the experimental period in which volunteers consumed the control meal was determined by Student's *t* test for paired data. Analysis of variance with persons as blocks and, sex, smoking habits, period, treatment x sex and treatment x smoking as factors, was used to compare the plasma and serum values found after consumption of the supplemented meals with those found after consumption of the control meal. Significance of the differences was assessed by Dunnett's test. As sex and smoking had no significant effect, these variables were excluded from the ANOVA model. Differences between the two types of spinach were assessed by orthogonal contrasts.

Plasma carotenoid concentrations were log-transformed to minimize correlation between mean values and standard errors. For these parameters, geometric means are presented with the standard error as percentage of the geometric means. Other variables are shown as least square means with their standard error. Plasma concentrations of carotenoids, normalized for serum cholesterol and triacylglycerol levels, were also analyzed for differences between the treatments (carotenoid concentration/(cholesterol + triacylglycerol concentration)).

All comparisons were at the two-sided 0.05 significance level, except for the difference between chopped and whole leaf spinach which was tested one-sided, based on the hypothesis that disruption of the spinach matrix would enhance the bioavailability of carotenoids, folate and vitamin C.

RESULTS

Three volunteers dropped out of the study before the end of the first experimental period because of lack of time to participate in the trial and four volunteers were not able to participate in all of the four experimental periods for various reasons (e.g. illness, business trip) (see Figure 1). Data of 31 males and 38 females were included in the statistical analyses. Sixty-five of these volunteers participated in all of the four experimental periods whereas three volunteers participated in three experimental periods and one volunteer in only two periods. Two volunteers did not receive the control meal. The average age (SD) of the participants was 42 (13) y and their mean Quetelet Index (SD) was 24.6 (2.3) kg/m^2. Ten of the 69 volunteers were smokers (5 females and 5 males, maximum 15 cigarettes/d).

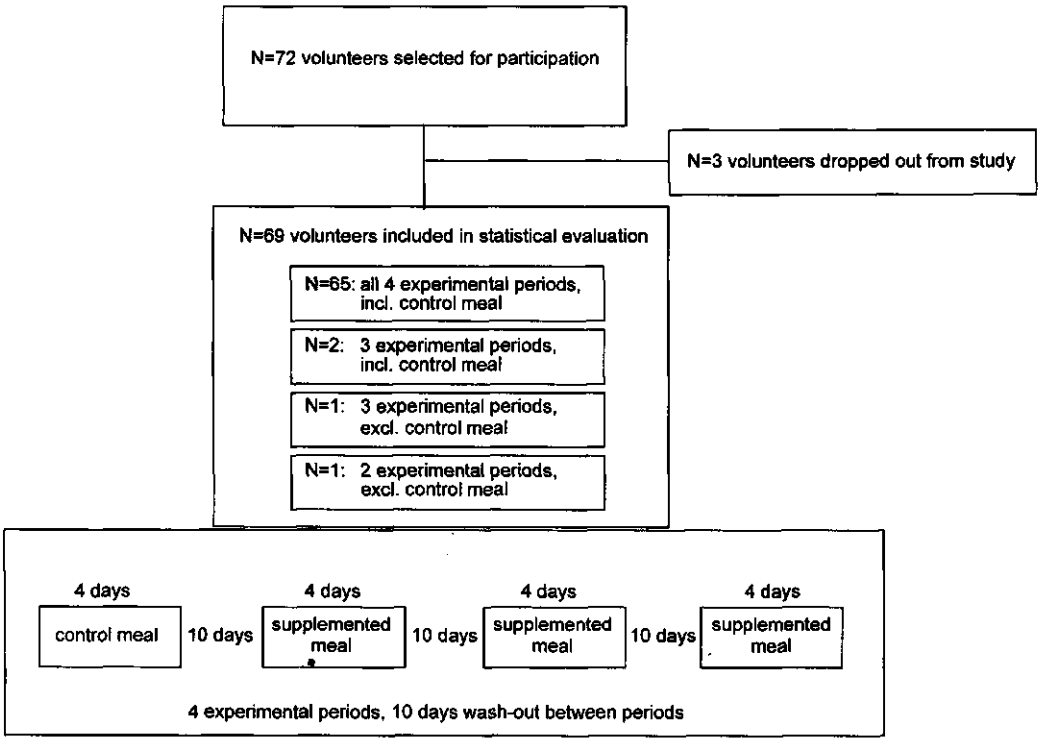

Figure 1 Experimental design of the study. The control meal contained no vegetables or carotenoids whereas the other test meals were supplemented with broccoli, green peas, whole leaf spinach, chopped spinach or synthetic β-carotene. Volunteers received three out of these supplemented meals. The combinations of supplemented meals and the order of the test meals was varied among the participants.

Table 2 shows the least square means of the plasma concentrations of carotenoids, folate and vitamin C and serum concentrations of total cholesterol and triacylglycerol before and after four days of consumption of the control meal without vegetables (low in carotenoids, folate and vitamin C) and after four days of the same meal supplemented with vegetables or β-carotene. As no vegetables and fruits were allowed to be consumed during the experimental periods, consumption of the control meal without any vegetables significantly reduced plasma carotenoid and vitamin C levels (mean (SE): β-carotene 0.069 (0.018) μmol/L; lutein 0.038 (0.0034) μmol/L; vitamin C 14 (1.5) μmol/L, $P<0.005$), whereas plasma folate concentrations were not significantly affected.

Unfortunately, consumption of the meal supplemented with β-carotene induced a carry-over effect in plasma concentrations of β-carotene. The plasma levels of β-carotene, found in the first and second test periods following consumption of the β-carotene

TABLE 2 Plasma levels of carotenoids, folate and vitamin C and serum lipid levels before and after four days consumption of a control meal and after four days consumption of a vegetable or synthetic β-carotene supplemented meal.

Variable	Type of test meal											
	Control (N=67)		Broccoli (N=31)		Green peas (N=31)		Whole leaf spinach (N=26)		Chopped spinach (N=26)		β-Carotene (N=28)	
	Baseline[1]	4 Days[2]	4 Days[2]	P	4 Days[2]	P	4 Days[2]	P	4 Days[2]	P	4 Days[2]	P
Folic acid (nmol/L)	20.9 (1.2)	21.4 (0.4)	22.8 (0.6)	0.23	23.1 (0.6)	0.087	23.4 (0.6)	0.048	25.7 (0.7)[4]	0.0001	--	--
β-Carotene (μmol/L)[3]	0.70 (0.09)	0.55 (4.1)	0.71 (5.7)	0.0019	0.69 (6.3)	0.017	0.62 (6.1)	0.46	0.63 (6.7)	0.34	3.40 (5.9)	0.0001
Lutein (μmol/L))	0.18 (0.02)	0.12 (2.0)	0.28 (3.2)	0.0001	0.28 (3.3)	0.0001	0.33 (3.5)	0.0001	0.37 (3.5)[4]	0.0001	0.12 (3.4)	0.70
Vitamin C (μmol/L)	67.2 (1.8)	49.6 (0.82)	63.5 (1.3)	0.0001	55.3 (1.3)	0.0018	66.3 (1.4)	0.0001	66.4 (1.5)	0.0001	49.9 (1.4)	1.00
Total cholesterol (mmol/L)	5.4 (0.14)	5.23 (0.04)	5.23 (0.06)	1.0	5.21 (0.06)	1.0	5.17 (0.06)	0.9	5.23 (0.06)	1.0	5.22 (0.06)	1.0
Triacylglycerol (mmol/L)	1.1 (0.08)	0.93 (0.02)	0.96 (0.03)	1.0	0.89 (0.03)	0.9	0.90 (0.04)	1.0	0.98 (0.04)	0.8	0.97 (0.04)	0.9

[1] Baseline values are expressed as mean (SEM), N=26 (N=17 for β-carotene, see below)

[2] Data are expressed as least square means (SE) (for carotenoids SE is expressed as % of the geometric means); P-values are based on analysis of variance and Dunnett's test for the comparison of the plasma level following consumption of the vegetable or β-carotene supplemented meal with that after the control meal.

[3] Due to a carry over effect in plasma concentrations of β-carotene, part of the results had to be excluded from the statistical evaluation (see results section): Control: N=43; Broccoli: N=23; Green peas: N=19; Whole leaf spinach: N=20; Chopped spinach: N=18; β-Carotene: N=24

[4] Significant difference between chopped spinach and whole leaf spinach (folate: P=0.013; lutein: P=0.010)

-- = Not determined

supplemented meal, were therefore excluded from the statistical evaluation (i.e. using a wash-out period of 38 days). A significant increase in plasma concentration of β-carotene was found after consumption of the β-carotene supplemented meal (5-fold increase (95% CI: 409, 648%)) as well as after the broccoli and green peas supplemented meals (28% (95% CI: 6.4, 55%) and 26% (95% CI: 2.6, 54%), respectively), whereas consumption of the spinach supplemented meals had no significant effect as compared to consumption of the control meal (Table 2). As the intake of β-carotene was different in each of the test meals, we calculated the plasma response per mg dietary β-carotene supplied per serving. Figure 2 shows the efficacy of each supplemented meal to raise the plasma level of β-carotene as compared to consumption of the control meal. Per mg β-carotene, broccoli and green peas induced a similar response in plasma concentration of β-carotene as consumption of the meal supplemented with synthetic β-carotene.

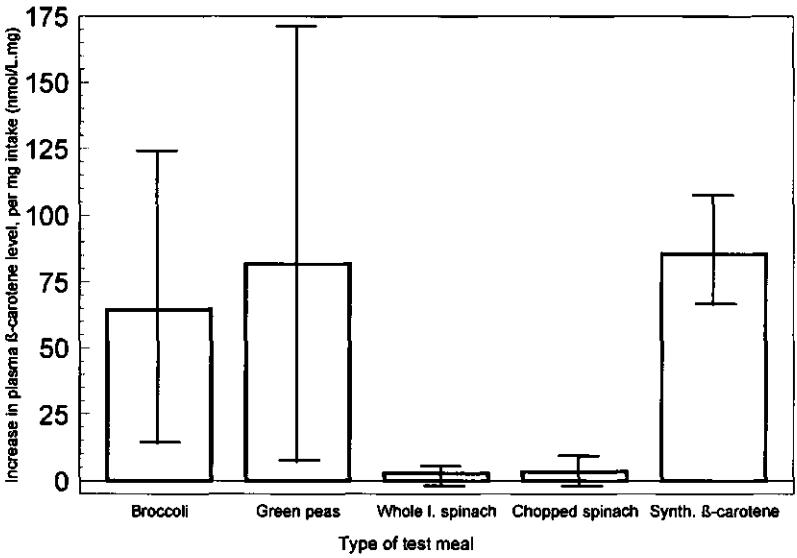

Figure 2 Plasma response of β-carotene after four days of supplementation with vegetables or β-carotene as compared to consumption of a low carotenoid control meal, expressed as increase per mg β-carotene intake from the vegetable or β-carotene supplemented meal (**mean ± 95% confidence interval**) (β-carotene intake/serving: broccoli: 2.43 mg; green peas: 1.72 mg; whole leaf spinach: 24.6 mg; chopped spinach: 23.8 mg; synthetic *all-trans* β-carotene: 33.3 mg).

Consumption of either of the vegetable supplemented meals resulted in a significantly increased plasma level of lutein as compared to consumption of the control meal (1.3-fold (95% CI: 104, 150%) for broccoli, 1.3-fold (95% CI: 108, 155%) for green peas, 1.7-fold (95% CI: 139, 197%) for whole leaf spinach, 2.0-fold (95% CI: 173, 239%) for chopped

spinach) (Table 2). Chopping of spinach enhanced this effect and plasma lutein levels after consumption of chopped spinach were significantly higher than those after whole leaf spinach (difference: 14% (95% CI: 3.7, 25%)). Figure 3 shows for each vegetable type the plasma lutein response per mg lutein present in the vegetable per serving. Per mg of intake, lutein seems more bioavailable from broccoli and green peas than from spinach.

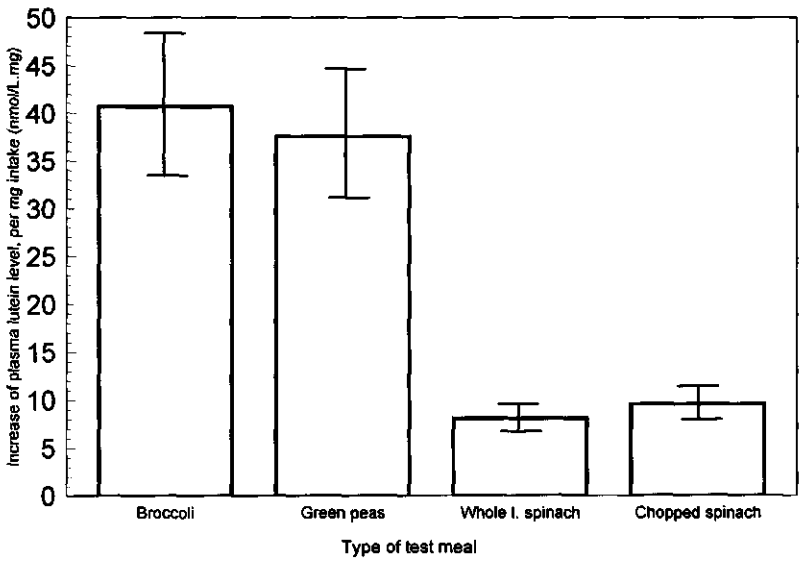

Figure 3 Plasma response of lutein after four days of supplementation with vegetables as compared to consumption of a low carotenoid control meal, expressed as increase per mg lutein intake from the vegetable supplemented meal (mean ± 95% confidence interval) (lutein intake/serving: broccoli: 3.78 mg; green peas: 4.22 mg; whole leaf spinach: 25.0 mg; chopped spinach: 26.0 mg).

As anticipated, because of the virtual absence of lycopene in the experimental meals, none of the vegetables or the β-carotene supplemented meals induced a significant change in lycopene levels in plasma (results not shown). No significant differences were found in serum lipid levels (Table 2) and normalizing plasma concentrations of carotenoids for serum lipids did not alter the results. Also, treatment effects were not significantly different between males and females and between smokers and non-smokers.

Folate levels in plasma were significantly increased by respectively 1.9 µmol/L (95% CI: 0.021, 3.8) and 4.2 µmol/L (95% CI: 2.3, 6.2) after four day consumption of the meal supplemented with whole leaf or chopped spinach as compared to the levels found after

consumption of the control meal (Table 2). In addition, a significant difference (2.3 µmol/L (95% CI: 0.8, 3.8)) was found between chopped and whole leaf spinach, such that the folate concentration was higher after consumption of the chopped spinach supplemented meal. None of the other vegetable supplemented meals increased folate levels in plasma significantly (Table 2). However, when expressed per mg of folate intake, the plasma response of folate following green peas consumption was larger than that following consumption of broccoli or whole leaf spinach (Figure 4).

As compared to four days of consumption of the control meal, all of the vegetable supplemented meals increased plasma concentration of vitamin C significantly, while the synthetic β-carotene supplemented meal had no effect (Table 2).

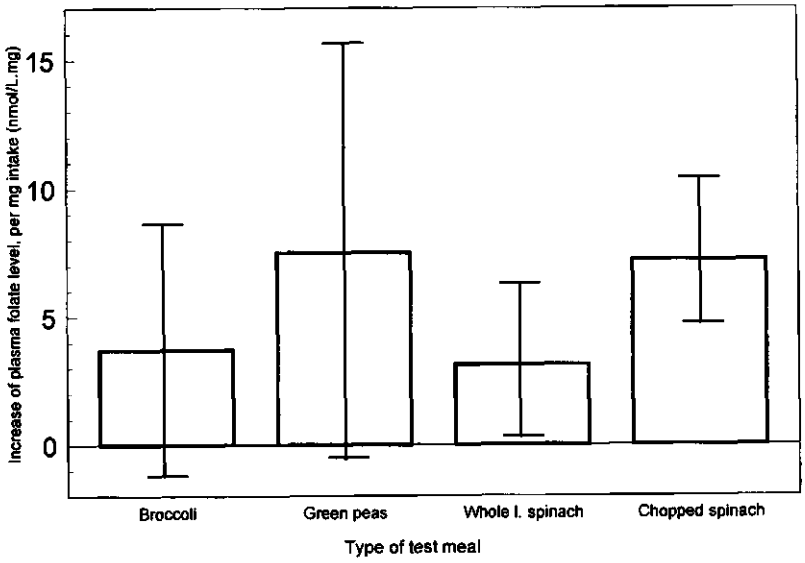

Figure 4 Plasma response of folate after four days of supplementation with vegetables as compared to consumption of a low carotenoid control meal, expressed as increase per mg folate intake from the vegetable supplemented meal (mean ± 95% confidence interval) (folate intake/serving: broccoli: 0.35 mg; green peas: 0.22 mg; whole leaf spinach: 0.60 mg; chopped spinach: 0.59 mg).

DISCUSSION

The present study investigated the effect of four days consumption of different vegetables on the plasma status of carotenoids, folate and vitamin C. Three-hundred g/d of vegetables was chosen as a relatively high, but acceptable amount with a reasonable chance of significant effects. In addition, the changes induced by the vegetables

supplemented meals were compared with those following consumption of a vegetable-free diet. Indeed, the results show that 300 g/d was sufficient to increase plasma levels of lutein and vitamin C for all vegetables in such a short time, whereas plasma levels of β-carotene were increased following consumption of broccoli or green peas but not spinach. The plasma concentration of folate was increased after consumption of spinach, which provided the largest amount of folate in this study. It should be taken into account that the changes induced by vegetable consumption are the sum of the decrease due to exclusion of any vegetables and fruits from the diet and the increase due to consumption of 300 g/d of vegetables. Furthermore, although the changes in plasma levels are used as a measure of relative bioavailability, it is not possible to extrapolate these changes into estimates of actually absorbed carotenoids, folate or vitamin C as the underlying kinetics of plasma and tissue distribution have not been assessed.

It is unlikely that four days is sufficient to reach new steady state plasma concentrations for the investigated micronutrients and antioxidants as previous studies have indicated that this would take approximately 2-6 weeks (Micozzi et al, 1992; Levine et al, 1996; Truswell & Kounnavong, 1997). However, previous studies have shown that differences in plasma levels of carotenoids (Micozzi et al, 1992) and folate (Brouwer et al, personal communication, 1997) after short term supplementation are related to the differences found after reaching a new steady state, although the absolute differences may deviate. Such a short-term protocol is advantageous as compared to a long-term protocol because it is less labour-intensive and compliance to instructions is easier during a short period. Furthermore, in contrast to a single-dose protocol, it is possible to apply more realistic circumstances and investigate the effect of the test meals as part of a normal diet.

Effect of vegetable consumption on plasma carotenoid levels

We found a substantial difference between the various vegetables in their efficacy to increase plasma levels of β-carotene. Despite a ten times higher β-carotene content of spinach, the total increase in plasma levels induced by broccoli or green peas exceded that of spinach. We calculated the plasma response per mg β-carotene intake as a measure of the efficacy (Figure 1), assuming a linear dose-response relation at the dosages provided. When expressed as percentage of the response induced by one mg synthetic β-carotene, the plasma response of β-carotene after consumption of broccoli or green peas was, per mg β-carotene supplied, 74% and 96% of the response following supplementation with β-carotene. Micozzi et al (1992) found that broccoli was 22-24% as effective to increase plasma levels of β-carotene as compared to encapsulated β-

carotene. In their study, the changes in plasma β-carotene levels were compared after six weeks intervention. As indicated above, the absolute percentages found after short term supplementation may deviate from those found in steady state plasma concentration. Both values are however higher than the percentage that we found in the present study for spinach, which was only 3-4% as effective as compared to the β-carotene supplemented meal. De Pee et al (1995) also showed that the relative bioavailability of β-carotene from green leafy vegetables is low. The present data indicates that this observation is specific for green leafy vegetables and should not be extrapolated to all green vegetables.

There may be several explanations for the observed differences in bioavailability of β-carotene from vegetables. The vegetables contained different amounts of β-carotene and the efficiency of β-carotene absorption or conversion into retinol may decrease with increasing intake. The first phenomenon would imply a relatively lower plasma response following spinach consumption as this vegetable contained more β-carotene than broccoli and green peas. However, the increase induced by broccoli or green peas consumption exceded that of spinach, also without correction for the difference in β-carotene intake (Table 2). A less efficient conversion to vitamin A can also not explain our findings as a larger β-carotene response would be expected from a recuded conversion of β-carotene absorbed from spinach.

We used different cooking methods and times and heat treatment is suggested to enhance the bioavailability of carotenoids by loosening their binding to proteins (Erdman et al, 1988). However, spinach was heated longer than broccoli and green peas and this difference does therefore not explain the relatively lower availability of β-carotene from spinach. Another explanation might be the interaction of β-carotene with other carotenoids. It has been suggested that the presence of lutein may decrease the bioavailability of β-carotene (Kostic et al, 1995; Van den Berg et al, 1998). However, the ratio β-carotene/lutein was higher and thus more favourable, for spinach than for broccoli and green peas. Other absorption modifiers, such as fibre (Rock & Swendseid, 1992) may explain the differences. Although the meals were designed to provide the same amount of fibre, the type of fibre may have varied as the ratios hemicellulose:cellulose:lignin are different among broccoli, green peas and spinach (Spiller, 1992). Another factor is probably typical characteristics of the vegetables. In plant leaves, carotenoids are present in chloroplasts and have a function in the process of photosynthesis by photoprotection and light collection (Cogdell & Gardiner, 1993). Little is known about the location of carotenoids in other parts of plants. De Pee et al (1998) recently found that β-carotene from fruits was more effective in increasing plasma levels of β-carotene and retinol than green leafy vegetables. If, like in fruits, carotenoids are present in chromoplasts of

broccoli (the flower) or green peas (the seeds), this may explain the higher β-carotene response as compared to spinach.

For lutein, the difference between spinach and the two other vegetables was less pronounced, although also for this carotenoid, broccoli and green peas were, per mg provided, more effective sources than spinach (Figure 3). Again, one explanation may be a difference in location of the carotenoids in the plant cells. In addition, the higher β-carotene/lutein ratio in spinach as compared to broccoli and green peas may have reduced lutein absorption (Kostic et al, 1995). The efficiency of lutein absorption from spinach may also have been reduced due to the larger amount of lutein present in the test meal.

Vegetable processing may improve the bioavailability of carotenoids, as has been indicated for β-carotene from carrots (Van Zeben & Hendriks, 1948; Hussein & El-Tohamy, 1990; Törrönen et al, 1996) and lycopene from tomatoes (Stahl & Sies, 1992; Gärtner et al, 1997; Porrini et al, 1998). Mechanical homogenization of the spinach before consumption resulted in a significantly higher plasma response of lutein. It did not, however, affect the plasma response of β-carotene. Lutein is more hydrophilic than β-carotene and this may have enhanced the release of lutein from the chloroplasts in the cytosol during disruption of the cell structure. The effect was moderate and the release of lutein was still lower than that from broccoli or green peas (Figure 3). This suggests that a difference in characteristics of the vegetables may be the major determinant.

The differences in relative carotenoid bioavailability between different vegetables are important for the interpretation of health benefits of carotenoid consumption. Most epidemiological studies do not take into account the apparently substantial variation in carotenoid bioavailability from different foods. Giovannucci et al (1995) showed that the association between intake of lycopene-rich foods and risk of prostate cancer varied among different foods. Differences in lycopene bioavailability may have been responsible for this observation (Gärtner et al, 1997). The present study shows that for β-carotene and lutein not only the extent of vegetable processing should be taken into account, but also the type of vegetable ingested. This may be an important reason for the rather low correlation coefficients (ca. 0.50) found in most studies between carotenoid intake and plasma levels (Campbell et al, 1994; Scott et al, 1996; Drewnowski et al, 1997).

Effect of vegetable consumption on plasma folate level

A significant increase in plasma concentration of folate was found after four days consumption of spinach, which contained the highest amount of folate, whereas the increases following broccoli or green peas consumption almost reached significance. These increases indicate that the different vegetables are valuable sources of folate.

When expressed per mg of folate intake the investigated vegetables also differ in their folate bioavailability as green peas induced a larger response per mg folate intake than broccoli and whole leaf spinach (Figure 4). The major part of folate in vegetables is present as polyglutamyl folate, which has to be converted enzymatically into monoglutamyl folate before absorption. This conversion is suggested to be one of the limiting steps during the uptake of folate from natural dietary folate sources (Bailey, 1988). Müller (1993) found that 32% of the total folate content was present as monoglutamyl folate in green peas. The percentage monoglutamyl folate in the spinach used in the present study was ca. 30% (Table 1). This suggests that other characteristics may account for the possible differences in folate bioavailability among the vegetables investigated. Possibly the differences in amount of folate supplied by the different vegetables have interfered with their effectiveness to increase plasma folate levels (Truswell & Kounnavong, 1997).

Disruption of the whole leaf matrix of spinach enhanced the bioavailability of folate. However, the finding that chopped spinach induced a larger plasma folate response than whole leaf spinach can not be attributed to a difference in mono/polyglutamyl folate ratio (Table 1). Our study shows that disruption of the matrix makes folate more accessible for absorption. Apparently, the disruption of the whole leaf spinach matrix in the gastro-intestinal tract is not complete and limits the bioavailability of folate.

Effect of vegetable consumption on plasma vitamin C level

Plasma vitamin C levels were increased after consumption of either of the vegetables and the differences in increases between the vegetables were related to differences in their vitamin C content. Due to saturation of plasma levels at about 80 µmol/L, the major factor predicting the plasma response of vitamin C following supplementation appears to be the prior vitamin C status of the volunteers (Levine et al, 1996). This concentration was not achieved in our study, which may explain the dose-response effect of vitamin C we found. Previous studies have shown that the bioavailability of vitamin C from vegetables is similar to that from a supplement (Mangels et al, 1993b). Therefore, it is not surprising that chopping of whole leaf spinach did not improve the bioavailability of vitamin C. Release of vitamin C from the food matix is apparently not a limiting step during absorption of vitamin C.

In conclusion, the present study shows that the bioavailability of β-carotene and lutein varies substantially among different types of vegetables. Processing of vegetables, such as mechanical homogenisation, can improve the bioavailability of lutein and folate. This

variation in nutrient bioavailability should be considered when the impact of vegetable consumption on health is assessed.

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4

Carotenoid bioavailability from tomatoes processed in different ways. Comparison of carotenoid response in triglyceride-rich lipoprotein fraction of plasma after a single consumption of tomato products and in plasma after four days of consumption

Karin H. van het Hof, Ben C.J. de Boer, Lilian B.M. Tijburg, Bianca R.H.M. Lucius, Itske Zijp, Clive E. West, Joseph G.A.J. Hautvast, Jan A. Weststrate

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ABSTRACT

Tomatoes are the main dietary source of lycopene and the bioavailability of lycopene from tomato paste is higher than that from fresh tomatoes. We investigated systematically the effect of mechanical homogenisation and heating on the bioavailability of carotenoids from canned tomatoes. Further, we compared the carotenoid response in triglyceride-rich lipoproteins (TRLs) after single consumption with the change in fasting plasma carotenoid concentrations after four days.

In a split plot design, 17 volunteers consumed tomatoes which had received minimal additional heating and 16 others consumed extensively heated tomatoes (1 h at 100°C). These tomatoes were not, mildly or severely homogenised. The tomato products were consumed daily (ca. 22 mg/d lycopene) during four days. Eleven participants provided postprandial blood samples on the first day and all gave fasting blood samples on the first day and after four days.

Homogenisation enhanced the lycopene response significantly ($P<0.05$) both in TRLs (mean area under the curves: 54.9, 72.0 and 88.7 nmol/L.h (SE 11.0) for not, mildly and severely homogenised tomatoes, respectively) and in plasma (mean changes: 0.19, 0.22 and 0.23 µmol/L (SE 0.009), respectively). Additional heating also enhanced the lycopene responses in TRLs and plasma, but the differences lacked significance. Similar effects as those for lycopene were found for β-carotene.

We conclude that the intactness of the cellular matrix of tomatoes determines the bioavailability of carotenoids and that matrix disruption by mechanical homogenisation and/or heat treatment enhances the bioavailability. The carotenoid response in plasma after four days intervention can be used to compare the bioavailability of carotenoids from different foods.

INTRODUCTION

High lycopene and tomato intakes have been found to be associated with a reduced risk of prostate cancer (Giovannucci et al, 1995). Interestingly, the association was strongest for tomato paste, which was the tomato product that showed the best correlation with serum lycopene levels. Differences in lycopene bioavailability among different tomato products may explain this latter observation. This is supported by the finding that the lycopene response in plasma or triglyceride-rich lipoproteins (TRLs) is higher after consumption of tomato paste than after consumption of fresh tomatoes (Gärtner et al, 1997, Porrini et al, 1998). The production of tomato paste from fresh tomatoes involves homogenisation and heat treatment. Previous studies have shown that a combination of these treatments enhances the bioavailability of carotenoids from vegetables (Van Zeben & Hendriks, 1948; Rock et al, 1998). However, up until now, the contribution of each of these processes has not been clear. As heat treatment can have a deleterious effect on the micronutrient content of vegetables, it is important to determine systematically the separate effect of homogenisation and heat treatment on the bioavailability of lycopene and β-carotene from tomatoes and the interaction between these two processes.

Various approaches have been used to investigate carotenoid bioavailability. Most studies have used carotenoid responses in plasma following 3 to 6 weeks supplementation as a measure of carotenoid bioavailability (e.g., Van Zeben et al, 1948; Micozzi et al, 1992; Törrönen et al, 1996; Rock et al, 1998). Recently, the carotenoid response in the TRL-fraction of plasma was suggested as a valuable model, as TRLs contain newly absorbed carotenoids (Van Vliet, 1996). A disadvantage of such a single dose protocol is the large number of blood samples that need to be drawn. However, longer term supplementation is also a burden on the volunteers and labour intensive. Therefore, we determined whether a short term intervention period would be a suitable approach to estimate carotenoid bioavailability from tomatoes processed in different ways. We compared the postprandial carotenoid response in the TRL fraction of plasma after single consumption with the carotenoid response in fasting plasma after four days consumption of the same tomato products. The tomato products had received different degrees of homogenisation and heat treatment to determine systematically the effect of these processing conditions on the bioavailability of carotenoids from canned tomatoes.

MATERIALS AND METHODS

Volunteers

Participants were recruited via advertisements in the weekly periodical of the laboratory, in local newspapers and on local radio and television stations. Volunteers were employees of Unilever Research Vlaardingen or inhabitants of the surrounding area. They were eligible when they met the following criteria: aged between 18 and 70 y; Body Mass Index between 19 and 30 kg/m^2; no excessive use of alcohol (males ≤ 28 units/wk, females ≤ 21 units/wk); intensive sporting activities ≤ 10 h/wk; smoking ≤ 5 cigarettes, cigars or pipes/d. Volunteers were apparently healthy, as assessed by questionnaire, and they did not use any medications except oral contraceptives or reported gastrointestinal disturbances. Their body weight was stable for at least the previous two months and they had not used dietary supplements (e.g. vitamins or minerals) during the month prior to the start of the study. Volunteers were exclused if they were adhering to a medically prescribed, slimming or vegetarian diet or if they were pregnant or lactating. Volunteers who were selected for participation in the postprandial measurement of the carotenoid response in TRLs, had normal fasting serum triglyceride concentrations (i.e. < 3.0 mmol/L) and normal whole blood haemoglobin concentrations (i.e. ≥ 8.0 mmol/L for males; ≥ 7.5 mmol/L for females).

Volunteers received information on the background and design of the study and they gave their informed consent before participation. The protocol of the study was approved by the Medical Ethical Committee of Unilever Nederland BV.

Study design

The study had a split-plot design with three degrees of homogenisation of the tomatoes (none, i.e. whole tomatoes; mild, i.e. blended tomatoes; severe, i.e. tomatoes blended under high pressure) and two levels of additional heat treatment (minimal, i.e. only heating before serving; extensive, i.e. one hour at 100° C before serving). The effect of homogenisation was tested within persons during three experimental periods and that of additional heat treatment between persons. Thirty-six volunteers were divided in two groups, stratified for gender.

The tomato products were served as part of a pasta meal at lunch time on four consecutive days and no other vegetables, fruits or tomato products were consumed during these days. These four day experimental periods were separated by a wash-out period of ten days during which volunteers returned to their habitual diet with a restriction for their consumption of vegetables, fruits and tomato products. Fasting blood samples

were taken before and at the end of each experimental period for analysis of plasma carotenoid concentrations and plasma total antioxidant activity.

A sub-group of 12 participants, 6 for each heat treatment group, participated in measurements of the postprandial carotenoid response in the TRLs following consumption of the tomato products. On the first day of each of the three experimental periods, they consumed the tomato-pasta meals in the morning, instead of at lunch time. After a fasting blood sample had been drawn, they consumed the meal within 30 min. Additional blood samples were taken 2, 3, 4.5, 6 and 8 h after start of consumption of the experimental meal for measurement of carotenoids, retinyl palmitate, triglycerides and total antioxidant activity in the TRL-fraction of plasma. A low fat, carotenoid-free lunch was provided after the 4.5 h blood sample.

We calculated that, based on previous studies, the number of volunteers included in the four days study would be sufficient to show a 35% difference in plasma lycopene response in the parallel comparison of the heat treatment effects. The choice of the number of volunteers for the measurement of the carotenoid response in TRLs was based on practical considerations as no data were available on the within person variation of lycopene responses in TRLs.

Tomato products, experimental meals and background diet

Starting material for the tomato products were peeled and canned whole tomatoes (*Lycopersicum esculentum*) (Lipton, Stockton, CA, USA). In the factory, these tomatoes had received a 55 min heat treatment at 100°C after canning to ensure microbiological safety. After reaching 100°C in the centre of the cans, they had been cooled to about 50°C within one hour. We did not use fresh tomatoes because the physical and chemical properties of fresh tomatoes may change during storage and that might have interfered with the effects of processing which we assessed in a cross-over study design over three weeks.

Mildly homogenised tomatoes were prepared on the experimental days by blending for 2 min (Ultra-Turrax T50, IKA-Labortechnik, Staufen, Germany). Severe homogenisation included blending for 2.5 min using the same blender, followed by processing in a high pressure homogeniser at 200 bar (APV-GAULIN homogeniser, type Lab 60-10 TBS, APV-GAULIN GmbH, Lübeck, Germany). Severely homogenised tomatoes were prepared in one batch before the start of the experiment and batches of 2.8-3 kg were stored at -20°C until use on the experimental days.

Additional heat treatment was given on the experimental days, just before serving. The minimally heated tomatoes were heated to approximately 80°C and served immediately

thereafter, whereas the extensively heated tomatoes were first boiled for one hour and then served.

Tomato products were served with macaroni, ham and a white sauce and a dessert of custard. The total energy content and macronutrient composition of the meals were the same for all volunteers and the total energy content was about 70% of an average Dutch main meal (Voorlichtingsbureau voor de Voeding, 1993) to ensure that everyone would be able to consume the complete meal. Participants consumed the experimental meals under supervision in the kitchen of our laboratory. They were free to choose their own foods during the rest of each experimental day. However, they were instructed not to consume products high in carotenoids, such as vegetables, fruits, fruit juices and tomato products, or high in vitamin A, such as liver products. Compliance was assessed by questionnaire.

During the postprandial studies, volunteers consumed no other foods until 8 h after consumption of the experimental meal, except for a standard lunch of low fat and carotenoid and vitamin A-free products 4.5 h after start of consumption of the experimental meal. This lunch was consumed under supervision as well.

Seven days before the start of each experimental period, volunteers received the same instructions with respect to consumption of carotenoid and vitamin A-rich products as during the experimental periods. We supplied them with frozen ready to eat meals (Iglo, Veldhoven, the Netherlands), which were low in carotenoids, to replace their hot main meal.

Analysis of tomato products and experimental meals

Duplicate portions of the complete experimental meals (3 samples per type), as consumed, were analysed for macronutrient and fibre content. The meals were formulated to provide 157 µg of preformed retinol (Holland et al, 1991). The carotenoid content of the tomato products (9 samples per type) was determined by HPLC on a ET 200/4 nucleosil 100-5CN column (Machery & Nagel, Duren, Germany). After extensive extraction (five times, until the last extract was colourless) with tetrahydrofurane/methanol (1:1, v/v), ethyl-β-apo-8'-carotenoate was added as internal standard. β-Carotene and lycopene were separated by gradient elution with n-heptane/4% iso-propanol 0-3 min 97.5:2.5 (v/v); 3-15 min change from 97:2.5 to 50:50 (v/v); 16-30 min 97.5:2.5 (v/v) at a flow rate of 1.0 mL/min and a column temperature of 20°C. The eluent was monitored by UV-Vis detection at 450 nm for β-carotene and at 470 nm for lycopene. In this system, α-carotene coelutes with β-carotene. As tomatoes contain no α-carotene (Khachik et al, 1992), the HPLC response is considered as β-carotene. Table 1 shows the macronutrient

and fibre content of the experimental meals and the carotenoid content of the tomato products.

Table 1 Composition of experimental meals, expressed per serving (mean (SD)).

	Additional heat treatment					
	Minimal			Extensive		
	Degree of homogenisation			Degree of homogenisation		
	None	Mild	Severe	None	Mild	Severe
Fat (g)[1]	22.8 (0.7)	22.8 (1.2)	22.6 (0.7)	23.0 (0.2)	23.1 (1.5)	22.7 (0.5)
Protein (g)[1]	27.0 (0.8)	27.9 (1.8)	28.1 (2.3)	27.3 (0.6)	27.4 (1.1)	27.3 (0.4)
Carbohydrate (g)[1]	59.0 (2.8)	58.3 (0.1)	59.7 (0.5)	58.6 (1.6)	58.8 (2.4)	58.4 (0.6)
Fibre (g)[1]	5.8 (0.5)	6.0 (0.5)	5.8 (0.8)	6.1 (0.6)	5.4 (0.3)	5.5 (0.5)
Lycopene (mg)[2]	20.9 (1.8)	21.3 (1.1)	21.2 (1.5)	23.4 (3.2)	20.7 (1.1)	20.5(1.2)
β-Carotene (mg)[2]	1.1 (0.16)	1.1 (0.17)	1.0 (0.14)	1.2 (0.19)	0.99 (0.17)	0.93 (0.11)

[1] Based on analysis of duplicate samples of experimental meals, as consumed (n=3)

[2] Based on analysis of duplicate samples of tomato products, as consumed (n=9)

The integrity of the cells in the differently processed tomato products was assessed by light microscopy. After addition of sodium azide (0.001%) for preservation of the samples, the samples were examined as wet preparations. Figure 1 shows photographs of representative samples of the homogenised tomato products. The majority of the cells were intact in the minimally heated tomatoes, whereas after severe homogenisation, and/or extensive heat treatment, many of the cell walls were no longer intact. Heat treatment had a less destructive effect than severe homogenisation, as more cells were still intact after extensive heat treatment only.

In vitro release of lycopene from tomato products

Mild extraction of lycopene was used as an *in vitro* test to estimate the *in vivo* release of this carotenoid from tomatoes. Ca. 1 g of tomato product was mixed thoroughly by using a vortex during 1 min with 3 mL n-heptane. After 30 min at room temperature in the dark, the sample was mixed again on a vortex for 1 min and subsequently centrifuged at 1000 x *g* for 3 min. The supernatant was collected, the residue was mixed with n-heptane for 1 min on a vortex and immediately thereafter centrifuged. Lycopene content of the extracts was determined by spectophotometry (UV 2101 PC, Shimadzu Corporation, Japan) at 470 nm, using an extinction coefficient of 3450.

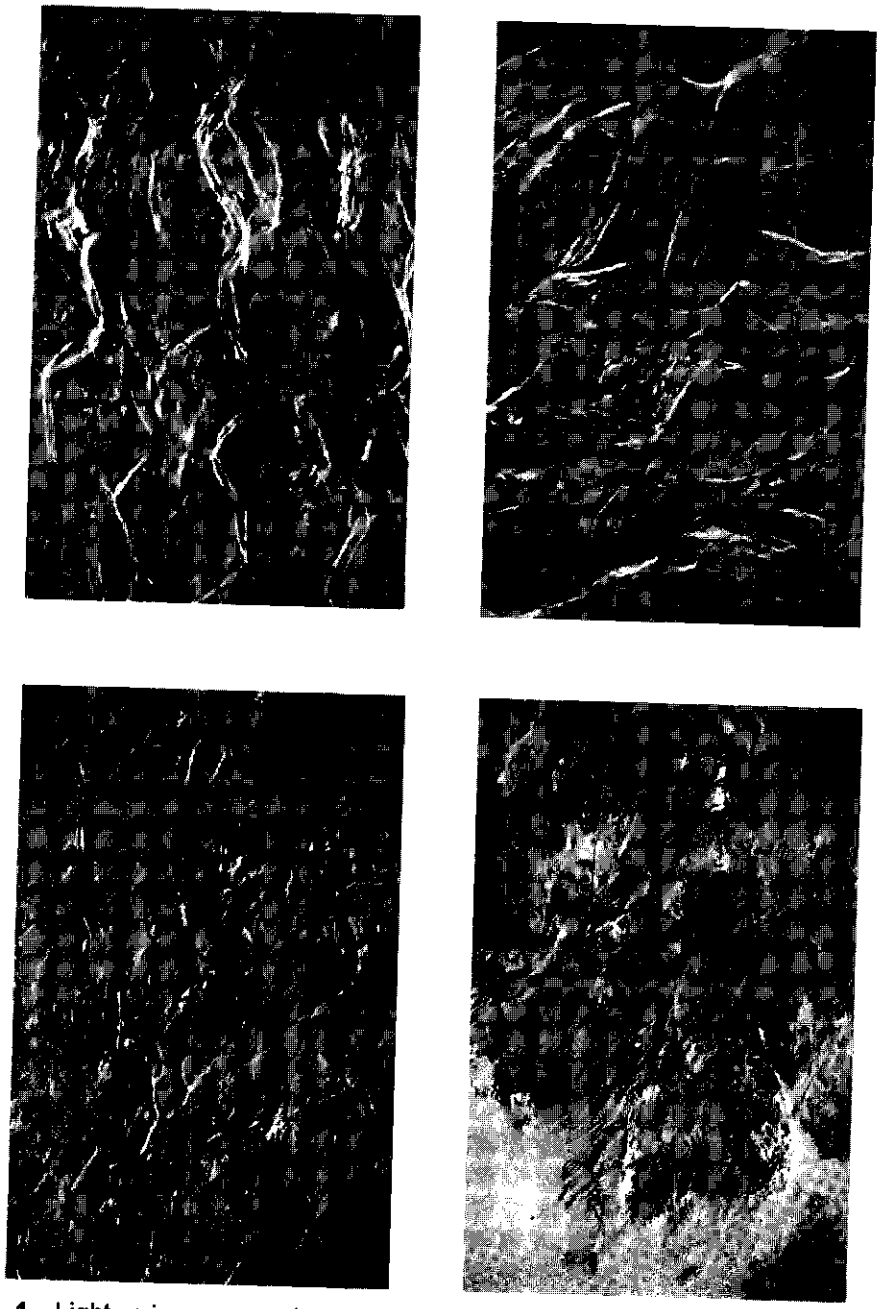

Figure 1. Light microscopy pictures of tomato products (bar=100 μm). A: mildly homogenised, minimally heated tomatoes; B: mildly homogenised, extensively heated tomatoes; C: severely homogenised, minimally heated tomatoes; D: severely homogenised, extensively heated tomatoes.

Collection and analysis of blood samples

Blood samples were obtained from fasting subjects before and after each of the four days experimental periods. In a subgroup, additional samples were collected for measurement of the carotenoid response in TRLs after consumption of the experimental meal. Blood samples were collected into sodium EDTA-coated tubes and plasma was separated by centrifugation at 1500 x g for 10 min at room temperature. Plasma samples were stored at -80°C until analysis or isolation of the TRL-fraction. Isolation of the TRL-fraction was performed as described by Van Vliet et al (1995). After thawing, 3.5 mL plasma was overlayered with 8 mL NaCl (0.9%, w/v; d=1.004) The samples were centrifuged for 1 h at 150,000 x g at 20°C in a Beckman L8-60M ultracentrifuge (Beckman Instruments, Pao Alto, CA, USA). The TRL-containing fraction was then removed (2.1 mL) and stored at -80°C until further analysis within 6 wk.

Prior to analysis of the carotenoid and retinyl palmitate content, the TRL-fraction was extracted with n-heptane/ether (3:1, v/v). Carotenoid concentrations in plasma and carotenoid and retinyl palmitate concentrations in TRLs were determined by HPLC on a nucleosil 100 5CN column (Machery & Nagel, Duren, Germany) with n-heptane as mobile phase at a flow rate of 0.7 mL/min. Ethyl-apo-8-carotenoate was used as internal standard. UV-Vis detection was used to monitor concentrations of lycopene at 470 nm, β-carotene at 450 nm and retinyl palmitate at 325 nm. Intra assay variation was 4.5% for lycopene and 3.9% for β-carotene in plasma.

The ferric reducing ability of plasma (FRAP) and the TRL-fraction was determined as a measure of total antioxidant activity, as described by Benzie & Strain (1996). Total cholesterol and triacylglycerol concentrations in plasma and triglyceride concentrations in the TRL-fraction were assessed by using commercially available colorimetric test kits (plasma cholesterol: CHOD-PAP, Boehringer, Mannheim, Germany; plasma triacylglycerol: GPO-PAP (Roche, Basel, Switzerland)/GPO-Trinder (Sigma, St. Louis, MO, USA); triglycerides in TRL: Unimate 5 TRIG kit (Roche, Basel, Switzerland)).

Statistical evaluation

The data obtained were normally distributed as determined by visual evaluation. The data were analysed using analysis of variance with persons (within heat treatment) and period as blocks and heat treatment (minimal or extensive) and degree of homogenisation (none, mild or severe) as factors in a split plot model. The significance of the treatment effects on the *in vitro* release of lycopene from the tomato products was determined by analysis of variance with heat treatment and degree of homogenisation as factors. If a significant interaction was found between the effect of heat treatment and degree of homogenisation, their effects were tested separately. For the carotenoid responses in the

TRL-fraction, the statistical analysis was performed with and without the triglyceride response included as covariable. The responses considered were changes in plasma concentrations and changes from baseline in TRL concentrations at each time point as well as the area under the curve of the concentrations in TRLs. Treatment effects on carotenoid responses in plasma and TRLs and antioxidant activity in TRLs were tested one-sided, based on the hypothesis that a more extensive heat treatment or more severe homogenisation would induce larger responses. For all other parameters (i.e., plasma lipid concentrations, antioxidant activity of plasma, triglyceride and retinyl palmitate concentrations in TRLs and *in vitro* lycopene release), differences among treatments were tested two-sided. P-values below 0.05 were considered significant.

Results are expressed as mean (SD) for descriptive variables and as least square mean (SE) for all other variables.

RESULTS

Volunteers

Thirty-three participants completed the study. One subject dropped out before the start of the study for unknown reasons and two others did not complete the study due to illness. Eleven volunteers participated in the measurement of the TRL- responses. Table 2 shows the descriptive characteristics of the participants. Only two of them smoked (≤ 5 cigarettes/d).

Table 2. Baseline characteristics of participants of the postprandial and four days study, per treatment group (mean (SD)).

	Postprandial study		Four days study	
	Additional heat treatment		Additional heat treatment	
	Minimal	Extensive	Minimal	Extensive
Males/Females (n)	2/4	3/2	6/11	7/9
Age (y)	40 (13)	39 (11)	44 (12)	44 (15)
Quetelet Index (kg/m^2)	23.8 (3.0)	24.4 (2.8)	24.2 (3.5)	25.1 (3.3)
Plasma Lycopene (µmol/L)	0.41 (0.23)	0.30 (0.070)	0.31 (0.17)	0.27 (0.096)
Plasma β-Carotene (µmol/L)	0.54 (0.27)	0.37 (0.16)	0.52 (0.27)	0.46 (0.25)
Plasma antioxidant activity (mmol/L)	0.97 (0.12)	1.1 (0.1)	1.0 (0.1)	1.0 (0.2)
Plasma total cholesterol (mmol/L)	4.9 (0.7)	5.3 (0.9)	4.9 (0.7)	5.4 (1.3)
Plasma triacylglycerol (mmol/L)	0.72 (0.2)	1.3 (0.5)	0.97 (0.5)	1.2 (0.5)

Note: There were no significant differences between heat treatment groups, except for the difference in baseline fasting plasma triacylglycerol concentration between the heat treatment groups of the postprandial study ($P<0.05$).

In vitro release of lycopene from tomato products

A significant difference was found in lycopene extractability from the different tomato products. Figure 2 shows the percentage of lycopene extracted after mild heptane treatment as compared to the contents measured after extensive extraction (see Table 1). Both additional heat treatment and homogenisation enhanced the release of lycopene during mild extraction. However, as 100% release was already reached by minimally heated, severely homogenised tomatoes, additional heat treatment did not further enhance the release of lycopene from the severely homogenised tomato products (Figure 2).

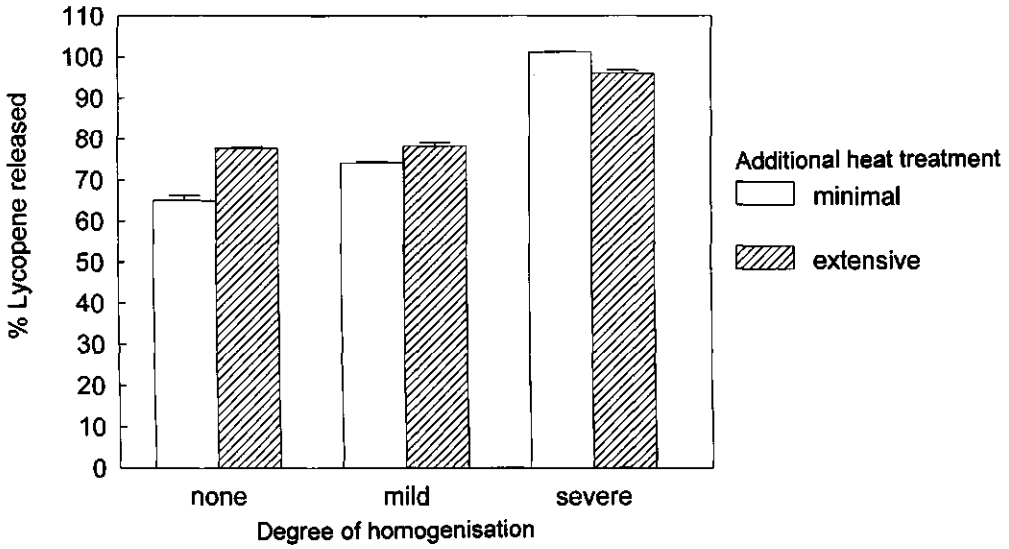

Figure 2. *In vitro* release of carotenoids from tomato products (lycopene content measured after mild extraction, expressed as proportion of the content measured after extensive extraction (n=3, mean ±SE)). The proportion of lyocene released was significantly different among all degrees of homogenisation for the minimally heated tomatoes and between severely homogenised and not or mildly homogenised for the extensively heated tomatoes ($P<0.05$). Additional heat treatment significantly enhanced the release of lycopene only for the unhomogenised and mildly homogenised tomatoes ($P<0.05$).

Carotenoid response in TRLs after a single consumption of tomato products

Figures 3 and 4 show the triglyceride, lycopene and total antioxidant activity response in the TRL-fraction of plasma after consumption of the experimental meals. The triglyceride responses were not significantly different among the treatments (Tables 3 and 4) and

inclusion of the triglyceride response as covariable did not change the treatment effects on carotenoid responses in TRLs. Therefore, here we consider the treatment effects on the carotenoid responses without correction for triglyceride concentrations.

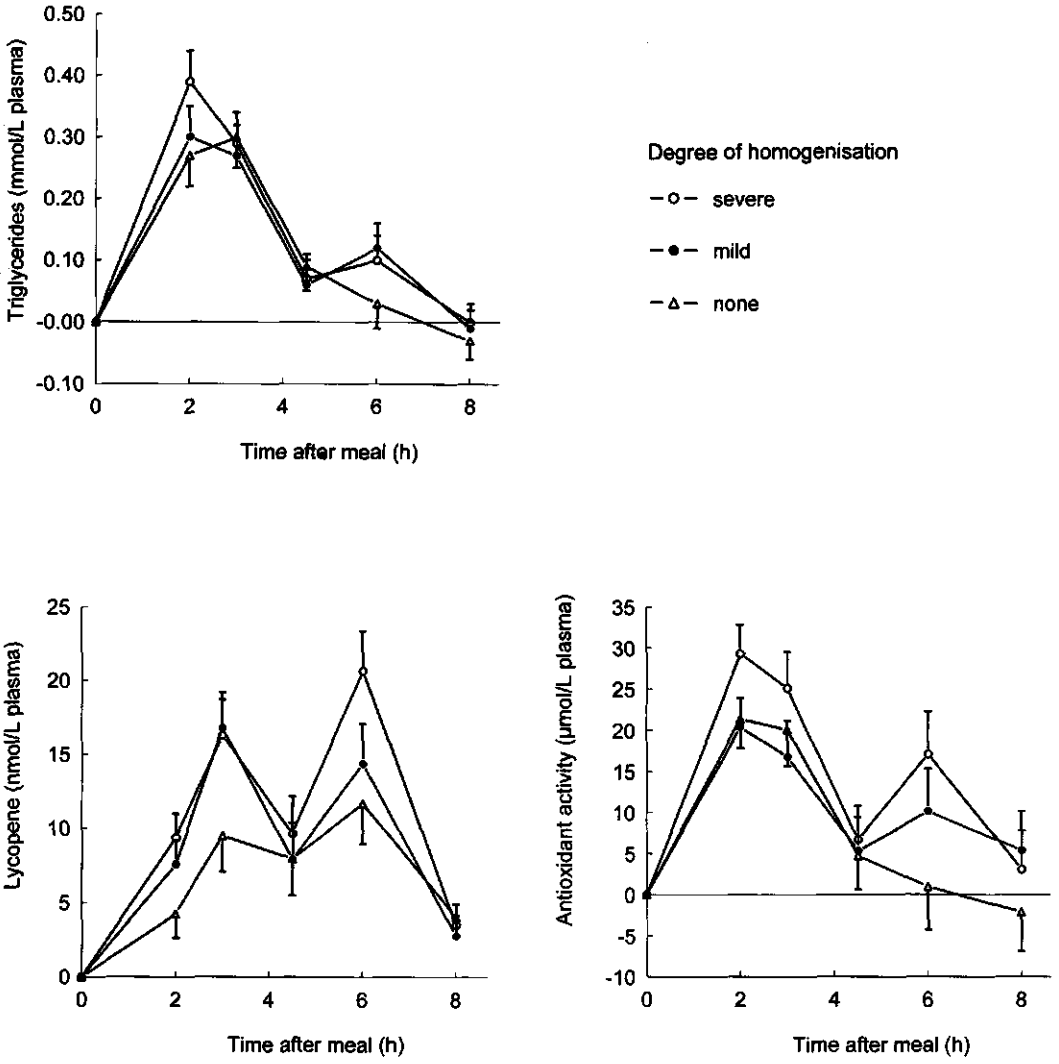

Figure 3. Mean changes (±SE) in concentrations of triglycerides and lycopene and antioxidant activity of triglyceride-rich lipoprotein fraction of plasma after single consumption of tomato products (21-23 mg lycopene/serving). Effect of homogenisation (n=33).

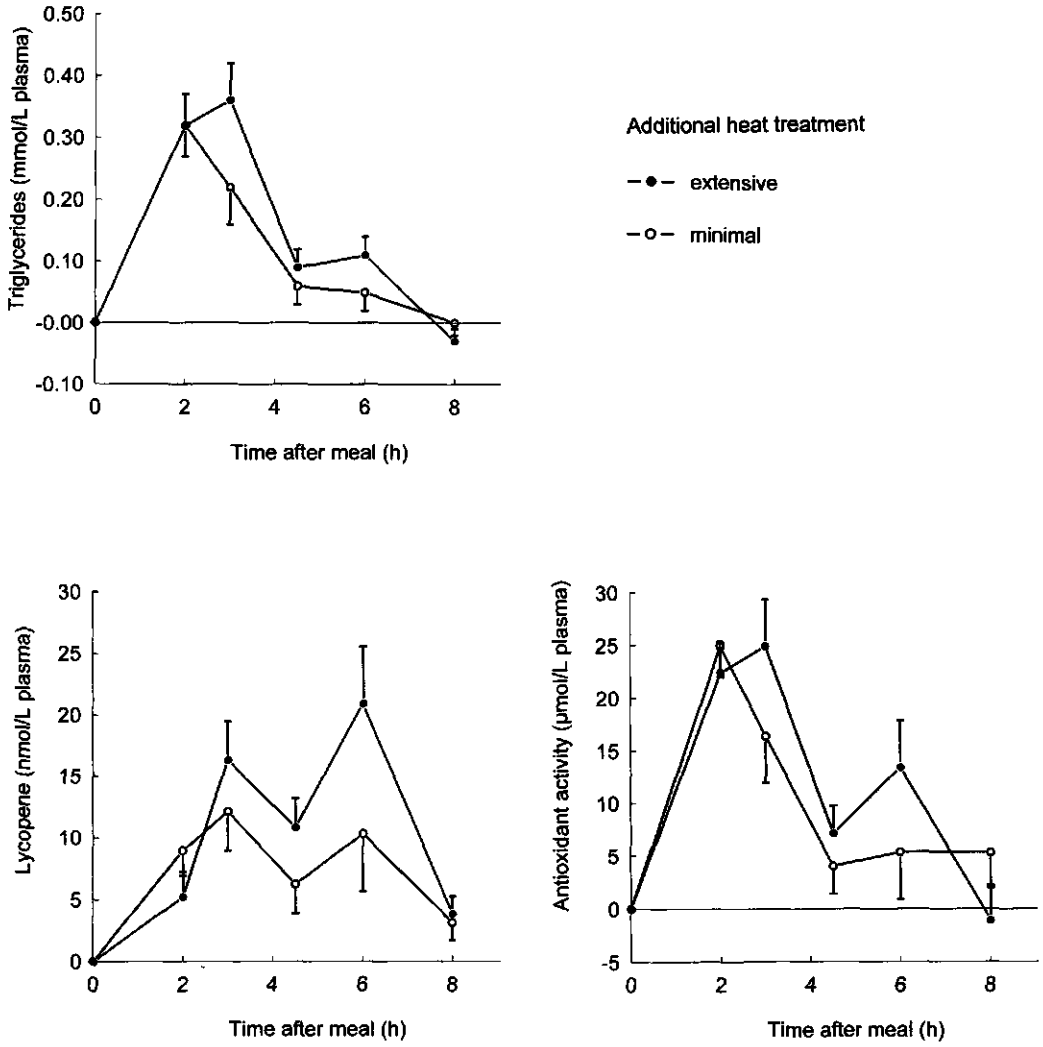

Figure 4. Mean changes (±SE) in concentrations of triglycerides and lycopene and antioxidant activity of triglyceride-rich lipoprotein fraction of plasma after single consumption of tomato products (21-23 mg lycopene/serving). Effect of heat treatment (n=16 or 17).

The area under the curves of the TRL-responses are shown in Tables 3 and 4 for the effects of homogenisation and heat treatment, respectively. In line with the enhanced *in vitro* release of lycopene, we found a significantly larger area under the curve of the TRL-

response of both lycopene and β-carotene after consumption of the severely homogenised tomatoes than after consumption of the whole tomatoes (Table 3). Additional heat treatment also induced a larger TRL-response of lycopene and β-carotene. However, the difference in areas under the curves was not significant (Table 4). The change in ferric reducing ability of the TRLs reflected the changes in carotenoid concentration. Homogenisation significantly improved the antioxidant activity of TRLs (Table 3), whereas the effect of additional heat treatment was not significant (Table 4). The responses of retinyl palmitate (results not shown) were not significantly different among the different treatments.

Table 3. Effect of homogenisation of tomatoes on the response of carotenoids and antioxidant activity in TRLs[1] and plasma after a single or four days consumption of the tomato products, respectively (mean (SE)).

		Degree of homogenisation		
		None	Mild	Severe
Postprandial TRL response[2]				
Lycopene (nmol/L.h)		54.9 (11.0)[a]	72.2 (11.0)[ab]	88.7 (11.0)[b]
β-Carotene (nmol/L.h)		2.51 (6.5)[a]	16.6 (6.5)[ab]	23.0 (6.5)[b]
Triglycerides (mmol/L.h)		0.94 (0.19)	1.09 (0.19)	1.23 (0.19)
Antioxidant activity (μmol/L.h)[3]		63.9 (16.5)[a]	82.6 (16.5)[ab]	118 (16.5)[b]
Four days plasma response[4]				
Lycopene (μmol/L)	Before	0.33 (0.009)	0.31 (0.009)	0.32 (0.009)
	Change	0.19 (0.009)[a]	0.22 (0.009)[b]	0.23 (0.009)[b]
Antioxidant activity (mmol/L)[3]	Before	1.02 (0.011)	1.02 (0.01)	1.04 (0.01)
	Change	0.072 (0.013)	0.046 (0.01)	0.039 (0.01)

[1] Triglyceride-rich lipoprotein fraction of plasma

[2] Area under the concentration curve, change from baseline

[3] Antioxidant activity determined as ferric reducing ability (see Methods section)

[4] Plasma response of β-carotene is shown in Table 5 because there was a significant interaction between homogenisation and heat treatment

[a,b] Values in the same row with different superscripts are significantly different ($P<0.05$)

Carotenoid response in plasma after four days consumption of tomato products

The effect of homogenisation and heat treatment on the change in fasting plasma lycopene concentrations following four days consumption of the differently processed tomatoes, were similar to those found for the lycopene response in TRLs after single consumption. We observed significantly larger plasma lycopene responses with

increasing degree of homogenisation (Table 3). Further, additional heat treatment tended to increase the plasma lycopene response, but this effect lacked significance (Table 4).

Table 4. Effect of heat treatment of tomatoes on the response of carotenoids and antioxidant activity in TRLs[1] and plasma after a single or four days consumption of the tomato products, respectively (mean (SE)).

		Additional heat treatment	
		Minimal	Extensive
Postprandial TRL response[2]			
Lycopene (nmol/L.h)		59.0 (16.6)	84.9 (16.6)
β-Carotene (nmol/L.h)		7.53 (8.8)	20.5 (8.8)
Triglycerides (mmol/L.h)		0.94 (0.21)	1.23 (0.21)
Antioxidant activity (μmol/L.h)[3]		78.6 (15.4)	97.9 (15.4)
Four days plasma response[4]			
Lycopene (μmol/L)	Before	0.32 (0.03)	0.32 (0.03)
	Change	0.20 (0.02)	0.22 (0.02)
Antioxidant activity (mmol/L)[3]	Before	1.02 (0.03)	1.04 (0.03)
	Change	0.054 (0.01)	0.051 (0.01)

[1] Triglyceride-rich lipoprotein fraction of plasma

[2] Area under the concentration curve, change from baseline

[3] Antioxidant activity determined as ferric reducing ability (see Methods section)

[4] Plasma response of β-carotene is shown in Table 5 because there was a significant interaction of degree between homogenisation and heat treatment

Note: none of the responses were significantly different between minimal and extensive additional heat treatment (α=0.05)

With respect to the plasma response of β-carotene, there was a significant interaction between the effects of the degree of homogenisation and those of additional heat treatment. Homogenisation enhanced the plasma response of β-carotene only if the tomatoes were not given additional heat treatment (Table 5). Furthermore, a significant effect of additional heat treatment was found only for whole tomatoes whereas it did not enhance the plasma β-carotene response induced by homogenised tomatoes.

The ferric reducing ability of plasma was not affected by any of the treatments and no significant differences were found among the different degrees of homogenisation or heat treatment (Tables 3 and 4). Total cholesterol and triacylglycerol concentrations in plasma remained unchanged (results not shown).

Table 5. Effect of homogenisation and heat treatment of tomatoes on β-carotene response in plasma after four days consumption of the the tomato products (mean (SE)).

	Additional heat treatment		Degree of homogenisation		
			None	Mild	Severe
β-Carotene (μmol/L)	Minimal	Before	0.52 (0.015)	0.50 (0.015)	0.49 (0.015)
		Change	0.009 (0.010)^{a,x}	0.044 (0.010)^{b}	0.035 (0.010)^{b}
	Extensive	Before	0.43 (0.007)^{a}	0.44 (0.007)^{ab}	0.45 (0.007)^{b}
		Change	0.043 (0.009)^{y}	0.040 (0.009)	0.022 (0.009)

^{a,b} Values in the same row with different superscripts are significantly different ($P<0.05$)

^{x,y} Values in the same column with different superscripts are significantly different ($P<0.05$)

DISCUSSION

The present study is the first to demonstrate the separate effects of homogenisation and heating on the bioavailability of carotenoids from tomatoes and the interaction between these treatments. It appears that both processes effectively enhance the carotenoid bioavailability from tomatoes, although the effect of heating was not always significant. The study design we used had less power to show significant effects of heat treatment as compared to that of homogenisation. The processing effects were apparent both in the carotenoid response in TRLs after single consumption and in fasting plasma after four days consumption of the tomato products. This indicates that, qualitatively, the plasma response of carotenoids after short term supplementation is a good model to compare the bioavailability of carotenoids from different foods. Disruption of the tomato matrix also enhanced the extractability of carotenoids from the tomatoes. The *in vitro* release of carotenoids from a food matrix can thus be used as a screening method for the effects of processing on carotenoid bioavailability.

Comparison of carotenoid responses in TRLs and plasma

The use of the postprandial carotenoid response in the TRL-fraction of plasma as a measure of carotenoid absorption is based on the fact that carotenoids present in chylomicrons originate directly from the enterocytes. In contrast, in experiments with repeated carotenoid consumption, changes in fasting plasma carotenoid concentrations are not only affected by the amount of carotenoids absorbed, but also by carotenoid metabolism, tissue distribution and transfer of carotenoids between lipoproteins in blood. As mentioned above, we found similar differences in lycopene responses in plasma and TRLs among the different treatments. This indicates that both approaches can be used to determine the effects of tomato processing on lycopene bioavailability. However, with

respect to β-carotene, the results were slightly different as a significant interaction was found between the degree of homogenisation and additional heat treatment for the response of β-carotene in plasma but not in TRLs. This suggests that the postprandial TRL-response is a less sensitive measure of β-carotene bioavailability than the plasma response after four days. It should be noted however, that the β-carotene intake was low (i.e., 1 mg, see Table 1).

Four days is not sufficient to achieve a new steady state in plasma carotenoid concentrations, as this takes at least three weeks (Micozzi et al, 1992). The differences we observed after four days will relate to those observed after longer intervention although quantitatively, they may differ. However, a short intervention period has several advantages. It is less labour-intensive and compliance to instructions is probably better during a short period. In contrast to a single dose design, it is possible to apply more realistic conditions and investigate the effect of the test meals as part of a normal diet. This latter aspect may be particularly relevant.

We found a second peak in carotenoid concentrations following the consumption of a second carotenoid-free, low fat meal after the 4.5 h blood sample. This was also reported by Borel et al (1998), who used a comparable design. In studies where only one meal was supplied, only one peak at 4-7 h was found (Van Vliet et al, 1995; O'Neill & Turnham, 1998; Van den Berg & Van Vliet, 1998). Borel et al (1998) explained their finding by the larger β-carotene content of their test meal as compared to the earlier studies (i.e., 120 mg vs ca. 15 mg respectively). In the present study, we supplied 21-23 mg lycopene (Table 1). The amount of fat in our test meals was less than that used by others (i.e., 23 g vs 43-50 g). Consequently, the triglyceride content of the TRLs was lower. This may have reduced our initial carotenoid responses due to a limited capacity of chylomicrons to take up carotenoids, as suggested by Borel et al (1996). Part of the initially absorbed carotenoids may have remained in the enterocytes and entered the blood stream following the uptake of the second meal.

Effects of homogenisation and heat treatment

The extent to which the food matrix, in which carotenoids are incorporated, is intact is an important determinant of carotenoid bioavailability as indicated by the present results. The first steps of carotenoid absorption include disruption of the food matrix, mechanically and by digestive enzymes, and the subsequent release of the carotenoids from this matrix and from protein complexes (Britton, 1995). Homogenisation and heat treatment disrupt cell membranes, whereas heat treatment has been suggested to disrupt further the protein-carotenoid complexes (Erdman et al, 1988). Previous studies have shown that homogenisation or a combination of homogenisation and heat treatment enhances

carotenoid bioavailability from vegetables in humans (Van Zeben & Hendriks, 1948; Törrönen et al, 1996; Gärtner et al, 1997; Porrini et al, 1998; Rock et al, 1998). Our results indicate that the difference in carotenoid bioavailability from tomato paste versus fresh tomatoes (Gärtner et al, 1997; Porrini et al, 1998) can be explained by alterations of the cellular matrix of tomatoes, due to the effect of both homogenisation and heat treatment.

The canned tomatoes used were heated for 55 min at 100°C during manufacturing. Further processing was still able to enhance the bioavailability of carotenoids from these tomatoes significantly, despite the processing steps which had previously been applied. As anticipated, homogenisation under high pressure was more effective in increasing carotenoid bioavailability than homogenisation under normal pressure. As shown in Figure 1, in part of the cells, the cell walls were still intact after mild homogenisation, whereas high pressure treatment destroyed the majority of the cell structures. The release of carotenoids from intact cells is thus indeed a limiting factor for carotenoid uptake. This confirms data from Van Zeben & Hendriks (1948), who found that homogenisation of cooked carrots enhanced the bioavailability of β-carotene as measured by changes in plasma concentrations.

Published data on the effects of heat treatment on the bioavailability of carotenoids from vegetables are not consistent. Previous studies in experimental animals showed no significant differences in β-carotene responses following consumption of raw or cooked homogenised carrots, unheated or heated carrot juice or carrot chromoplasts (Poor et al, 1993; Zhou et al, 1996). As the cellular matrix of the carrots had been disrupted, these results may indicate that heat treatment is only effective for unhomogenised carrots. That would be in line with our observation of an enhanced plasma β-carotene response by additional heating of whole tomatoes but not homogenised tomatoes. Also, the amount of *cis*-isomers of β-carotene may have interfered with these findings as heat treatment can induce in *cis-trans* isomerisation in tomatoes (Nguyen & Schwartz, 1998). The responsiveness of plasma concentrations of all-*trans* β-carotene to supplementation is larger than that of *cis*-isomers (Gaziano et al, 1995; Tamai et al, 1995). However, although we could not quantify the presence of *cis*-isomers of β-carotene, visual inspection of the chromatograms from reversed phase HPLC analysis indicated that the amount did not change with processing (data not shown).

The increases in carotenoid concentrations in TRLs were accompanied by an increase in the total antioxidant activity of the TRL-fraction of plasma (Figures 3C and 4C). In contrast, no significant differences were found in fasting plasma after four days. Uric acid accounts for 60% of the variation in the ferric reducing ability of plasma (Benzie & Strain, 1996; Cao & Prior, 1998). As TRLs do not contain uric acid or other endogenous

antioxidants, the sensitivity of this fraction of plasma to show differences in antioxidant uptake may be larger than that of plasma itself. The implication of such a postprandial increase in ferric reducing ability for the overall antioxidant status remains however to be established.

Conclusion

In conclusion, the cellular matrix of tomatoes, which can be disrupted by mechanical homogenisation and/or heat treatment, determines the bioavailability of carotenoids. The carotenoid response in plasma after four days consumption can be used to compare the bioavailability of carotenoids from different foods. This conclusion is based on the finding that the treatment effects shown were similar to those found in postprandial changes of carotenoids in TRLs.

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Antioxidant fortified margarine increases the antioxidant status

Karin H. van het Hof, Lilian B.M. Tijburg, Hilde S.M. de Boer, Sheila A. Wiseman, Jan A. Weststrate

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ABSTRACT

Objective: To assess the effect of supplementation with an antioxidant fortified margarine on the body's antioxidant status and on parameters of oxidative damage to lipids.

Design: Single blind, placebo controlled trial, two treatment groups balanced for sex, age and Quetelet Index.

Setting: Unilever Research Laboratorium, The Netherlands.

Subjects: Thirty-one healthy adult volunteers accomplished the study. Volunteers were recruited among inhabitants of the surrounding area of the research laboratory.

Interventions: Volunteers consumed during four weeks either 15 g/d of an antioxidant fortified margarine (providing 121 mg vitamin C, 31 mg vitamin E, 2.7 mg αcarotene and 5.3 mg β-carotene) or an ordinary margarine. Fasting blood samples were taken before and at the end of the study.

Results: Consumption of the antioxidant fortified margarine significantly increased the levels of the supplied antioxidants in plasma and LDL as compared to the changes found after consumption of the control margarine, with the largest increases found in LDL levels of α-carotene (15.5-fold increase, 95% CI: 8.4-fold to 27.8-fold) and β-carotene (4.3-fold increase, 95% CI: 2.2-fold to 7.9-fold). This increased antioxidant status in the antioxidant fortified margarine group resulted in a significantly increased total antioxidant activity of LDL and resistance of LDL to oxidation (lag time and rate of oxidation) as compared to baseline but not in comparison to the changes found in the control group.

Conclusion: Consumption of moderate doses of vitamin E, vitamin C, α-carotene and β-carotene, supplied in a full-fat margarine and consumed as part of a normal diet, effectively increases the blood levels of these antioxidants.

INTRODUCTION

Elevated plasma concentrations of low-density lipoprotein (LDL) cholesterol are associated with accelerated atherogenesis and increased risk of cardiovascular disease (Lipid Research Clinics Program, 1984). There is increasing evidence from experimental studies that in addition to the level of LDL-cholesterol, also the susceptibility of the LDL particle to oxidative modification may play a crucial role in atherogenesis (Steinberg et al, 1989; Witztum & Steinberg, 1991; Esterbauer & Ramos, 1995). As a consequence it is plausible that antioxidants act in preventing atherosclerosis. Several epidemiological studies have indeed indicated that a higher intake or increased plasma levels of the antioxidants vitamin E, vitamin C and β-carotene are associated with a reduced risk of cardiovascular disease (Stampfer et al, 1993; Rimm et al, 1993; Kardinaal et al, 1993; Riemersma et al, 1991; Gale et al, 1991; Knekt et al, 1994; Kushi et al, 1996) and a recent intervention study demonstrated that α-tocopherol supplementation reduced the rate of non-fatal myocardial infarction in patients with coronary atherosclerosis (Stephens et al, 1996). In addition, dietary intake of flavonoids, antioxidants which mainly occur in vegetables, tea and wine, have recently also been shown to be inversely associated with mortality from coronary heart disease (Hertog et al, 1993; Knekt et al, 1996).

The evidence from animal studies that antioxidants possess anti-atherogenic properties has been summarized by Daughterty and Roselaar (1995). In addition to animal studies, the effect of high doses of vitamin E and β-carotene on the resistance of LDL to oxidation has been investigated extensively in humans in short-term intervention studies. Supplementation with 100 to 1600 mg vitamin E per day has consistently been shown to increase the resistance of LDL to oxidation ex vivo (Dieber-Rotheneder et al, 1991; Jialal & Grundy, 1992; Princen et al, 1992; Reaven et al, 1993; Jialal et al, 1995; Jialal & Grundy, 1993) and lower doses also seem to be effective. Princen et al (1995) showed that vitamin E intake at a dose as low as 25 mg/d (2.5 times the recommended daily allowance, (Food and nutrition board, 1989)) significantly increased the duration of the lag phase before LDL oxidation ex vivo. On the other hand, Jialal et al (1995) found that only vitamin E doses of 400 mg/d and higher were effective. Supplementation with high doses of β-carotene or vitamin C does not seem to increase the resistance of LDL to oxidation (Reaven et al, 1993).

Atherogenesis is a multifactorial disease and combinations of antioxidants may be particularly effective in reducing the risk of cardiovascular disease. Synergistic effects may occur between different antioxidants because of differences in lipophilicity and thus the site of free radical scavenging activity may be in the LDL particle or intracellularly. Synergism has been found between vitamin C and E due to regeneration of vitamin E by vitamin C

(Sato et al, 1990). However, there is currently little knowledge on the beneficial effects of combinations of antioxidants and in particular little is known of the effects of lower doses of mixtures of these compounds that can be achieved by dietary means.

Therefore, the effect of consumption of moderate doses of a combination of antioxidants, incorporated into a habitually used food product, on the body's antioxidant status and on parameters of oxidative damage to lipids was assessed. A full-fat margarine, fortified with 31 mg/d vitamin E, 121 mg/d vitamin C, 2.7 mg/d α-carotene and 5.3 mg/d β-carotene, was used as vehicle to supply the antioxidants. These daily amounts equalled two to three times the recommended daily intake for vitamin E and C (Food and Nutrition Board, 1989) and about two times the average intake of β-carotene (Yong et al, 1994) and are in line with optimal intake levels recently proposed by Lachance (1996).

SUBJECTS AND METHODS

Volunteers

Non-smoking subjects, healthy as assessed by a medical investigation, between 18 and 65 years old, were eligible for participation in the study. The volunteers did not use vitamin C, E, carotenoid, selenium or zinc supplements. Subjects were not using a medically prescribed diet or slimming regime and had been weight-stable for at least one month prior to the start of the study. Females were not pregnant or lactating. Volunteers were recruited from employees of our laboratory and from inhabitants of Vlaardingen and the surrounding district. Subjects gave their written informed consent prior to participation.

Study design

After a two week run-in period during which time subjects consumed 15 g/d of an ordinary (control) margarine, 32 volunteers either continued to consume the control margarine or started to consume 15 g/d of an antioxidant fortified margarine, providing 121 mg vitamin C, 31 mg vitamin E, 2.7 mg α-carotene and 5.3 mg β-carotene, during the next four weeks. Volunteers were allocated to either of the two treatment groups, taking into account an equal distribution of sex, age and Quetelet Index. Due to the difference in colour of the margarines, the study was only blind to the analysts analysing the blood samples. The effects of consumption of the antioxidant fortified margarine on plasma and LDL antioxidant status, LDL-resistance to oxidation and markers of in vivo oxidative damage to lipids were evaluated. In addition, we calculated the contribution of individual antioxidants to parameters of the total antioxidant activity of plasma and LDL. For this part of the study, we used the initial values measured in plasma and LDL of volunteers from the present study

and of volunteers from a study which was executed at the same time (Van het Hof et al, 1997). The protocol of the study was approved by our local Research Ethics Committee.

Based on data of Abbey et al (1993a), we calculated that a number of 16 volunteers per group would be sufficient to detect a difference of 13% in changes in resistance of LDL to copper-induced oxidation (lag phase) between both groups (α=0.05; β=0.10).

Experimental margarines

The full-fat margarines were prepared in our laboratory. Ascorbic acid (L(+)-ascorbic acid, Merck, Darmstadt, Germany), α-tocopherol (L-α-tocopherol, Merck, Darmstadt, Germany) and natural palm carotene, containing α-carotene and β-carotene (30% suspension in corn oil, Quest International, Naarden, The Netherlands), were added to the antioxidant fortified margarine. Fat content (82%) and fatty acid composition were similar in the control and antioxidant fortified margarine. Volunteers consumed 15 g/d of either the control or antioxidant fortified margarine. This daily amount of the antioxidant fortified margarine contained 121 mg ascorbic acid, 31.1 mg α-tocopherol, 2.7 mg α-carotene and 5.3 mg β-carotene. The control margarine contained no ascorbic acid, no carotenoids and 0.97 mg α-tocopherol/15 g margarine. The daily amounts of vitamins C and E provided by the fortified margarine equalled two and three times the RDA of vitamin C and vitamin E respectively (Food and nutrition board, 1989) whereas the daily amount of β-carotene was similar to the amount advised by the National Cancer Institute in the United States (Lachance, 1988).

Lifestyle and dietary intake

Volunteers were requested to maintain their habitual lifestyle during the entire experiment. They were instructed to replace their habitual margarine on bread products by the supplied margarine and not to use the margarine for baking or frying. Changes in dietary intake of vitamin C, E and carotenoids were assessed by a self-completed food frequency questionnaire at the start and at the end of the study. The food frequency questionnaire was validated for carotenoid intake (Weststrate & Van het Hof, 1995) and vitamin C intake (Pearson's coefficient of correlation between estimated intake and plasma levels r=0.60, P=0.0001).

Plasma and LDL-analyses

Fasting venous blood samples were taken once before and once at the end of the experimental period and collected into Na_2EDTA coated tubes for plasma and into plain tubes for serum preparation. Plasma and serum were prepared by low-speed centrifugation. Plasma used for the analysis of ascorbic acid was treated with trichloro-acetic acid (150 mmol/L final concentration) immediately after preparation to prevent degradation of ascorbic

acid. Plasma samples for LDL isolation were stabilized with 6 g/L sucrose, immediately frozen in liquid nitrogen and stored at -70 0C. It has been established that freezing LDL in this way does not influence oxidation parameters (Ramos et al, 1995). LDL was isolated from thawed plasma by discontinuous density gradient ultracentrifugation for 24 hours at 4 0C (Redgrave et al, 1975). The LDL fraction (density 1.019 - 1.063 g/ml) for the analysis of LDL α-tocopherol, α-carotene, β-carotene and lipid hydroperoxides was stored at -70 0C. Immediately following LDL isolation, LDL protein content was determined using bovine serum albumin (Fraction V, Sigma, St. Louis, Mo., USA) as the standard (Markwell et al, 1978) and in vitro oxidation studies were carried out thereafter. Serum was stored at -20 0C for the analysis of lipids.

α-Tocopherol, α-carotene and b-carotene were determined in plasma and LDL. After addition of appropriate internal standards (α-tocopherol-acetate and ethyl-apo-β'-carotenoate), extraction was performed with n-heptane and n-heptane/diethyl ether (1:1, v/v). Following evaporation of the solvents, the residue was dissolved in eluent and α-tocopherol, α-carotene and b-carotene were separated by reversed phase HPLC using 201 TP54 Vydac column (Separations Group, Hesperia CA, USA) and methanol/tetrahydrofuran/ammonium acetate solution (1 g/L) (95:5:2, v/v) as mobile phase at a flow rate of 0.8 ml/min and a column temperature of 13 0C (Craft et al, 1992; Epler et al, 1992). Vitamin C concentration in trichloroacetic acid-treated plasma was determined fluorimetrically as ascorbic acid plus dehydroascorbate as described by Vuilleumier and Keck (1989). Albumin, bilirubin, uric acid, total cholesterol, LDL-cholesterol, HDL-cholesterol and triacylglycerol were determined in serum using enzymatic colorimetric methods (Boehringer Mannheim, Germany). Total antioxidant activity in plasma (Miller et al, 1993) and in LDL (Miller et al, 1995) were determined by the ABTS/ferryl myoglobin assay using reagents of Randox Diagnositics (County Antrim, UK). Results are expressed relative to Trolox (Randox Diagnositics, County Antrim, UK). The inter assay variation was 3% for plasma total antioxidant activity. Malondialdehyde was determined in plasma after complexation with diethylthiobarbituric acid. The malondialdehyde-thiobarbituric acid adduct was separated from interfering substances by HPLC essentially as described previously (Wong et al, 1987) and the adduct was detected fluorimetrically using an excitation wavelength of 538 nm and emission wavelength of 554 nm. LDL lipid hydroperoxides were determined as previously described (Wieland et al, 1992). The resistance of LDL to oxidation was determined by monitoring the production of conjugated dienes during copper-mediated LDL oxidation as described previously (Princen et al, 1992). To minimize effects due to between assay variability, LDL samples from the same subject, before and after the treatment period, were analyzed in the same run. The inter assay variation was 10% for the lag phase and 4% for the maximum rate of oxidation.

Statistical analyses

Data were analyzed using SAS computer software (Statistical Analysis System Institute, 1987). Plasma and LDL concentrations of α-carotene and β-carotene were log-transformed to minimize correlation between mean values and standard errors within the treatment groups. All comparisons were made at the two-sided 0.05 significance level. Data was analyzed using ANOVA with treatment, time, subject (within treatment) and the treatment*time interaction as effects. Means are presented with standard error (CVM if log-transformation was applied). Treatment*time effects are presented as difference between the time changes within the treatment groups with 95% confidence interval. In case of log-transformation these values are expressed as percentage of the time change within the control group. Plasma concentrations of vitamin E, α- and β-carotene, normalized for serum cholesterol and triacylglycerol levels, were also analyzed for differences in changes between the treatments (vitamin E, α- or β-carotene concentration/(cholesterol + triacylglycerol concentration).

Stepwise multiple linear regression analysis was used to analyze the relation between plasma total antioxidant activity as dependent variable and plasma concentrations of vitamin C, vitamin E, α-carotene, β-carotene, albumin, bilirubin and uric acid as independent variables as well as the relation between total antioxidant activity of LDL and LDL concentrations of vitamin E, α-carotene and β-carotene. Stepwise multiple linear regression analysis was also used to analyze the relation between the resistance of LDL to oxidation (duration of lag phase and maximum rate of oxidation) and the concentrations of the above mentioned, lipophilic antioxidants in LDL. The total number of observations included was 49 to 60, depending on the number of missing data for each parameter.

RESULTS

Fifteen men and sixteen women completed the study. The volunteer who withdrew from participation in the study did so for medical reasons not related to the experimental treatment. Table 1 shows descriptive statistics of the volunteers who completed the study. The volunteers ranged in age from 18 to 57 years.

Compliance to treatment

Treatment compliance of the volunteers, as monitored by assessment of unused tubs of margarine, was very good. More than 99.5% of the provided margarine was consumed in both treatment groups. The average intake of margarine was 15.2 g/d (SEM: 0.03).

Changes in dietary intake of vitamin E, vitamin C and carotenoids were calculated without the antioxidants supplied by the fortified margarine. The estimated intake of most of

the antioxidants investigated showed a significant decrease during the study in both treatment groups (Table 1). However, no significant differences in changes between the treatment groups were found.

Table 1 Mean[1] volunteers' descriptive characteristics, baseline serum lipid levels and dietary intake of antioxidants in control and antioxidant fortified margarine group.

		Control group (n=16)	Antioxidant fortified group (n=15)	Difference (95% CI)[2]	P value
Male/female sex (n)		8/8	7/8		
Age (y)		38 (12)	36 (12)		
Body weight (kg)		74.15 (9.97)	73.80 (11.44)		
Body height (m)		1.75 (0.10)	1.75 (0.10)		
Quetelet Index (kg/m^2)		24.19 (2.39)	24.12 (2.92)		
Total cholesterol (mmol/L)		5.39 (0.82)	5.34 (0.78)		
HDL-cholesterol (mmol/L)		1.48 (0.43)	1.44 (0.39)		
LDL-cholesterol (mmol/L)		2.46 (0.51)	2.42 (0.67)		
Triacylglycerol (mmol/L)		0.96 (0.36)	1.00 (0.36)		
Antioxidant intake[3]					
Vitamin E (mg/d)	Wk -4 to 0	11.0 (0.44)	12.5 (0.45)		
	Wk 0 to 4	9.8 (0.44)	10.4 (0.45)[4]	-0.93 (-2.8, 0.90)	0.31
Carotenoids (mg/d)	Wk -4 to 0	7.4 (0.55)	6.5 (0.57)		
	Wk 0 to 4	5.5 (0.55)[4]	4.8 (0.57)[4]	0.13 (-2.2, 2.4)	0.91
Vitamin C (mg/d)	Wk -4 to 0	88.8 (4.7)	102.0 (4.9)		
	Wk 0 to 4	71.4 (4.7)[4]	78.3 (4.9)[4]	-6.2 (-26, 13)	0.53

[1] Data are expressed as mean (SD) for descriptive characteristics and serum lipid levels and as mean (SE) for antioxidant intake

[2] Calculated as differences between changes from week -4 to 0 (4 wk before start of study) to week 0 to 4 (during study) in the two groups

[3] Estimated by use of a food frequency questionnaire

[4] Value in week -4 to 0 is significantly different from value in week 0 to 4 ($P<0.05$)

Note: none of the descriptive characteristics or serum lipid levels were significantly different between the two groups

Plasma and LDL antioxidant status

Table 2 shows the effect of consumption of antioxidant fortified margarine on plasma concentrations of vitamin E, α-carotene, β-carotene, vitamin C, albumin and uric acid and the total antioxidant activity of plasma. LDL concentrations of vitamin E, α-carotene and β-carotene and total antioxidant activity of LDL are shown in Table 3. Daily consumption of the antioxidant fortified margarine significantly increased plasma levels of vitamin E by 16% as compared to the consumption of a control margarine. In LDL vitamin E concentration increased by 22%, but this increase was not significantly different from that found in the control group. Intake of the antioxidant fortified margarine resulted in a 15% increase in the

plasma vitamin C level. The effect of consumption of the antioxidant fortified margarine was most striking with regard to blood levels of α-carotene and β-carotene. As compared to the control group, α-carotene levels showed a 14.5-fold increase in plasma and a 15.5-fold increase in LDL. β-Carotene concentration was increased 3.4-fold in plasma and 4.3-fold in LDL.

Table 2 Mean[1] *plasma concentrations of antioxidants and total antioxidant activity in* plasma before and after four weeks of consumption of antioxidant fortified or control margarine.

		Control group (n=16)	Antioxidant fortified group (n=15)	Difference (95% CI)[2]	P value
Vitamin E (μmol/L)	Wk 0	18.72 (0.36)	20.06 (0.37)		
	Wk 4	19.31 (0.36)	23.81 (0.37)[4]	3.16 (1.65, 4.66)	0.0002
α-Carotene (μmol/L)[1]	Wk 0	0.040 (8.5%)	0.038 (8.8%)		
	Wk 4	0.046 (8.5%)	0.66 (8.8%)[4]	1452% (989, 2113)	0.0001
β-Carotene (μmol/L)[1]	Wk 0	0.13 (5.9%)	0.12 (6.1%)		
	Wk 4	0.14 (5.9%)	0.57 (6.1%)[4]	343% (246, 466)	0.0001
Vitamin C (μmol/L)	Wk 0	56.93 (1.89)	67.55 (1.95)		
	Wk 4	56.61 (1.89)	77.43 (1.95)[4]	10.20 (2.3, 18.1)	0.01
Albumin (g/L)	Wk 0	50.74 (0.41)	50.17 (0.43)		
	Wk 4	50.48 (0.41)	49.49 (0.43)	-0.41 (-2.13, 1.31)	0.63
Uric acid (mmol/L)	Wk 0	0.283 (0.0054)	0.295 (0.0056)		
	Wk 4	0.291(0.0054)	0.298 (0.0056)	-0.0055 (-0.028, 0.017)	0.63
Total antioxidant activity (mmol/L)[3]	Wk 0	1.51 (0.01)	1.51 (0.01)		
	Wk 4	1.47 (0.01)[4]	1.47 (0.01)[4]	0.0 -0.04, 0.04)	1.00

[1] Data are expressed as mean (SE) except for α-carotene and β-carotene. Data for α-carotene and β-carotene are presented as mean (CVM) because these concentrations were log-transformed before statistical analysis

[2] Calculated as differences between changes from week 0 to week 4 in the two groups

[3] Expressed relative to Trolox (Randox Diagnositics, County Antrim, UK)

[4] Value at week 4 is significantly different from value at week 0 ($P<0.05$)

Because the mean change in serum levels of total cholesterol and triacylglycerol were not significantly different between the treatment groups (data not shown), the differences between the treatment groups in changes of plasma concentration of vitamin E, α- and β-carotene normalized for serum lipids (data not shown) were comparable to the differences between changes of plasma concentrations as such.

Plasma concentrations of the endogenous components with antioxidant activity, albumin and uric acid, were not affected by the treatments (Table 2).

Consumption of the antioxidant fortified margarine did not significantly alter the total antioxidant activity of plasma as determined by the ABTS/ferryl myoglobin assay (Table 2). The total antioxidant activity of LDL from the antioxidant-treated subjects was significantly higher at the end of the study as compared to the initial value, but this change was not significantly different from the change in the subjects who had consumed control margarine (Table 3).

Malondialdehyde and lipid hydroperoxides were measured as markers of oxidative stress in vivo as these compounds are breakdown products of lipid peroxidation. Supplementation with the margarine fortified with vitamin C, vitamin E, α-carotene and β-carotene did not affect the concentration of malondialdehyde in plasma (difference between changes (95% CI): -0.09 mmol/L (-0.34, 0.16)) nor that of lipid hydroperoxides in LDL (difference between changes (95% CI): 0.12 nmol/L LDL (-1.23, 1.46)).

The resistance of LDL to copper-induced oxidation was assessed by measurement of the lag phase before oxidation and the maximum rate of oxidation. The lag phase before oxidation was significantly increased after four weeks of consumption of antioxidant fortified margarine as compared to baseline values. No significant difference in changes was found however between the two treatment groups. The maximum rate of oxidation decreased significantly when the antioxidant fortified margarine was consumed, and the difference of this change compared to the change found in the control group was of borderline significance.

To establish the contribution of individual antioxidants to the total antioxidant activity, we determined the regression line between the status of vitamin C, vitamin E, α-carotene, β-carotene, albumin (g/L), bilirubin (mmol/L) and uric acid (mmol/L) in plasma and LDL and the total antioxidant activity of plasma and LDL. For this purpose, we combined the initial values from the present study (n=31) (Tables 2 and 3) and from a study executed in our laboratory in the same period (n=29) (Van het Hof et al, 1997). Total antioxidant activity of plasma was mainly determined by albumin and uric acid with an explained variance of 0.19 (equation: total antioxidant activity of plasma = 0.969 + 0.00904 $[albumin]_{plasma}$ + 0.000244 $[uric\ acid]_{plasma}$). No correlation was found between plasma total antioxidant activity and plasma concentrations of vitamin E, α-carotene, β-carotene or vitamin C.

The regression line of the total antioxidant activity of LDL appeared to be affected by each of the supplied antioxidants (equation: total antioxidant activity of LDL = -0.651 + 2.541 $[\alpha\text{-tocopherol}]_{LDL}$ + 0.0108 $[\beta\text{-carotene}]_{LDL}$ - 0.0555 $[\alpha\text{-carotene}]_{LDL}$, R^2=0.85). Vitamin E was the main contributor to the total antioxidant activity of LDL, explaining 79% of the variance. A higher level of α-carotene in LDL, however, was associated with a decreased total antioxidant activity of LDL.

Table 3 Mean[1] concentrations in LDL of antioxidants, total antioxidant activity of LDL and resistance of LDL to oxidation before and after four weeks of consumption of antioxidant fortified or control margarine.

		Control group (n=16)	Antioxidant fortified group (n=15)	Difference (95% CI)[2]	P value
Vitamin E (µmol/L)	Wk 0	6.15 (0.39)	6.18 (0.41)		
	Wk 4	6.16 (0.39)	7.57 (0.41)[4]	1.38 (-0.26, 3.02)	0.10
α-Carotene (µmol/L)[1]	Wk 0	0.024 (13.2%)	0.020 (14.2%)		
	Wk 4	0.026 (13.2%)	0.352 (14.2%)[4]	1547% (842, 2780)	0.0001
β-Carotene (µmol/L)[1]	Wk 0	0.109 (12.1%)	0.073 (12.9%)		
	Wk 4	0.101 (12.1%)	0.362 (12.9%)[4]	434% (220, 790)	0.0001
Total antioxidant activity (mmol/L)[3]	Wk 0	15.15 (0.73)	17.82 (0.76)		
	Wk 4	15.63 (0.73)	21.00 (0.76)[4]	2.70 (-0.38, 5.78)	0.08
Lag phase of oxidation (min)	Wk 0	99 (2)	101 (2)		
	Wk 4	101 (2)	109 (2)[4]	5 (-4, 14))	0.28
Maximum rate of oxidation	Wk 0	17.65 (0.41)	18.51 (0.44)		
(nmoles dienes/min/mg LDL protein)	Wk 4	18.04 (0.41)	17.24 (0.44)[4]	-1.66 (-3.41, 0.095)	0.06

[1] Data are expressed as mean (SE) except for α-carotene and β-carotene. Data for α-carotene and β-carotene are presented as mean (CVM) because these concentrations were log-transformed before statistical analysis

[2] Calculated as differences between changes from week 0 to week 4 in the two groups

[3] Expressed relative to Trolox (Randox Diagnositics, County Antrim, UK)

[4] Value at week 4 is significantly different from value at week 0 ($P<0.05$)

In addition, the relation between the LDL antioxidant levels and the resistance of LDL to oxidation was assessed by stepwise multiple linear regression analysis. The concentration of α-carotene in LDL appeared to be inversely associated with the lag phase before oxidation of LDL, with an explained variance of only 9% (equation: lag phase = 104.2 - 0.15 $[\alpha\text{-carotene}]_{LDL}$). The LDL levels of vitamin E, α-carotene and β-carotene were not significantly associated with the maximum rate of oxidation.

DISCUSSION

In the present study the effects of 4-weeks supplementation with an antioxidant fortified margarine on the body's antioxidant status and LDL oxidizability were investigated. The fortified margarine contained a mixture of different fat-soluble (vitamin E, α-carotene, β-carotene) and water-soluble antioxidants (vitamin C) in amounts equivalent to two to three times the recommended intakes or average daily intake. The results show that daily consumption of this antioxidant fortified margarine induces significant increases in both plasma and LDL levels of the supplied antioxidants.

Supplementation with 121 mg/d of vitamin C resulted in a 15% increase of plasma levels of vitamin C. Due to a tight regulation of the vitamin C status, the response to supplementation with vitamin C is dependent on the vitamin C status of the body before supplementation (Levine et al, 1996). Depending on the vitamin C status of the volunteers and the size of the doses (600 mg/d to 2 g/d), increases in plasma concentrations of 25% to 275% have been found previously (Reaven et al, 1993; Jialal & Grundy, 1993; Abbey et al, 1993b Salonen et al, 1991). Final concentrations reported in these studies were between 75 and 85 mmol/L and thus very similar to the concentration of 77 mmol/L we found at the end of our study in the antioxidant fortified margarine group. This indicates that consumption of moderate amounts of vitamin C is sufficient to achieve saturation levels of plasma vitamin C and that, quite surprisingly, a full-fat margarine is a good carrier for supplementation with water-soluble compounds.

In addition to vitamin C which functions as a water-soluble antioxidant, we supplied moderate amounts of fat-soluble antioxidants, i.e. vitamin E, α-carotene and β-carotene. The plasma levels of vitamin E increased by 16% after supplementation with 31 mg/d vitamin E, which is comparable to the 20% increase after supplementation during two weeks with 25 mg/d of vitamin E that has been reported previously (Princen et al, 1995).

An important finding of the present study is that margarine is an excellent vehicle for carotenoid supplementation. Our data show that 2.7 mg/d of α-carotene and 5.3 mg/d of β-carotene incorporated in margarine resulted in respectively 14.5-fold and 3.4-fold increases in plasma levels of these carotenoids. It has been shown in previous studies that, in

comparison with plasma levels of vitamin E and vitamin C, plasma carotenoid concentrations are most sensitive to supplementation (Jialal & Grundy, 1993; Abbey et al, 1993b; Salonen et al, 1991; Zenhua et al, 1991). The increases in carotenoid concentrations observed in the present study also exceed the 2.6-fold increase in α-carotene and 1.9-fold increase in β-carotene levels in plasma found by Carughi et al (1994) after 4 weeks supplementation with slightly higher doses of carotenoids (3.5 mg/d α-carotene and 8.5 mg/d β-carotene). Abbey et al (1993b) reported a 5-fold increase in plasma β-carotene after 3 months of supplementation with β-carotene at a dose of 18 mg/d. All of these data pertain to well-nourished individuals. De Pee et al (1995) showed that in anaemic women, 12 weeks supplementation with 3.5 mg/d β-carotene resulted in a 3.9-fold increase in serum β-carotene levels and a 1.4-fold increase in serum retinol levels.

Interestingly, the increase of plasma levels of α-carotene exceeded that of β-carotene. The final concentration of α-carotene in plasma and in LDL was similar to that of β-carotene, although the intake of α-carotene was only 50% of that of β-carotene. This is in contrast with Carughi et al (1994) who found that the ratio of final plasma concentrations of α-carotene and β-carotene was the same as the supplemented ratio. One possible explanation for our results is that the bioavailability of α-carotene is higher than that of β-carotene. This is however not likely because Van den Berg (personal communication) found no difference after consumption of a single dose of either α- or β-carotene in chylomicron concentration response. It is also possible that the metabolism of α-carotene is slower than that of β-carotene. Both α- and β-carotene have provitamin A activity and in vitro experiments have shown that retinal formed from α-carotene was only 29% of the amount formed from equal amounts of β-carotene (Van Vliet et al, 1996). These results support the suggestion of a lower turn-over rate of α-carotene as compared to β-carotene. It is also possible that the rate of oxidative degradation of β-carotene is higher than that of α-carotene or that a quantitative difference in tissue distribution of α- and β-carotene has contributed to the difference in blood levels, but there are no data available to support these hypotheses.

Despite the observed increase in blood levels of individual antioxidants after supplementation with the antioxidant fortified margarine, no significant treatment effect on total antioxidant activity of plasma was found. The total antioxidant activity of plasma reflects the "sum" of endogenous and dietary antioxidants in plasma and is mainly determined by the concentration of albumin and uric acid (Miller et al, 1993; Wayner et al, 1987). However, only 19% of the variation in total antioxidant activity of plasma appeared to be explained by the variation in concentration of these two endogenous antioxidants. Apparently, variation in plasma levels of other than the measured antioxidants determine

the between subject variation in the total antioxidant activity of plasma. Plasma concentrations of albumin and uric acid were unchanged in both treatment groups. The magnitude of the changes in dietary antioxidant concentrations in plasma may have been too small to significantly increase the total antioxidant activity of plasma in the antioxidant fortified margarine group.

In addition to measurement of the total antioxidant activity of plasma, the total antioxidant activity of the LDL particles was assessed. LDL mainly contains fat-soluble components and we found a significant contribution of all of the supplied fat-soluble antioxidants to LDL antioxidant activity at baseline. LDL antioxidant activity increased significantly in the antioxidant fortified margarine group compared to baseline and the difference with the change found in the control group almost reached significance (P=0.08). This indicates that supplementation with a combination of the fat-soluble antioxidants vitamin E, α- and β-carotene, in dosages comparable to those used in the present study, may increase the protection of LDL to oxidative stress in vivo.

Despite significant increases in the antioxidant levels in plasma and LDL and the LDL antioxidant activity, no alteration of the lipid peroxidation products malondialdehyde in plasma and lipid hydroperoxides in LDL was found. It is however conceivable that effects of antioxidant supplementation on lipid peroxidation are detectable only in subjects exposed to increased levels of oxidative stress, e.g. in heavy smokers. Allard et al (1994) showed that daily supplementation with 20 mg β-carotene for four weeks reduced lipid peroxidation in smokers but not in non-smokers. Our results are in agreement with those of Allard et al (1994) and with those of Abbey et al (1993b), who also found no change of malondialdehyde levels in LDL of non-smoking subjects after six months of dietary supplementation with a combination of α-tocopherol (200 mg/d), β-carotene (18 mg/d) and vitamin C (900 mg/d).

Consumption of the antioxidant fortified spread for four weeks tended to increase the resistance of LDL to oxidation as compared to the changes found in the control group. Both the lag time as well as the maximum oxidation rate were significantly affected as compared to baseline in the fortified margarine group. This is in line with Princen et al (1995) who also found that supplementation with 25 mg/d vitamin E significantly increased the lag time of LDL oxidation as compared to baseline. In the latter study, the maximum rate of oxidation was however only affected if 400 mg/d or more of vitamin E was consumed. This lower sensitivity of the oxidation rate to increased antioxidant status was also observed by Jialal et al (1995). In their study, the oxidation rate was affected only after supplementation with 800 mg/d vitamin E, whereas the lag time was significantly increased as compared to baseline after supplementation with 400 mg/d but not after supplementation with 60 or 200 mg/d vitamin E. It is possible that the greater effectiveness of antioxidant supplementation found

in the present study is due to the combination of antioxidants supplied to the volunteers. Not only did the vitamin E status increase significantly but also the concentrations in plasma and LDL of α- and β-carotene and the plasma concentration of vitamin C were increased in the antioxidant fortified margarine group. For the lag time, however, an inverse correlation was found with α-carotene level in LDL. The α-carotene concentration explained only 9% of the variation in the duration of the lag phase and this may be the reason that the increase in LDL concentration of α-carotene in the fortified margarine group did not abolish the protective effect of the other antioxidants. The role of α-carotene in the protection of LDL against oxidation in vitro has not yet been investigated, whereas for β-carotene Esterbauer et al (1991) have shown that this carotenoid is used sequentially to vitamin E as protective barrier to LDL oxidation. Furthermore they showed that vitamin C, when added to LDL, was oxidized even before vitamin E and β-carotene. Previous studies in which different antioxidants were supplemented showed no additional protective effects of β-carotene and vitamin C as compared to supplementation with vitamin E alone (Princen et al, 1992; Reaven et al, 1993; Jialal & Grundy, 1993). However, the pharmacological doses of vitamin E used in these studies may have masked the protective effects of the other antioxidants.

Vitamin C can protect against oxidative stress both by its role as antioxidant defence against aqueous radicals (Jialal & Grundy, 1991) as well as by regenerating vitamin E from the vitamin E radical at the water-lipid interface of LDL as has been observed in vitro (Sato et al, 1990). The role of α- and β-carotene as antioxidants is however still not elucidated. The inverse correlation we found between the level of α-carotene in LDL and both the LDL antioxidant activity and the duration of the lag phase before LDL oxidation does not support the hypothesis that this carotenoid protects against cardiovascular disease by increasing intrinsic LDL antioxidant protection. However, the resistance of LDL to oxidation in vitro is only one suggested biomarker of the risk of cardiovascular disease and the role of carotenoids in the process of atherogenesis has recently been suggested to be mediated by conversion into retinoic acid rather than via protection of LDL against oxidation (Shaish et al, 1995).

In conclusion, the results of our study clearly show that consumption of moderate doses of vitamin E, vitamin C, α-carotene and β-carotene, supplied at two to three times the RDA in a full-fat margarine and consumed as part of a normal diet, effectively increases the blood levels of these antioxidants to achieve threshold plasma levels that have been suggested for optimal health (Gey et al, 1995).

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6

Amount of fat in the diet affects bioavailability of lutein esters but not of α-carotene, β-carotene and vitamin E in humans

Annet J.C. Roodenburg, Rianne Leenen, Karin H.van het Hof, Jan A.Weststrate, Lilian B.M. Tijburg

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ABSTRACT

Background: The fat-soluble vitamin E and carotenoids are regarded as being protective against chronic diseases. Little is known about the effect of dietary fat on their bioavailability.

Objective: To asses the effect of the amount of dietary fat on plasma concentrations of vitamin E and carotenoids after supplementation with these compounds.

Design: During two 7-d periods, four groups of 14-15 volunteers received daily with a low-fat hot meal, one of 4 different supplements: vitamin E (50 mg), α- plus β-carotene (8 mg), lutein esters (8 mg lutein) or placebo. The supplements were provided in a low-fat or high-fat spread which were supplied in random sequence during either of the two experimental periods.

Results: As anticipated, significant increases in plasma concentrations of vitamin E, α- and β-carotene or lutein were found in the supplemented groups as compared to the placebo group. The amount of dietary fat consumed with the hot meal (3 g vs 36 g) did not affect the increases in plasma concentrations of vitamin E (low vs high-fat, 20% vs 23% increase) or α-carotene and β-carotene (low vs high-fat, α-carotene: 315% vs 226% increase and β-carotene: 139% vs 108% increase). The plasma lutein response was higher when lutein esters were consumed with a high-fat meal (207% increase) than with a low-fat meal (88% increase).

Conclusion: Optimal uptake of vitamin E and α- and β-carotene requires a limited amount of fat whereas the amount of fat required for absorption of lutein esters is greater.

INTRODUCTION

Epidemiological studies have indicated that an increased intake of vitamin E or carotenoids is associated with a reduced risk of cardiovascular disease (Stampfer et al 1993, Rimm et al 1993; Gey, 1995) or cancer (Van Poppel & Goldbohm, 1995; Willet & Trichopoulos, 1997, Heinonen et al 1998). Vitamin E and carotenoids have biological activity as antioxidants (Sies & Stahl, 1995) which may mediate these putative beneficial effects.

Important dietary sources of vitamin E are oils and fats (FASEB, 1995; Eitenmiller, 1997) and the richest dietary sources of carotenoids are fruits and vegetables (Chug-Ahuja et al, 1993, Mangels et al, 1993). Vitamin E and carotenoids are fat-soluble compounds and their absorption involves solubilization in bile salts and incorporation into micelles. Actual absorption is believed to occur by passive diffusion along with dietary fat. The presence of dietary fat is thought to be important for micelle formation in the small intestine and it may therefore also be crucial for absorption of vitamin E and carotenoids (Kayden and Traber, 1993; Parker, 1996; Cohn, 1997).

There is little information available on the influence of dietary fat on vitamin E absorption in humans and results from animal studies are conflicting. Dimitrov et al (1991) concluded from a small study in humans (n=6) that dietary fat enhanced vitamin E absorption. However, in some studies with rats, the amount of dietary fat present did not influence the apparent absorption of vitamin E (Brink et al 1996; Tijburg et al 1997). In contrast, vitamin E absorption, measured as lymphatic appearance of radiolabelled vitamin E in rats, was increased with higher intakes of saturated fat (Gallo-Torres et al 1971).

Most of the research on the effect of dietary fat on carotenoid bioavailability in humans has focussed on β-carotene (Jayarajan et al 1980; Dimitrov et al, 1988; Prince & Frisoli, 1993; Shiau et al 1994; Jalal et al, 1998). These studies indicated that the presence of fat was essential for the absorption of β-carotene. However, in some studies, the influence of dietary fat was assessed in comparison with its complete absence at the time of β-carotene ingestion (Dimitrov et al, 1988; Prince & Frisoli, 1993). Although this situation may be applicable to the very low-fat diets of some populations in developing countries, it is not representative for a western diet. For comparison, an average hot meal in the Netherlands contains as much as 40 g of fat (Voorlichtingsbureau voor de Voeding, 1992).

The present experiment was designed to investigate the effect of the amount of dietary fat on the plasma response to supplementation with vitamin E or carotenoids. We compared two amounts of dietary fat that are achievable in a western diet.

SUBJECTS AND METHODS

Subjects

Participants were recruited from the local population by means of advertisements in newspapers. Sixty non-smoking subjects (23 men, 37 women) aged between 18 and 70 years and with a reported body mass index (BMI) between 19 and 30 kg/m^2, were selected for participation. They did not use medication, apart from oral contraceptives, or follow a medically prescribed diet or weight-loss regime. They did not use any supplements containing vitamin C, vitamin E, carotenoids, calcium or iron. Their body weights had been stable for ≥ 1 month prior to the start of the study. None of the female volunteers were pregnant or lactating. The selected subjects were apparently healthy as evaluated by a medical history questionnaire.

Two females were withdrawn from the experiment: one because her fasting plasma cholesterol level was > 8.5 mmol/L and one because of intercurrent illness not related to the treatment. Data from a third subject were not used because of suspicion of prior carotenoid supplement use (plasma α-carotene: 285 nmol/L; plasma β-carotene: 2068 nmol/L).

The protocol of the study, which had been approved by our local Medical Ethical Committee, was fully explained to the volunteers and they gave their written informed consent before participation.

Experimental design

The study was carried out according to a split-plot design. Each subject was randomly assigned to one of four experimental groups after stratifying for sex, age and BMI. One group received a placebo during two 7-d experimental periods. The other three groups received supplements of vitamin E or α- plus β-carotene or lutein esters. The placebo and antioxidant supplements were provided in a low-fat or high-fat spread. All subjects consumed these low-fat and high-fat spreads in a cross-over design during either of the two experimental periods. They were randomly allocated over the treatment sequences. The experimental periods were separated by a wash-out period of five weeks during which no intervention was carried out.

The spread was consumed daily with a low-fat hot meal in the evening. The amount of fat, carotenoids and vitamin E consumed during the rest of the experimental days was restricted.

Volunteers were instructed to maintain their habitual pattern of physical activity and lifestyle during the entire study period, including the wash-out period. In addition, they were not allowed to take any supplements containing vitamin C, vitamin E, carotenoids, calcium or iron.

Before and after each experimental period, body weight was measured and fasting venous blood samples were collected. At the end of the first experimental period, body height was measured also.

Dietary restrictions

All volunteers were asked to keep to strict dietary prescriptions to limit intake of fat, carotenoids and vitamin E during the experimental periods. They were instructed to eat a breakfast and a low-fat lunch with negligible amounts of vitamin E and carotenoids, a maximum of only 0.25 g of fat after lunch and in the late evening, and no fat for 2 hours before and after the supplemented hot meal. From previous work, it was deducted that this range of 2 hours would minimalize the influence of fat from other dietary sources than the experimental meal (Van Amelsvoort et al, 1989). After lunch the subjects were not allowed to consume any products containing vitamin E or carotenoids. Besides the low-fat hot meal and experimental spread, the volunteers received an additional low-fat spread without β-carotene and vitamin E (Promise Ultra®, VandenBergh Foods Co., Lisle, Illinois, USA) to use with their lunch during the experimental periods. Compliance to the instructions was assessed by asking the volunteers about their diet during the experimental periods, consumption of experimental spread and hot meals, and by weighing the amount of spread left over in the tubs.

Experimental spreads

The supplementary dose of vitamin E was 50 mg/d α-D-tocopherol (Sigma, St. Louis, MO, USA, purity 67%), equivalent to 5 times the recommended daily allowance of 10 mg/d (RDA for males aged 22-50 y, National Research Council, 1989; Voedingsraad, 1989). The carotenoid-enriched spreads provided either 8 mg/d α- and β-carotene (equivalent to 15 μmol/d α- and β-carotene together; 30% suspension in oil, Vegex® Carotene, Quest International Ireland Ltd., Cork, Ireland), or 8 mg/d lutein (as lutein esters, equivalent to 15 μmol/d lutein, 3.2% suspension in oil, Vegex® Lutein OS30, Quest International Ireland Ltd., Cork, Ireland). The daily amount of carotenoids added to the spread equalled about 1-1.3 times the daily carotenoid intake in the US (6 mg, Chug-Ajuha et al 1993) and the Netherlands (7 mg, Goldbohm et al, 1998). The control spread contained no added carotenoids or vitamin E.

Volunteers were required to eat daily 50 g of the experimental spread, which contained about 3% fat (w/w) during the low-fat period and about 80% fat (w/w) during the high-fat period. In this way, a maximum contrast between the low and high-fat intervention, together with an acceptable meal, could be achieved.

The spread was freshly prepared in our laboratory for each experimental period. Fat content and fatty acid composition were kept similar in the control and supplemented spreads for the low-fat spreads and high-fat spreads.

Experimental meals

Volunteers were instructed to consume the spread together with the low-fat hot meal which was provided for consumption at home. These low-fat hot meals consisted of ca. 70 g of prepared lean meat, ca. 160 g of low-carotenoid vegetables containing negligible amounts of vitamin E. Consumption of low-fat gravy and potatoes was allowed ad libitum. These low-fat hot meals were designed to contain less than 5 g fat. The low-fat spread contained about 1.5 g fat and the high-fat spread provided an additional amount of about 40 g. Thus, total fat intake per meal was calculated to be < 6.5 g for the low-fat treatment and < 45 g for the high-fat treatment.

In order to correct for the effects of meal preparation on the amounts of fat, carotenoids and vitamin E consumed, duplicate portions were collected. Following the instructions given to the volunteers, representative meals were prepared for each different study group and each period. The average amount of potatoes (266 g) and low-fat gravy (29 g) used by the subjects was calculated based on the quantities used, as reported by the volunteers on a questionnaire. The samples were stored under argon at -80°C until analysis.

Laboratory analyses

Plasma

Fasting venous blood samples were collected before and after each intervention period. EDTA-plasma was prepared and stored at -80°C. Plasma total cholesterol and triacylglycerol concentrations were determined spectrophotometrically by enzymatic methods using commercially available test kits (Boehringer, Mannheim, Germany). Carotenoids in plasma were extracted with heptane/dichloromethane (5:1, v/v). After addition of ethyl-β-apo-8'-carotenoate (gift from Hoffmann-LaRoche, Switzerland) as internal standard, the sample was injected onto a Suplex column pkb-100, 25 cm x 4.6 mm ID, 5 μm (Supelco Inc., Bellefonte, PA, USA), and eluted with 93.5% methanol/acetonitrile/toluene (A) (55/44/2, v/v/v) and 6.5% water containing 0.1% ammonium acetate (B). Ten minutes after sample injection, a linear gradient started from 93.5% A and 6.5% B to 100% A at 45 min. Between 45-47 min the eluent returned to 93.5% A and 6.5 % B, which was maintained until 55 min. The flow rate was 1.0 mL/min. Detection of carotenoids was performed by UV-vis dectection at 450 nm. Plasma α-

tocopherol was determined by HPLC on a Lichrospher RP-18 (5μm) Merck column (Merck, Darmstadt, Germany). α-Tocopheryl-acetate was used as internal standard (Merck, Darmstadt, Germany). The mobile phase consisted of methanol/isopropanol/water (50/50/8, v/v/v) and the flow rate was 0.6 mL/min. α-Tocopherol was detected by UV-vis detection at 292 nm and α-tocopheryl acetate at 284 nm.

Experimental meals

The duplicate samples of the meals were analysed for content of fat, fatty acids, α-tocopherol, α- and β-carotene and lutein. The values in Table 1 are based on the chemical analyses and expressed as daily intake from an average hot meal. The fat content was determined after freeze drying the duplicate samples, followed by extraction with dichloromethane. Fatty acid composition was determined as described previously (Tijburg et al 1997). Extraction of vitamin E and carotenoids from the meals containing the high-fat spread was performed with heptane/diethylether (3:1, v/v). These extractions were repeated three times, whereas in case of the low-fat meals diethylether/methanol (5:2, v/v) was used. These extractions were repeated two times with diethylether. α-Tocopherol and carotenoids were analysed by reversed phase HPLC using a C30 S-5μm column, 4.6 x 150 mm (YMC, Inc., Wilmington, NC, USA). Ethyl-β-apo-8'-carotenoate and α-tocopheryl acetate were used as internal standards. The column was eluted with 95% methanol/tert-butylmethylether/1.5% aq ammonium acetate (A) (83/15/2, v/v/v) and tert-butylmethyl ether/5% methanol/1.5% aq ammonium acetate (B) (90/8/2, v/v/v). Ten minutes after sample injection, a linear gradient started from 95% A and 5% B to 55% A and 45% B at 22 min. Between 22-34 min a linear gradient was used from 55% A and 45% B to 5% A and 95% B which remained until 39 min, from 39-44 min the eluens returned to 95% A and 5% B, which was maintained until 50 min. The flow rate was set at 1.0 mL/min. Carotenoids were detected by UV-vis detection at 450 nm, α-tocopherol at 292 nm and α-tocopheryl-acetate at 284 nm.

Statistics

The present study was carried out according to a split-plot design. Different supplement conditions were tested against the between subject error and the effect of the amount of fat against the within subject error.

The changes over the experimental periods of the variables measured in the supplemented groups were compared with those in the control group. The following factors were used in the analysis of variance: amount of fat, type of supplement, subject, gender, experimental period and their interaction. In addition, the analysis was executed for each

TABLE 1 Average nutrient composition of the different hot meals that were consumed daily[1].

	Type of meal							
	Control		Vitamin E		α-Carotene & β-Carotene[2]		Lutein[2]	
	Low-fat	High-fat	Low-fat	High-fat	Low-fat	High-fat	Low-fat	High-fat
Fat (g)	3.1	36.0	3.1	34.1	2.6	36.0	3.1	34.4
Fatty acids (%)								
Saturated	59.7	22.2	61.7	22.5	59.0	22.3	60.4	22.2
Monounsaturated	24.4	25.3	23.3	25.4	23.8	25.4	23.7	25.2
Polyunsaturated	14.7	51.6	12.6	51.4	15.0	51.1	13.2	51.9
α-Tocopherol (mg)	1.2	0.8	59.7	56.0	1.2	0.9	1.4	1.1
α-Carotene (mg)	<0.02	<0.02	<0.02	<0.02	2.2	2.1	<0.02	<0.02
β-Carotene (mg)	0.02	0.02	0.02	0.02	4.4	4.2	<0.02	<0.02
Lutein (mg)	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	8.0	7.6

[1] Based on chemical analysis of duplicate portions of an average daily hot meal, including the experimental margarine. Values for the two study periods were similar and therefore averaged

[2] α-Carotene and β-carotene were present as free carotenoids, whereas lutein was present as lutein esters

amount of fat and type of supplement separately. Significant deviations from the control group were determined by the Dunnett-test.

To compare the relative plasma response after α-carotene, β-carotene and lutein supplementation, the changes in each of the plasma carotenoid concentrations were expressed relative to their intake. For each level of fat intake, differences in relative response of α-carotene vs β-carotene, in the group that received the α- plus β-carotene supplement, were determined with a paired t-test. Differences between lutein response in the lutein group and α-carotene or β-carotene response in the group that received the α- plus β-carotene supplement were determined by an unpaired t-test.

All comparisons were made at a two-sided level (α=0.05).

RESULTS

Baseline characteristics and compliance

There were more women than men participating in the study (Table 2). Mean BMI and age at baseline (SD) were 25.0 (3.2) kg/m^2 and 46.4 (13.4) y, respectively. There were no significant differences among the four groups (data not shown). Compliance, as assessed by means of compliance forms and weighing of spread left over in the tubs, was excellent. All subjects consumed all of the meals during the two experimental periods and < 3% of the spread was left over.

Body weight, plasma cholesterol and triacylglycerol

In all groups, body weight and plasma concentrations of cholesterol and triacylglycerol decreased during each of the two 7-d intervention periods (Table 2). However, there were no significant differences between the supplemented groups and the control group, except for the difference in change of plasma cholesterol concentrations between the lutein supplemented group and the control group. With respect to the differences between the low-fat and high-fat treatments, significant differences were found only for the vitamin E supplemented group. The decrease in body weight was smaller following the high-fat treatment whereas the decrease in plasma triacylglycerol concentration was smaller after the low-fat treatment.

Plasma α-tocopherol and carotenoids

As plasma α-tocopherol and carotenoid concentrations are associated with plasma lipid concentrations (Traber et al 1994), they were corrected for plasma cholesterol and triacylglycerol concentrations prior to statistical analysis. Table 3 illustrates the uncorrected baseline concentrations and changes in concentrations of plasma α-tocopherol and carotenoids after their supplementation with the low-fat or high-fat meals.

TABLE 2 Mean (SEM) body weight and plasma lipid concentrations: baseline values and changes after 7 d consumption of a low- or high-fat meal not supplemented (control) or supplemented with vitamin E or carotenoids.

Males/Females (n)		Intervention group							
		Control 5/10		Vitamin E 6/8		α-Carotene & β-Carotene 6/8		Lutein 6/9	
		Low-fat	High-fat	Low-fat	High-fat	Low-fat	High-fat	Low-fat	High-fat
Body weight (kg)	Before	76.0 (3.9)	75.8 (3.8)	74.8 (1.9)	74.8 (1.8)	73.9 (3.7)	74.3 (3.8)	72.7 (3.4)	72.6 (3.3)
	Change	-1.5 (0.2)	-1.3 (0.3)	-1.4 (0.2)[y]	-0.8 (0.2)	-1.3 (0.3)	-1.2 (0.2)	-1.2 (0.2)	-0.9 (0.2)
Total cholesterol (mmol/L)	Before	5.32 (0.34)	5.29 (0.34)	5.23 (0.24)	5.02 (0.22)	5.08 (0.21)	4.97 (0.23)	4.90 (0.23)	4.84 (0.23)
	Change	-0.52 (0.08)	-0.53 (0.08)	-0.75 (0.10)	-0.40 (0.13)	-0.53 (0.11)	-0.57 (0.08)	-0.48 (0.10)	-0.32 (0.08)[a]
Triacylglycerol (mmol/L)	Before	1.46 (0.16)	1.38 (0.13)	1.52 (0.18)	1.75 (0.22)	1.54 (0.17)	1.45 (0.11)	1.36 (0.13)	1.39 (0.14)
	Change	-0.26 (0.08)	-0.30 (0.04)	-0.03 (0.13)[x]	-0.49 (0.16)	-0.27 (0.16)	-0.35 (0.08)	-0.18 (0.10)	-0.30 (0.09)

[a] Different from control (same fat treatment) ($P < 0.05$)

[x,y] Low-fat different from high-fat treatment, within intervention group (x: $P < 0.05$; y: $P < 0.01$)

TABLE 3 Mean (SEM) plasma concentrations of α-tocopherol and carotenoids: baseline values and changes after 7 d consumption of a low or high-fat meal not supplemented (control) or supplemented with vitamin E or carotenoids[1].

		Intervention group							
		Control (n=15)		Vitamin E (n=14)		α-Carotene & β-Carotene (n=14)		Lutein (n=15)	
		Low-fat	High-fat	Low-fat	High-fat	Low-fat	High-fat	Low-fat	High-fat
α-Tocopherol (μmol/L)	Before	23.9 (1.9)	23.8 (1.6)	25.1 (2.1)	24.4 (2.2)	--	--	--	--
	Change	-4.4 (0.6)	-4.7 (0.6)	5.0 (0.8)[a]	5.5 (1.2)[a]	--	--	--	--
α-Carotene (nmol/L)	Before	80 (9)	74 (6)	--	--	106 (11)	120 (14)	77 (16)	76 (12)
	Change	-14 (2)	-13 (2)	--	--	334 (22)[a]	271 (16)[a]	-9 (3)	-9 (2)
β-Carotene (nmol/L)	Before	291 (37)	281 (31)	--	--	339 (55)	358 (51)	338 (46)	341 (54)
	Change	-71 (9)	-73 (12)	--	--	472 (50)[a]	386 (40)[a]	-69 (13)	-68 (12)
Lutein (nmol/L)	Before	146 (17)	146 (15)	--	--	182 (31)	183 (28)	180 (20)	176 (20)
	Change	-39 (7)	-33 (5)	--	--	-55 (9)	-49 (5)	158 (25)[ax]	365 (38)[a]
β-Cryptoxanthin (nmol/L)	Before	184 (29)	191 (28)	--	--	165 (21)	187 (27)	179 (22)	191 (25)
	Change	-36 (9)	-48 (12)	--	--	-34 (6)	-40 (6)	-32 (6)	-34 (9)
Lycopene (nmol/L)	Before	357 (44)	353 (36)	--	--	316 (47)	311 (50)	277 (34)	246 (34)
	Change	-89 (16)	-88 (24)	--	--	-88 (18)	-83 (18)	-75 (14)	-62 (13)

[1] Statistical evaluation has been performed on values corrected for plasma cholesterol and triacylglycerol concentration. However, because they are more informative, uncorrected means are shown in the table. The only significant difference that disappeared after correction for plasma cholesterol and triacylglycerol concentrations was the higher increase in plasma α- and β-carotene concentrations with the low-fat treatment as compared to the high-fat treatment ($P < 0.05$, for the uncorrected values).

[a] Different from control (same fat treatment) ($P < 0.001$)

[x] Low-fat different from high-fat treatment, within intervention group ($P < 0.001$)

Plasma concentrations of α-tocopherol, α- and β-carotene and lutein increased significantly after consumption of spread supplemented with vitamin E, α- and β-carotene or lutein when compared to the control spread. Decreases in plasma α-tocopherol and carotenoid concentrations in the control group and in plasma lycopene and β-cryptoxanthin concentrations in all groups, were demonstrated. Changes in plasma concentrations of lycopene and β-cryptoxanthin in the supplemented groups were not significantly different from those in the control group.

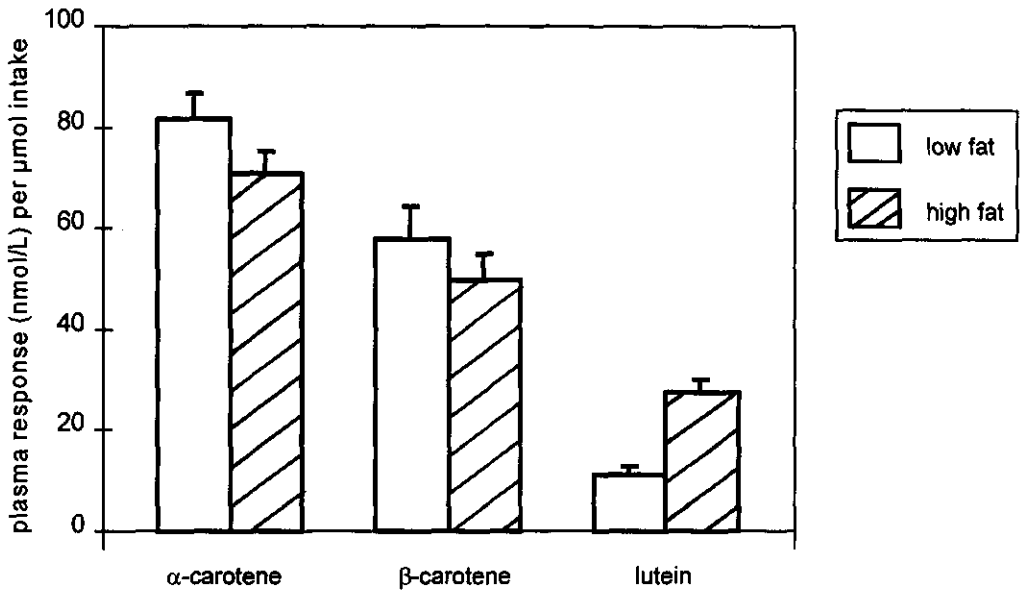

FIGURE 1 Plasma carotenoid responses (mean ± SEM) following 7-d consumption of a low or high-fat meal supplemented with carotenoids, expressed as increase per µmol carotenoid supplemented: (α-carotene intake: 4.1 and 3.8 µmol/d; β-carotene intake: 8.1 and 7.8 µmol/d; lutein intake: 14.0 and 13.3 µmol/d, for low and high-fat respectively). For both the low- and high-fat treatment, the relative plasma responses were significantly different among the different carotenoids (α-carotene vs β-carotene; α-carotene vs lutein; β-carotene vs lutein: $P < 0.001$). Only for lutein the relative responses between the low-fat and the high fat treatment were signifiantly different ($P < 0.001$).

There was no significant effect of the amount of fat on the response in plasma α-tocopherol concentration after consumption of the vitamin E supplemented meals (Table 3). Table 3 also shows that in case of the meals supplemented with α- and β-carotene, the increases in plasma α- and β-carotene concentrations following the low-fat meal were

slightly larger than after the high-fat meal. This could at least in part be explained by a larger amount of α- and β-carotene in the low-fat meal (Table 1, Figure 1). The difference in plasma response of α- and β-carotene between the low-fat and the high-fat meal was however not statistically significant after correction for plasma cholesterol and triacylglycerol concentrations. The increase in plasma lutein concentration was significantly larger after the high-fat treatment when compared with the low-fat treatment.

As α- and β-carotene and lutein esters were consumed in about the same amounts (Table 1), we compared the responses in plasma carotenoid concentrations, relative to their intakes. For both the low- and high-fat treatment, these relative responses were significantly higher for α-carotene and β-carotene than for lutein and the relative response for α-carotene was significantly (20%) higher than for β-carotene (Figure 1). In addition, the relative response for lutein was significantly higher on the high fat diet than on the low fat diet (Figure 1).

DISCUSSION

The present study shows that the bioavailability of vitamin E, α-carotene and β-carotene was similar when consumed with a low-fat meal (ca. 3 g fat) or a high-fat meal (ca. 36 g fat), whereas for lutein esters, the bioavailability was significantly lower when consumed as part of a low-fat meal. Bioavailability was assessed as changes in plasma concentrations after 7-d supplementation with vitamin E, α- and β-carotene or lutein esters.

The reductions observed in plasma concentrations of carotenoids other than those supplied and in plasma α-tocopherol concentration in the control group are most probably the result of a good compliance to the dietary restrictions. The volunteers were instructed to refrain from foods rich in vitamin E and carotenoids and the experimental meals contained virtually no carotenoids and little vitamin E. Body weight and plasma lipid levels also decreased during the two study periods, but there were no major differences among the groups and between the low-fat and high-fat treatments.

Analysis of the composition of the meals demonstrated that the fat content of both the low and high-fat meals was lower (3 g and 36 g, respectively) than expected (6.5 g and 45 g, respectively) (Table 1). The slightly smaller amount of vitamin E and carotenoids in the high-fat meals when compared to the low-fat meals may be due to the larger amount of unsaturated fatty acids in the high-fat meals. It is likely that the supplemented antioxidants were used to prevent oxidation of these polyunsaturated fatty acids. It cannot be excluded this has also reduced the bioavailability of the antioxidants from the high-fat meals (Gallo-

Torres et al 1971, Tijburg et al 1997). Although it is impossible to quantify this effect in the present study, this may explain the slightly lower plasma response after α- and β-carotene supplementation with the high-fat meal as compared to the low-fat meal.

Vitamin E

Based on the absorption mechanism of vitamin E, the presence of dietary fat is generally believed to be necessary for its intestinal uptake (Kayden and Traber, 1993; Cohn, 1997). We conclude from the present results that the small amount of fat in the low-fat meal (ca. 3 g) was already sufficient to ensure the uptake of vitamin E. A previous cross-over study with only six human volunteers showed a larger increase in plasma α-tocopherol concentrations when 5-d vitamin E supplementation was followed 6-8 h later by a high fat intake (≥ 45 g fat) as compared to a low fat intake (about 6 g fat) (Dimitrov et al 1991). However, this study was rather small and could be flawed by the fact that the vitamin E intake with the high-fat treatment was higher than with the low-fat treatment (Dimitrov et al 1991). In the present study it was shown that when diets were equalized for vitamin E content, a small amount of fat was sufficient to facilitate vitamin E absorption.

α-Carotene and β-carotene

Previous studies have found that the presence of dietary fat is crucial for the absorption of β-carotene. However, in these studies, the importance of dietary fat was assessed in comparison with its complete absence at the moment of ingestion of β-carotene (Dimitrov et al, 1988; Prince & Frisoli, 1993). Recently, Jalal et al (1998) reported a significantly larger increase in serum retinol in vitamin A deficient children if a sweet potato snack (providing about 4.5 mg/d β-carotene) was ingested with 18 g fat when compared to 3 g fat. In addition, Jayarajan et al (1980) found no difference in improvement of the vitamin A status when 5 g or 10 g of dietary fat was added to spinach (providing 1.2 mg/d β-carotene), whereas 0 g fat resulted in a smaller increase. Although there were differences in food matrices in these studies, the data suggest that there could be a minimum amount of dietary fat in a meal of about 3-5 g, needed to ensure carotene absorption. In the present study, the fat content of the low-fat meal with which α- and β-carotene were ingested, contained about 3 g fat. Apparently, this amount of fat was just sufficient for the uptake of α- and β-carotene.

Lutein

The influence of dietary fat on lutein bioavailability differed from that of vitamin E and α- and β-carotene. About 3 grams of dietary fat was not sufficient to ensure absorption of the

same magnitude as that found when about 36 g fat was present. This result was unexpected as lutein is less lipophilic than α- and β-carotene and a larger impact of the amount of dietary fat would have been expected for the more lipophilic carotenoids. However, the lutein used in this study was esterified, mainly with palmitic acid (Granado et al 1998). Although the partition coefficient between fat and water for the lutein esters is unknown (Straub, 1987), it is likely that lutein esters are more lipophilic than α- and β-carotene. It can thus be speculated that in the presence of only small amounts of fat, the emulsification in the intestine of lutein esters is less than that of α- and β-carotene.

After 4 mo of supplementation with lutein esters (15 mg/d), some lutein monopalmitate was detected in plasma by Granado et al (1998). However, it is generally assumed that most of the lutein esters are hydrolysed prior to or during absorption and that free lutein is absorbed, as this is the case for cholesteryl esters (Tso, 1994) and β-cryptoxanthin esters (Wingerath, 1995). Hydrolysis of esters is mediated by esterases and possibly also lipases. The excretion of these enzymes by the pancreas is regulated by the presence of fat in the stomach and duodenum. In addition, the activity of esterases and lipases is substantially enhanced if the amount of fat is sufficient to form lipid/aqueous interfaces in the duodenum (Tso, 1994). Our results suggest that either the release of esterases and lipases and/or the formation of lipid/aqueous interfaces was hampered at low-fat intake, resulting in a reduced uptake of lutein esters.

It has previously been shown that the bioavailability of lutein esters is similar to lutein when supplemented to a normal diet (Herbst et al 1997). Results from the present study suggest, however, that this may depend on the amount of fat that is consumed with the lutein esters. If lutein is present as free lutein, as it is the case in lutein-rich vegetables such as spinach, broccoli, kale and green peas (Khachik et al, 1991), the amount of fat needed to be present may be smaller than that needed for optimal uptake of lutein esters.

Comparison of relative plasma responses of carotenoids

The study design, with similar amounts of α- and β-carotene and lutein allowed to compare plasma responses. The analysed daily intake of carotenoids varied slightly (Table 1). The relative plasma response was calculated by dividing the plasma responses measured by the amount of carotenoids supplied daily, thus assuming a linear dose-response relationship (Figure 1). Substantial differences in relative plasma responses among the carotenoids were demonstrated. Previous studies also found that the relative plasma response of lutein was less than that of β-carotene after 3-4 weeks supplementation (Castenmiller et al, 1999; Van het Hof et al, *submitted*). In line with Van het Hof et al (1998), the relative plasma response of α-carotene exceeded that of β-

carotene after supplementation with palm oil carotenoids. These differences may result from differences in absorption, metabolism and/or tissue distribution between the carotenoids. Kostic et al (1995) suggested that when β-carotene and lutein were supplemented simultaneously, the lower plasma response of lutein may be due to competition for absorption between the carotnoids. Although this interaction cannot be excluded, the present study shows that the plasma response after lutein supplementation was lower than that of β-carotene, independent from the simultaneous presence of β-carotene.

The present study illustrates that enrichment of food products with α-carotene, β-carotene, lutein and vitamin E can be an effective way to enhance the plasma status of these antioxidants and thus possibly reduce the risk of degenerative diseases (Gey 1995, Van Poppel & Goldbohm 1995, Stampfer et al 1993, Rimm et al 1993, Heinonen et al 1998). Fat containing products are suitable carriers for these fat-soluble antioxidants and they need to contain only a small amount of fat to ensure uptake of these fat-soluble minor components. The minimal amount of fat needed depends however on the physico-chemical characteristics of the fat-soluble compounds.

In conclusion, the present study shows that consumption of a low-fat or high-fat meal, enriched with vitamin E, α-carotene and β-carotene or lutein esters, enhances the plasma concentrations of these antioxidants. Simultaneous ingestion of only small amounts of fat with vitamin E, supplied as α-D-tocopherol, guarantees good bioavailability of vitamin E. Likewise, a small amount of fat is sufficient to optimise the uptake of α- and β-carotene, whereas a comparable amount of lutein esters requires a larger amount of fat.

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Sucrose polyester and plasma carotenoid concentrations in healthy subjects

Jan A. Weststrate and Karin H. van het Hof

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ABSTRACT

A double-blind, placebo-controlled cross-over study of the effects of the non-absorbable fat analogue, sucrose polyester (SPE; 12.4 g/d) on plasma concentrations of five different carotenoids and vitamin E in 21 volunteers, and a double-blind, placebo-controlled parallel comparison study in 53 subjects of the effect of 3 g/d SPE on plasma concentrations of two different carotenoids were undertaken. SPE-containing margarine added to the main meal was used. SPE (12.4 g/d) reduced plasma β-carotene concentrations by 0.13 μmol/L (34%, P=0.0001) and concentrations of lycopene by 0.14 μmol/L (52%, P=0.0001). Smaller but significant reductions were found for plasma concentrations of β-cryptoxanthin, lutein, zeaxanthin and vitamin E. SPE (3 g/d) reduced plasma concentrations of β-carotene by 0.094 μmol/L (20%, P=0.0001), and concentrations of lycopene by 0.12 μmol/L (38%, P=0.0001). Even at low doses, SPE strongly reduces plasma carotenoid concentrations. This finding merits careful consideration in assessing the long-term health effects of SPE-containing consumer foods.

INTRODUCTION

Excessive consumption of total fat and saturated fat increases the risk for coronary heart diseases, obesity, some types of cancer, and possibly gallbladder disease (US Surgeon General, 1988). Therefore, health authorities in many countries have advised individuals to reduce their total fat intake (WHO, 1990; Committee on Medical Aspects of Food Policy, 1991). The food industry has recognized that the perceived health benefits of a diet reduced in fat offer opportunities for reduced-fat products. Food companies are therefore active in developing low- or zero-energy fat substitutes, produced both from existing food sources and from chemical synthesis. The latter compounds, so called fat analogues, are designed to mimic natural fats and oils but are partially or totally undigested. An interesting synthetic fat analogue is the nontoxic sucrose polyester (SPE), which has generated much interest among consumers, industry and regulatory authorities. SPEs are fatty acyl esters of sucrose, in which the fatty acyl chains are of the types familiarly found in edible oils and fats. The chemical and physical properties of SPEs closely parallel those of triacylglycerols. Currently available information indicates that SPEs are neither broken down nor absorbed as such in the gastrointestinal tract and that they are thus a truly nonenergetic fat substitute.

Replacement of traditional fatty foods by foods containing SPE may be an effective means to reduce total fat and energy intakes of the population. Several studies have shown that SPEs reduces fat intake at the expense of carbohydrates (Rolls et al, 1992) and at the expense of total energy intake (Glueck et al, 1982; Cotton et al, 1993; Hulshof et al, 1993). Other studies showed a reduction in cholesterol absorption (Jandacek et al, 1990) or in plasma cholesterol levels (Glueck et al, 1983) when SPEs were present in the diet. These positive nutritional effects are obvious reasons for introduction of SPEs in food products. Currently, however, SPE is not yet cleared by the FDA for use in typical consumer goods.

Apart from the technical and nutritional benefits, consumption of SPE may produce some potential adverse health effects. These effects have to be addressed before a decision can be made on the introduction of SPE in the food supply. One of the possible adverse effects is the sequestering in the gut of essential fat-soluble compounds and subsequent elimination in the stool along with intact SPE. Although SPE was found to reduce the body's vitamin E status (Koonvitsky et al, 1991), no adverse effect was reported for the status of vitamins D and K (Jones et al, 1991a; Jone et al, 1991b).

To our knowledge no results have been published of the effect of SPE consumption on carotenoid status. Carotenoids are a class of fat-soluble compounds that have antioxidant properties and occur naturally in vegetables and fruits (Gerster, 1993). In addition to a

role for some carotenoids, particularly β-carotene, as safe precursors of vitamin A, it has been suggested that carotenoids may protect against the development of cancer (Gerster, 1993), coronary heart disease (Gerster, 1991), cataracts and age-related maculopathy (Sarma et al, 1994).

We report on two 4-wk studies of the effect of consumption of realistic daily doses of SPE-containing margarine on blood concentrations of carotenoids.

SUBJECTS AND METHODS

Subjects

The protocols of both studies were approved by our local Research Ethics Committee. Volunteers were recruited from employees of our laboratory and from inhabitants of Vlaardingen and surroundings. Subjects gave their written informed consent before participation.

Eligible subjects were healthy as assessed by a medical examination. The volunteers did not use a medically prescribed diet or weight-loss regime and had been weight-stable for ≥ 1 month before the start of the study. Females were non-pregnant and non-lactating.

Subject characteristics for both studies are shown in Table 1. Twenty-one volunteers entered the high-dose crossover study, nine males and two females were assigned to the group starting with the SPE treatment and nine males and one female to the group starting with the control treatment. Only three volunteers smoked. The volunteers ranged in age from 23 to 55 years and their Quetelet Index at the start of the study was between 20.4 and 27.2 kg/m^2. In the low-dose study, 53 volunteers participated. Twelve male and 14 female volunteers were assigned to the SPE group and 12 males and 15 females to the control group. None of the volunteers in the SPE-group smoked, whereas four volunteers in the control group smoked. Age ranged from 19 to 64 years and Quetelet Index was between 20.0 and 28.4 kg/m^2.

Experimental design

For the high-dose study we used a randomised double-blind placebo-controlled 4-wk crossover design to evaluate the effect on plasma cholesterol, triacylglycerol, vitamin E, β-carotene, lycopene, β-cryptoxanthin, lutein and zeaxanthin concentrations, of consumption at the main meal of 31 g margarine/d, containing 12.4 g SPE. The order of the treatments was randomised and balanced for sex as much as possible. No washout period between the two treatments was included. SPEs are unabsorbed and no metabolites of the preceding treatment were expected to circulate in the subject's body during the following treatment period. We calculated that 19 volunteers would be sufficient

to detect a difference of 15% between both treatments in plasma β-carotene concentrations with a power of 90% at a confidence of 5%.

Six months after the first study we carried out a low-dose SPE study. In this study a randomised double-blind parallel comparison trial of 4 wk was used to assess the effect on plasma cholesterol, triacylglycerol and β-carotene and lycopene concentrations of consumption at the main meal of 7.5 g margarine/d, containing 3 g SPE. (These carotenoids were found to be most responsive to SPE in the high-dose study.) Volunteers were randomly assigned to the control or SPE group, ensuring an equal distribution of both sexes over the groups. Both groups were matched for mean age, Quetelet Index and mean prestudy plasma concentration of total carotenoids. Using data from the first study we calculated that 23 volunteers per group would provide a power of 90% to detect at a confidence of 5% a difference of 20% in changes of plasma β-carotene concentration between both groups.

Table 1 Descriptive characteristics of volunteers in the high-dose sucrose polyester (SPE) (whole group) and low-dose SPE groups[1].

Characteristic	High dose (n = 18 m, 3 f)	Low dose	
		SPE group (n = 12 m, 14 f)	Control group (n = 12 m, 15 f)
Age (y)	39.1 (10.5)	37.9 (12.4)	37.8 (11.1)
Body weight (kg)	77.7 (8.4)	72.3 (9.5)	73.6 (9.5)
Body height (m)	1.80 (0.096)	1.73 (0.083)	1.75 (0.11)
Quetelet index (kg/m^2)	24.0 (1.8)	24.0 (0.073)	24.0 (1.8)
Plasma total carotenoids (extinction at 447 nm)[2]	na[3]	0.25 (0.073)	0.26 (0.086)
Number of smokers[4]	3	0	4

[1] Mean (SD)

[2] Assessed as described by Oliver and Kafwembe (1993)

[3] Not assessed

[4] Maximum of seven cigarettes per day

Experimental foods

Subjects were instructed to consume the margarine together with the main meal. We expected that the main meal, because of its vegetable content, would contain the largest amount of carotenoids compared to the other meals of the day. The concurrent ingestion of SPE and carotenoids was hypothesized to maximize the effect of a single dose of SPE on plasma carotenoid concentrations. Margarines and SPEs were prepared in our laboratory and tubs containing the appropriate amount of margarine were labelled with a

blind product code. Table 2 shows the composition characteristics of the margarines. No carotenoids were added to the margarines. Both types of margarine were identical in appearance. Compliance with treatment was assessed by asking the volunteers about margarine consumption and by weighing left-overs from the tubs.

Table 2 Composition of experimental margarines, based on analytical data.

	High dose margarines		Low dose margarines	
Component	SPE	Control	SPE	Control
		% by wt		
Total fat	80.4	80.6	80.7	81.4
Sucrose polyester	40.0	0	40.3	0
Digestible fat	40.4	80.6	40.4	81.4
Saturated fatty acids	6.8	25.2	7.0	22.1
Monounsaturated fatty acids	18.3	38.5	22.9	43.7
Polyunsaturated fatty acids	15.3	16.9	10.5	15.6
α-Tocopherol	0.0124	0.0108	0.00922	0.00896
Water	19.6	19.4	19.3	18.6

Lifestyle and dietary intake

Volunteers were requested to keep their normal lifestyle during the entire experiment. Changes in dietary intake of carotenoids and vitamin E were assessed from a self-completed food frequency questionnaire that was completed at the end of each period of the crossover study and at the start and end of the parallel study. The food-frequency questionnaire contained 114 items, which represented the main sources of carotenoids and vitamin E and vitamin supplements. The difference in β-carotene, other carotenoids and vitamin E intake was estimated as far as possible by available food-composition data (Voorlichtingsbureau van de Voeding, 1989; TNO Nutrition, 1990; Reed Mangels et al, 1993).

Measures

In the high-dose crossover study, fasting venous blood samples were taken before the study started and at 3 and 4 wk of each treatment. At each point in time, two blood samples were taken, one d apart, to obtain more accurate estimates of true plasma carotenoid concentrations of the individual. Plasma samples for analysis of plasma concentrations of β-carotene, lycopene, β-cryptoxanthin, lutein, zeaxanthin and α-tocopherol were stored under argon at -70°C until analyzed, but no longer than 4 mo (Craft et al, 1988). After extraction, β-carotene and lycopene were separated by HPLC on a Nucleosil 5-$N(CH_3)_2$ column (Machery & Nagel, Duren, Germany) with *n*-heptane as the

mobile phase. The chromatographic system for α-tocopherol, β-cryptoxanthin, lutein and zeaxanthin consisted of a Nucleosil 5CN column and a mobile phase of *n*-heptane/dichloromethane/isopropanol (90:10:0.2, v/v) for 5 min followed by *n*-heptane/dichloromethane/isopropanol (90:10:2, v/v) for 20 min at a flow rate of 1 mL/min. Total cholesterol and triacylglycerol in plasma were determined by enzymatic methods using commercial test kits from Boehringer (Mannheim, Germany). Body weight was assessed before the start of the high-dose study and after 4 wk of each treatment, with the subjects wearing light indoor clothing and no shoes and after voiding. In the low-dose parallel comparison study, a fasting venous blood sample was taken prior to the study for plasma concentration of total carotenoids (Oliver & Kafwembe, 1993), to match control and treatment groups for total plasma carotenoids concentration at randomisation. In the low-dose study, additional blood samples were taken once before the start and once after the treatment. Only β-carotene, lycopene, total cholesterol and triacylglycerol were determined. Body weight was assessed before and after the treatment.

Statistical analyses

In the high-dose study the mean of the analyses of the two blood samples was used in the statistical analyses. Statistical analyses were performed on plasma concentrations of carotenoids, cholesterol and triacylglycerol as well as on normalized plasma concentrations of carotenoids. Normalized plasma concentrations of carotenoids were calculated as follows: [carotenoid concentration/(cholesterol + triacylglycerol concentration)]. Adjustment of plasma carotenoid concentrations for concentrations of cholesterol and other lipids may provide a better reflection of dietary intake of carotenoids (Willet et al, 1983). However, expression of the plasma carotenoid concentrations as normalized levels revealed no different effects of SPE compared with the results of the unadjusted concentrations. Only the results of the unadjusted plasma carotenoid concentrations are therefore shown. For the statistical evaluation of the treatment effect, plasma carotenoid concentrations in the high-dose study and plasma lycopene concentrations in the low-dose study were log-transformed to minimize correlation between mean and SD of the analysed variables. However, descriptive statistics are shown for untransformed data. Body weight after 4 wk; plasma carotenoid, vitamin E, cholesterol and triacylglycerol concentrations after 3 and 4 wk; and dietary intake of carotenoids and vitamin E during the 4 wk were compared by analysis of variance for differences between the treatments. Both treatment and period effects along with any interaction were assessed. Student's *t*-test for paired data was performed on the difference between plasma concentrations after 3 and 4 wk of SPE treatment, to assess whether a plateau was reached in the change of plasma carotenoid and vitamin E

concentrations. In the parallel comparison design, the mean change in body weight and plasma concentrations after 4 wk and the mean change in dietary intake of β-carotene and lycopene during the treatment period compared with the 4 wk before the start of the experiment in the SPE group was compared by Student's *t*-test with the mean change in body weight, plasma concentrations, and dietary intake of β-carotene and lycopene found in the control group. Differences in baseline concentrations between sexes were also compared by Student's *t*-test. Analysis of variance was used to assess the effect of sex on the treatment effect. To assess the validity of the food-frequency questionnaire, Pearson's correlation coefficients between baseline dietary intake and plasma concentrations of β-carotene and lycopene were calculated from the data of the low-dose study because of the larger number of volunteers. SAS computer software was used for all statistical calculations (Statistical Analysis System Institute, 1987). All comparisons were made at the two-sided 0.05 significance level.

RESULTS

In both studies, all volunteers completed the entire experiment. In the high-dose study none of the volunteers used dietary supplements. In the low-dose study, one volunteer in the SPE group consumed four times a week a supplement containing vitamin A, C, D and E and 6 mg β-carotene per capsule. In the control group three volunteers used supplements, but none of these contained carotenoids. There was no effect of SPE on body weight, neither in the crossover study (P=0.50) nor in the parallel comparison investigation (P=0.15).

Compliance with treatment

The mean daily intakes of margarine in the high-dose study were 31.3 g (range 30.6 - 31.8 g) and 31.4 g (range: 30.7 - 31.9 g) for the SPE and control treatments, respectively. Mean SPE intake was 12.4 g/d. In the low-dose study, the mean daily intake of margarine was 7.4 g (range: 7.2 - 7.6 g) for both the SPE and control treatments. Mean SPE intake was 3 g/d. All volunteers reported to have consumed the experimental margarines for ≥ 26 d in the high-dose study, and for ≥ 27 d in the low-dose study. In the high-dose study a maximum of three main meals was used by a particular volunteer without adding the SPE-containing margarine. In the low-dose study this occurred for a maximum of two meals.

Dietary intake of carotenoids and vitamin E

The mean estimated intake of carotenoids and vitamin E did not differ significantly between the treatments in the high-dose study (carotenoids: P=0.3, vitamin E: P=0.2). In the low-dose study, the change in estimated mean intake of β-carotene in the control group was not significantly different from the change in the SPE group (P=0.3). In both groups, estimated intake of lycopene did not change during the study. β-Carotene and lycopene intakes correlated significantly with plasma concentration of β-carotene (r=0.27, P=0.05) and lycopene (r=0.39, P=0.004).

Plasma carotenoid and serum lipid concentrations

Table 3 shows plasma carotenoid concentrations for both studies. All plasma carotenoid concentrations were significantly reduced after 4 wk consumption of the SPE-containing margarine compared with the control margarine for both the high- and the low-dose study. In the high-dose study plasma cholesterol and vitamin E concentrations were also significantly lowered by SPE. The greatest reductions in the high-dose study were observed for β-carotene (-0.13 μmol/L, P=0.0001; 95% CI, -0.088, -0.18 μmol/L) and for lycopene (-0.14 μmol/L, P=0.0001; 95% CI, -0.070, -0.20 μmol/L). These differences correspond to a decrease of 34% and 52%, respectively, compared with the control margarine. The reductions of the hydroxylated carotenoids, β-cryptoxanthin, lutein and zeaxanthin were somewhat smaller: 23%, 20% and 18%, respectively. Plasma vitamin E concentrations decreased by 13%. Figure 1 shows the individual responses in the high-dose study for β-carotene and for lycopene only. Nearly all individuals showed a decrease in plasma concentrations of these carotenoids. The reductions were already significantly different between the SPE-containing and the control margarine after 3 wk. A significant further reduction in the fourth week compared with the value in the third week was shown only for zeaxanthin (-0.0023 μmol/L, P=0.02).

The results of the high-dose study showed that β-carotene and lycopene were most susceptible to the effect of SPE. In the low-dose study, therefore, only these carotenoids were assessed. Also in this study, SPE significantly reduced plasma concentrations of β-carotene and lycopene. Plasma concentrations of β-carotene and lycopene were reduced by -0.094 μmol/L (P=0.0001; 95% CI, -0.031, -0.16 μmol/L) and -0.12 μmol/L (P=0.0001; 95% CI, -0.070, -0.17 μmol/L). These reductions correspond with 20% and 38%, respectively. Figure 2 shows individual responses in the low-dose study for both treatments, separately for males and females and smokers and non-smokers. As expected, responses in the control were evenly distributed around the baseline. In the SPE group nearly all subjects had reduced plasma levels compared with baseline. No

significant differences were observed in the response and baseline concentrations of β-carotene and lycopene between males and females.

The serum total cholesterol concentration decreased significantly by ca. 6% after consumption of the high dose of the SPE- containing margarine compared with the control margarine. Serum triacylglycerol concentrations were not affected by the treatment in either study.

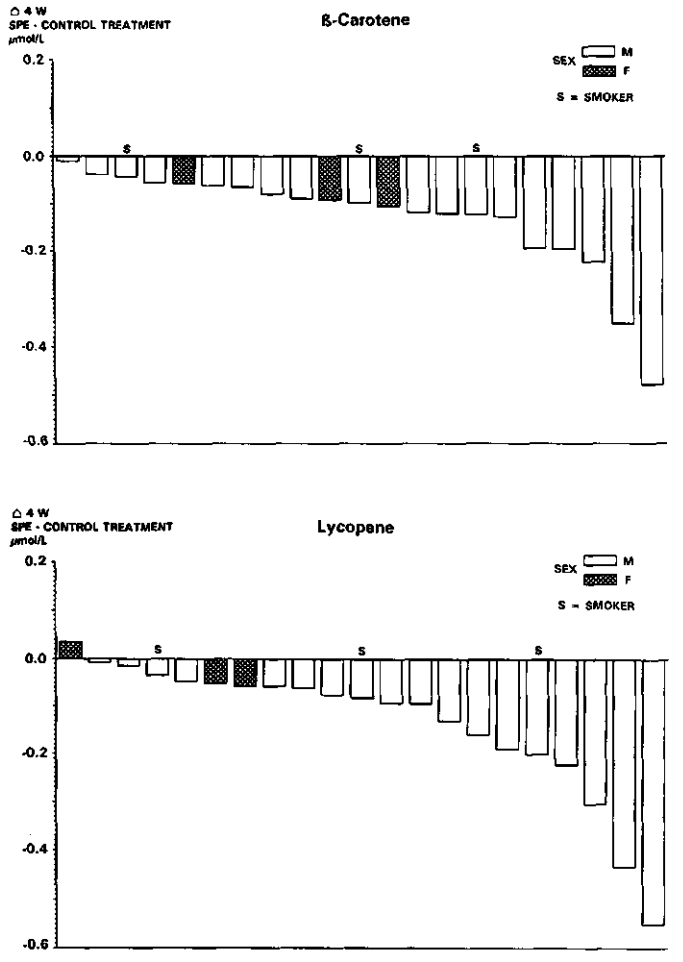

FIGURE 1 Individual differences in plasma β-carotene and lycopene concentrations of volunteers consuming a margarine containing 12.4 g/d of a nonenergy-containing fat substitute, sucrose polyester (SPE), compared with a margarine without SPE (control). Each bar represents an individual difference between the SPE and control treatments.

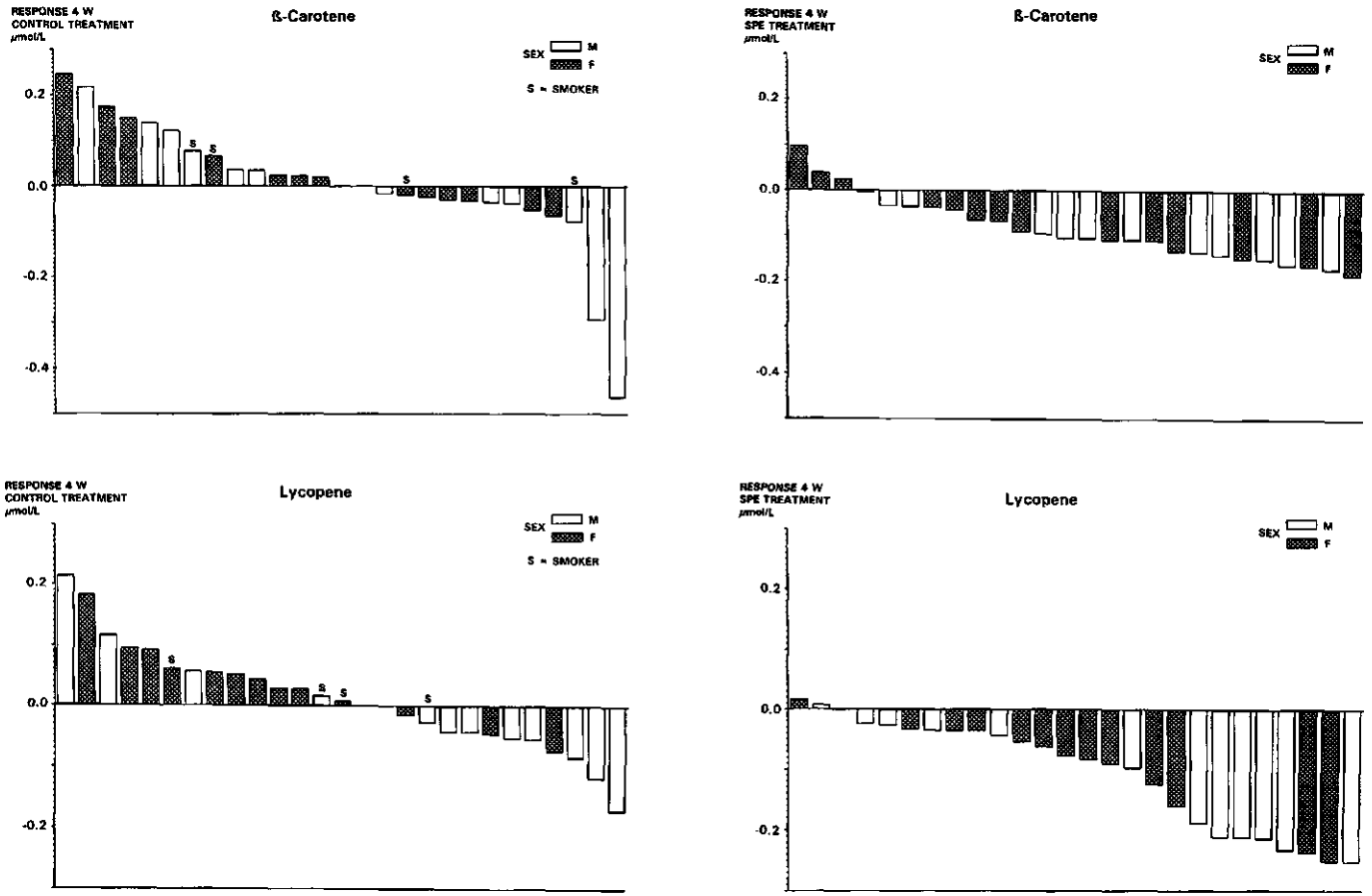

FIGURE 2 Individual differences in plasma β-carotene and lycopene concentrations of volunteers consuming either a margarine containing 3 g/d of a nonenergy-containing fat substitute, sucrose polyester (SPE), or a margarine without SPE (control). Each bar represents an individual difference between plasma concentrations at the end of the experiment and the start.

Table 3 Plasma concentrations of β-carotene, lycopene, β-cryptoxanthin, lutein, zeaxanthin, α-tocopherol, and cholesterol before and after 4 wk of consumption of a high or low dose of either sucrose polyester (SPE) or control margarine[1].

	Baseline value,	SPE treatment		Control treatment		Test for difference SPE vs control treatment	
Study variable	whole group	Week 0	Week 4	Week0	Week 4	Difference (95% CI)	P[2]
High-dose study							
(12.4 g SPE/d; n=21)							
β-Carotene (μmol/L)	0.38 (0.046)		0.25 (0.028)		0.38 (0.046)	-0.13 (-0.18, -0.088)	0.0001
Lyocpene (μmol/L)	0.22 (0.018)		0.13 (0.016)		0.27 (0.042)	-0.14 (-0.20, -0.070)	0.0001
β-Cryptoxanthin (μmol/L)	0.25 (0.037)		0.18 (0.026)		0.24 (0.034)	-0.055 (-0.086, -0.025)	0.0002
Lutein (μmol/L)	0.16 (0.012)		0.12 (0.010)		0.15 (0.012)	-0.030 (-0.048, -0.012)	0.0003
Zeaxanthin (μmol/L)	0.024 (0.0020)		0.016 (0.0010)		0.020 (0.0013)	-0.0035 (-0.0054, -0.0015)	0.001
α-Tocopherol (μmol/L)	24.8 (0.76)		23.8 (0.80)		27.2 (0.87)	-3.53 (-4.27, -2.79)	0.0001
Cholesterol (mmol/L)	4.68 (0.15)		4.53 (0.16)		4.81 (0.16)	-0.28 (-0.38, -0.18)	0.0001
Low-dose study							
(3 g SPE/d; SPE: n=26;							
control: n=27)							
β-Carotene (μmol/L)		0.46 (0.044)	0.37 (0.046)	0.58 (0.061)	0.58 (0.060)	-0.094 (-0.16, -0.031)	0.004
Lyoopene (μmol/L)		0.32 (0.031)	0.21 (0.024)	0.32 (0.029)	0.33 (0.034)	-0.12 (-0.17, -0.070)	0.0001
Cholesterol (mmol/L)		5.16 (0.18)	5.00 (0.19)	5.06 (0.22)	4.93 (0.24)	-0.028 (-0.18, 0.24)	NS

[1] Mean (SEM)

[2] Values are based on ANOVA for the high-dose study and on Student's *t*-test for the low-dose study

DISCUSSION

The present study clearly shows that daily consumption of even a very small quantity of the non-absorbable fat substitute SPE (3 g) strongly reduces plasma concentrations of carotenoids in healthy volunteers. We observed that the magnitude of the effect of a small dose of 3 g SPE/d is comparable to the one found with a higher dose of 12.4 g/d. This suggests that no linear dose-response relation exists and that SPE is a powerful sequestrant of lipophilic carotenoids. Our findings are important in view of the strong suggestions that carotenoids may protect against the development of cancer, coronary heart disease and cataracts and age-related maculopathy (Gerster, 1991; Gerster 1993; Sarma et al, 1994).

We believe that the observed effects cannot be ascribed to a reduction in the dietary intake of carotenoid-rich foods during the SPE treatment. Both studies had a double-blind, placebo-controlled design and our dietary intake data, validated against plasma carotenoid concentrations, indicated no differences between both treatments in the estimated dietary intake of carotenoids and vitamin E.

Baseline plasma concentrations of β-carotene and lycopene were different between both studies. This might be related to seasonal differences in the intake of carotenoid-rich vegetables or fruits. The high-dose study was performed during winter, whereas the low-dose study was executed in June.

It might be argued that the particular set-up of our studies reflects a worst-case scenario for the effect of a single ingestion of SPE on plasma carotenoid concentrations, because the main meal is the most important source of carotenoids of the day. The simultaneous presence of SPE and carotenoids in the lumen of the gut would maximize the chance for the carotenoids to become retained in the nonabsorbable fat phase and to be excreted in the faeces. We believe that a realistic consumption scenario of SPE in typical consumer goods will never exclude the possibility of concurrent ingestion of dietary carotenoids and such low amounts of SPE as used by our low-dose study.

The substantial drops of between 18% to 52% of the plasma concentrations of carotenoids we found in the high-dose SPE study suggest that a large part of the dietary carotenoids in the main meal was solubilised and retained in SPE rather than in the dietary fat fraction. The variability in response of plasma carotenoid concentrations to SPE was small in both studies, indicating that we have reliably estimated the effects of SPE.

Rock et al (1992) found a reduction of ca. 50% in the plasma concentrations of carotenoids after 12-13 d of a low-carotenoid diet. The changes we found were generally smaller than those reported by Rock et al. In the high-dose study we observed the largest

reductions in plasma concentrations of the most lipophilic carotenoids, β-carotene and lycopene. This indicates that partition in the gut of carotenoids between the lypophilic SPE phase and the more lypophobic bile salt-, phospholipids-, fatty acid- and monoacylglycerol-containing micellar phase is improved when carotenoids contain more polar groups.

We observed that 12.4 g SPE/d reduced plasma β-carotene levels from an average concentration of 0.37 μmol/L after the control margarine to 0.24 μmol/L. This is a greater difference than that measured between cancer patients and control subjects in prospective studies involving measurement of plasma β-carotene concentrations (Wald et al, 1988; Comstock et al, 1991). Also, an amount of 3 g/d of SPE was sufficient to reduce plasma concentration of β-carotene to the concentrations found in those who developed cancer (Comstock et al, 1991).

The results of our high-dose study suggested that after 4 wk of SPE the reduction in plasma concentrations of most carotenoids had reached a plateau. Other investigators have reported similar responses of blood carotenoid concentrations when SPE was given over 3 mo to volunteers (DH Thurnham, personal communication, 1993).

In conclusion, we showed that SPE, in relatively small quantities of 3-12 g/d, causes substantial decreases in blood concentrations of major carotenoids. In view of the evidence that carotenoids may have positive effects on health, decreases of the magnitude we observed are undesirable.

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8

Comparison of the bioavailability of natural palm oil carotenoids and synthetic β-carotene in humans

Karin H. van het Hof, Christine Gärtner, Anton Wiersma, Lilian B.M. Tijburg, Jan A. Weststrate

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ABSTRACT

Palm oil carotenoids are a mixture of α- and β-carotene which are used as a food colorant. They may also be applied as a functional foods ingredient because of the provitamin A activity of α-carotene and β-carotene and their proposed beneficial roles in prevention of chronic diseases. We executed an incomplete balanced cross-over study with 69 healthy adult volunteers to compare palm oil carotenoids with synthetic β-carotene in their efficacy to increase plasma levels of carotenoids. Four days supplementation with natural palm oil carotenoids (7.6 mg/d α-carotene, 11.9 mg/d all-*trans* β-carotene, 7.5 mg/d *cis* β-carotene) or synthetic β-carotene (23.8 mg/d all-*trans* β-carotene, 4.4 mg/d *cis* β-carotene), added to a mixed meal, resulted in significant increases in plasma levels of the supplied carotenoids as compared to consumption of a low-carotenoid meal (i.e. 7.2-fold increase in α-carotene and 3.5-fold increase in all-*trans* β-carotene following palm oil carotenoids; 6.9-fold increase in all-*trans* β-carotene following synthetic β-carotene). As the carotenoid content differed between the treatments, we calculated the relative plasma responses per mg β-carotene intake. These were similar for the two supplements, suggesting that the presence of α-carotene does not affect the bioavailability of β-carotene from palm oil. We conclude that four days supplementation with palm oil carotenoids or synthetic β-carotene improves the plasma β-carotene status substantially, whereas α-carotene is additionally delivered by the palm oil supplement.

INTRODUCTION

Carotenoids may play a beneficial role in human health beyond their provitamin A function. Several biological activities of carotenoids have been demonstrated *in vitro* or *in vivo*, such as scavenging of free radicals, singlet oxygen quenching (Burton & Ingold, 1984; Sies & Stahl, 1995), enhancement of intercellular communication (Zhang, 1991) and immunomodulatory effects (Santos et al, 1996). Among the major carotenoids present in the human body (i.e. α-carotene, β-carotene, lycopene, lutein, zeaxanthin, α-cryptoxanthin and β-cryptoxanthin), β-carotene has been studied most extensively. Interest in other carotenoids is growing, particularly since several intervention studies showed no protective effect of supplementation with high doses of β-carotene (ATBC Cancer Prevention Study Group, 1994; Hennekens et al, 1996; Omenn et al, 1996). Recent epidemiological studies have indicated beneficial effects of α-carotene (Ziegler et al, 1996), lycopene (Giovannucci et al, 1995) and lutein (Seddon et al, 1994).

In the light of the emerging interest in carotenoids other than β-carotene, it is important to increase the knowledge on the bioavailability and metabolism of these carotenoids. Palm oil is a rich source not only of β-carotene, but also of α-carotene and palm oil carotenoids are currently used as a food colorant. Ziegler et al (1996) recently postulated that α-carotene may be more effective in reducing the risk of lung cancer than β-carotene. In line with their observation, *in vitro* and animal studies have shown that α-carotene is a more potent inhibitor of cancer cell proliferation than is β-carotene (Murakoshi et al, 1992; Levy et al, 1995). Little is known about the bioavailability of α- and β-carotene from palm oil and it may well be that the two carotenoids compete for absorption as has been suggested for lutein and β-carotene (Kostic et al, 1995; Van den Berg & Van Vliet, 1998).

The objective of the present study was to compare the changes in plasma concentrations of α- and β-carotene following 4-day consumption of palm oil carotenoids with the changes following consumption of synthetic β-carotene alone.

MATERIALS AND METHODS

Volunteers

A total of 72 apparently healthy volunteers, aged 18-65 y, were enrolled in the study. They did not use dietary supplements (e.g. vitamins, minerals, carotenoids), consume a medically prescribed or weight loss diet or use excessive amounts of alcohol (i.e., ≤ 21 glasses/wk for females; ≤ 28 glasses/wk for males; ca. 10 g alcohol/glass), and they smoked maximally 15 cigarettes/d. The women were not pregnant or lactating. Volunteers

were employees of our laboratory or inhabitants of the Vlaardingen area and they gave their written informed consent before participation.

Study design

In an incomplete cross-over design of four experimental periods, 72 volunteers received a palm oil carotenoid supplement, a synthetic β-carotene supplement, no supplement (control) or one of four other supplements. The results of the latter treatments are outside the scope of this paper and will be reported separately. All volunteers received the control treatment during one of the experimental periods. The treatments were supplied randomized over the volunteers and in randomized order. The carotenoid supplements were added to a standard hot meal which was consumed at lunch time on four consecutive days. Fasting plasma levels of carotenoids were assessed at the end of the four days. These experimental periods were separated by 10 days of wash out, during which volunteers returned to their habitual diet. Volunteers were instructed not to consume any vegetables, fruits, fruit juices or red sauces (e.g. tomato ketchup, pizza) during the experimental periods. Compliance was assessed by questionnaire and the experimental meals were consumed under supervision in the laboratory.

Carotenoid supplements

The palm oil carotenoids (Vegex Natural Carotene, 30% suspension in oil, Quest International, Ireland) or synthetic all-*trans* β-carotene (β-carotene 30% FS (E160a), 30% suspension in oil, Hoffmann-La Roche, Switzerland) were consumed with a standard meal. This was a pasta meal with ham and a white sauce and custard for dessert. The carotenoids were added to the sauce at the end of the preparation and the sauce remained heated until being served to the volunteers (ca. 80 °C, 5-30 min). Energy and fiber content and macronutrient composition of the meal were similar to that of an average Dutch main meal (Voorlichtingsbureau voor de Voeding, 1993) (Table 1). Carotenoid extraction from the control and supplemented sauces was done according to Hart & Scott (1995). Extracts were appropriately diluted in HPLC solvent A (methanol/acetonitrile/2-propanol (54/44/2, v/v/v)). Analysis was performed on a 5 µm Suplex pKb 100 column (250 x 4.6 mm, Supelco, Bellefonte, PA), using a step gradient: 0-10 min 97% solvent A and 3% water, 10-25 min 100% solvent A, with a flow rate of 1 ml/min and detection at 450 nm. Peaks were identified spectrophotometrically by diode array detection (model 168, Beckman, Munich) and by coelution with synthetic reference carotenoids. Response factors determined for our HPLC system were used to calculate the carotenoid contents of the sauces. Reference carotenoids were either a gift from Makhteshim Chemical Works (Beer Sheva, Israel) (lycopene) and Hoffmann-La Roche (Basel, Switzerland) (lutein,

zeaxanthin) or purchased from Sigma (Deisenhofen, Germany) (α-carotene). All other chemicals were obtained from Merck (Darmstadt, Germany). Results of the analyses are shown in Table 1. The synthetic β-carotene supplement contained small amounts of *cis*-isomers, probably due to isomerisation during heating of the sauce. As we intended to supply similar amounts of total carotenoids, the β-carotene content of the palm oil carotenoids supplemented meal was lower than that of the synthetic β-carotene supplemented meal.

TABLE 1 Macronutrient and carotenoid content of the experimental meals[1].

Component	Type of experimental meal		
	Control	Palm oil carotenoids[2]	Synthetic β-carotene[3]
Fat (g)	19.8 (1.3)	21.6 (0.7)	21.8 (1.1)
Carbohydrate (g)	82.1 (5.4)	84.5 (2.6)	84.1 (3.1)
Protein (g)	32.4 (0.7)	32.4 (1.5)	33.8 (1.7)
Fiber (g)	16.0 (1.1)	16.1 (1.1)	15.7 (0.5)
α-Carotene (mg)	–[4]	7.6 (0.7)	–[4]
all-*trans* β-Carotene (mg)	–[4]	11.9 (1.3)	23.8 (3.1)
9-*cis* β-Carotene (mg)	–[4]	5.3 (0.8)	0.48 (0.04)
13-*cis* β-Carotene (mg)	–[4]	2.2 (1.0)	3.9 (0.8)

[1] Values are expressed as mean (SD) per daily serving (*n*=3-5)

[2] Quest International, Ireland

[3] Hoffmann-La Roche, Switzerland

[4] < 0.3 mg/serving

Note: the amounts of lutein, zeaxanthin and lycopene were each < 0.3 mg/serving in all of the meals

Plasma and serum analyses

Blood samples, obtained while subjects were fasting, were collected at the end of each experimental period into tubes coated with sodium EDTA and tubes containing a serum separator for serum preparation. Plasma and serum were prepared by low-speed centrifugation (1500 x g for 10 min at 4°C). Plasma was stored at -70°C under argon for analysis of carotenoids, and serum at -20°C for analysis of lipids.

Extraction of carotenoids from plasma was performed as described by Wingerath et al (1995). Dry carotenoid residues from plasma extraction were redissolved in HPLC solvent

A (methanol/acetonitrile/2-propanol (54/44/2, v/v/v)) and analysis was performed using the same HPLC system as described above.

Total cholesterol and triacylglycerol concentration in serum were assessed by using commercially available colorimetric test kits (CHOD-PAP, Boehringer, Mannheim, Germany and GPO-PAP (Roche, Basel, Switzerland)/GPO-Trinder (Sigma, St. Louis, USA), respectively).

Statistical evaluation

Analysis of variance with persons as blocks and sex, smoking habits, period, treatment, treatment x sex and treatment x smoking as factors, was used to compare the plasma and serum values found after consumption of the supplemented meals with those found after consumption of the control meal. Significance of the differences was assessed by Dunnett's test (α=0.05). As sex and smoking had no significant effect, these variables were excluded from the ANOVA model. Differences between the two carotenoid supplements were assessed by orthogonal contrasts (α=0.05). Plasma carotenoid concentrations were log-transformed to minimize correlation between mean values and standard errors and the geometric means are presented with the standard error as percentage of these means.

RESULTS

Three volunteers dropped out of the study before the end of the first treatment period because of lack of time to participate in the trial and four volunteers were not able to participate in each of the experimental periods for various reasons (e.g. illness, business trip). Two of these latter volunteers did not receive the control treatment. Data of 31 males and 38 females were included in the statistical analyses, n=67 received the control treatment, n=31 the palm oil carotenoids supplement and n=28 the synthetic β-carotene supplement. The average age (SD) of the volunteers was 42 (13) y and their mean Quetelet Index (SD) was 24.6 (2.3) kg/m^2. Ten of the 69 volunteers were smokers (maximum 15 cigarettes/d).

Carotenoid concentrations in plasma as determined at the end of the experimental periods, are shown in Table 2. Unfortunately, the carotenoid supplements induced a carry-over effect in plasma concentrations of α- and β-carotene. The plasma levels of α- and β-carotene, found in the first and second test periods following consumption of the supplements, were therefore excluded from the statistical evaluation (i.e., using a wash-out period of 38 days). For α-carotene, this extended wash-out period was only applied following consumption of the palm oil carotenoids supplement. No carry-over effect was

found for lutein, zeaxanthin and lycopene. The numbers presented in Table 2 are based on the data actually included in the statistical evaluation.

TABLE 2 Plasma carotenoid concentrations[1] after four days consumption of meals low in carotenoids (control meal) or supplemented with carotenoids.

	Control		Palm oil carotenoids		Synthetic β-carotene	
	N	Mean (CVM)	N	Mean (CVM)	N	Mean (CVM)
α-Carotene (µmol/L)	55	0.028 (3.7%)[a]	31	0.23 (5.4%)[b]	24	0.028 (6.0%)[a]
all-*trans* β-Carotene (µmol/L)	43	0.14 (4.5%)[a]	28	0.60 (5.9%)[b]	24	1.1 (6.6%)[c]
13-*cis* β-Carotene (µmol/L)	39	0.009 (8.6%)[a]	28	0.023 (11%)[b]	24	0.034 (12%)[c]
Lutein (µmol/L)	67	0.12 (2.2%)	31	0.13 (3.6%)	28	0.12 (3.8%)
Zeaxanthin (µmol/L)	67	0.031 (1.8%)	31	0.033 (2.9%)	28	0.031 (3.1%)
Lycopene (µmol/L)	67	0.12 (3.5%)	31	0.13 (5.7%)	28	0.14 (6.1%)

[1] Plasma carotenoid concentrations were log-transformed to minimize correlation between mean values and standard errors and the geometric means are presented with the standard error as percentage of these means (CVM)

[a,b,c] Means with different superscripts are significantly different ($P<0.05$)

Note: due to a carry over effect of the carotenoid supplements, we excluded part of the data on α-carotene and β-carotene (see Results section). The values presented are based on the actual data included in the statistical evaluation.

As compared to the control meal, consumption of the carotenoid supplements resulted in significantly increased plasma levels of all-*trans* β-carotene (mean (95% CI): 345% (267, 439) for palm oil carotenoids; 686% (539, 867) for synthetic β-carotene) and 13-*cis* β-carotene (154% (76.8, 265) for palm oil carotenoids; 265% (149, 536) for synthetic β-carotene), whereas only consumption of the palm oil carotenoids supplemented meal induced a significant increase in plasma concentration of α-carotene (716% (590, 865)). Plasma concentrations of lutein, zeaxanthin and lycopene (Table 2) and serum lipid levels (data not shown) remained unchanged. Data of 9-*cis* β-carotene are not presented in Table 2 because almost half of the plasma concentrations measured were below the detection level (<0.002 μmol/L). However, after consumption of the palm oil carotenoids supplement, the percentage of volunteers with plasma concentrations of 9-*cis* β-carotene within the detectable range was significantly larger than after consumption of the control or synthetic β-carotene supplemented meals (84% for the palm oil carotenoids supplemented meal and 55% for the other two meals, P<0.005). None of the treatment effects were significantly different between males and females and smokers and non-smokers.

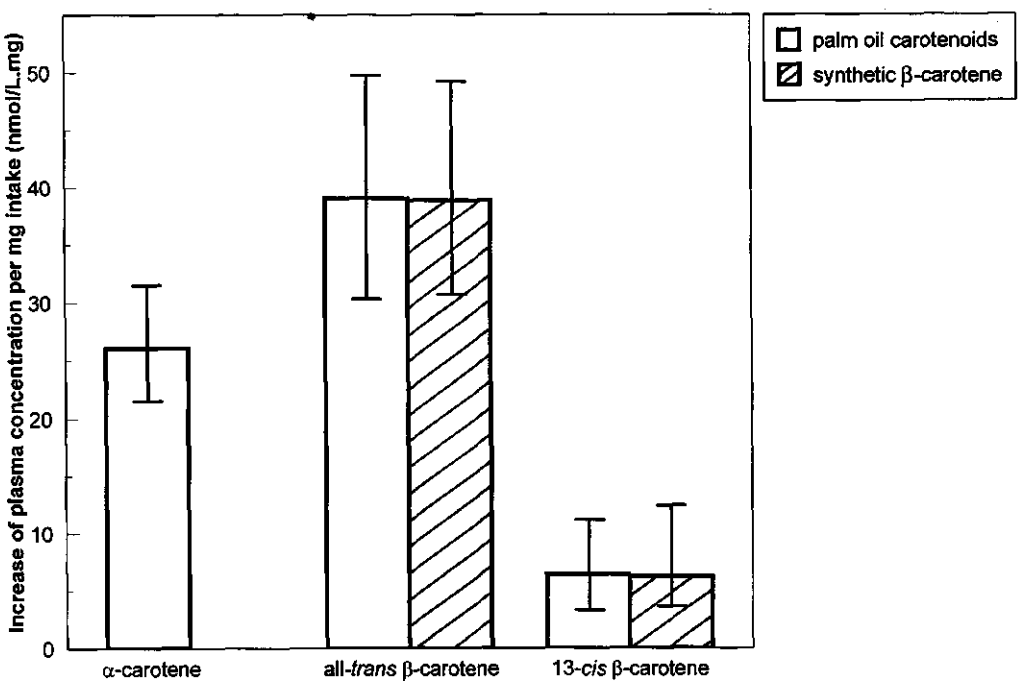

Figure 1 Plasma carotenoid response to four days consumption of palm oil carotenoids or synthetic β-carotene, as compared to a low-carotenoid diet, expressed per mg carotenoid intake (mean, 95% confidence interval).

Consumption of synthetic β-carotene resulted in a higher plasma level of all-*trans* β-carotene and 13-*cis* β-carotene than consumption of palm oil carotenoids (Table 2). On the other hand, consumption of palm oil carotenoids resulted in a 7-fold higher plasma level of α-carotene than consumption of the synthetic β-carotene supplement, which contained no α-carotene at all (Tables 1 and 2). The differences in increases of plasma levels of all-*trans* β-carotene and 13-*cis* β-carotene between the palm oil carotenoids and synthetic β-carotene supplement could be explained by the differences in composition of the two supplements. This is illustrated in Figure 1, which shows the increases in plasma concentrations of these carotenoids per mg carotenoid intake after consumption of the supplemented meals as compared to those after consumption of the control meal.

DISCUSSION

Four days supplementation with palm oil carotenoids or synthetic β-carotene, added to a standard hot meal, resulted in significantly increased plasma levels of the supplied carotenoids. The magnitude of the differences between the two supplements in increases of plasma concentrations of all-*trans* β-carotene and 13-*cis* β-carotene appeared to be proportional to the differences in level of intake (Figure 1).

No effect was found on plasma levels of lutein, zeaxanthin and lycopene. The standard meal contained no detectable amounts of these carotenoids (Table 1) and volunteers avoided carotenoid-containing foods during the rest of the experimental days. Hence it is unlikely that competition for uptake has occurred between these carotenoids and the supplemented carotenoids. In addition, these results suggest that four days supplementation with α- and/or β-carotene does not affect circulating lutein or lycopene levels and tissue uptake or metabolism of these carotenoids.

Our findings of a significantly increased plasma carotenoid status following supplementation with purified α-carotene and/or β-carotene are in line with other studies and the magnitude of the increases were within the ranges expected (Brown et al, 1989; Micozzi et al, 1992; Rock & Swenseid, 1992; Carughi & Hooper, 1994; Törrönen et al, 1996; Canfield et al, 1997). The relative differences in plasma levels of all-*trans* β-carotene and 13-*cis* β-carotene between the two carotenoid supplements were in line with the differences in composition of the supplements (Figure 1). Apparently, at this level of intake a proportional relation exists between intake of these carotene isomers and their plasma response. Other studies also found a proportional association between carotenoid intake and plasma responses after supplementation with 12 to 90 mg β-carotene (Dimitrov et al, 1986; Brown et al, 1989; Micozzi et al, 1992). In addition, our findings

reveal that all-*trans* and 13-*cis* β-carotene were equally available from the natural palm oil carotenoids supplement and the synthetic β-carotene supplement. Apparently, simultaneous ingestion of α-carotene had no effect on the response of plasma β-carotene levels following supplementation with palm oil carotenoids.

The present data do not allow to speculate about the effect of β-carotene on the bioavailability of α-carotene because we did not include supplementation with α-carotene only. Although the palm oil carotenoids supplement increased the plasma level of α-carotene significantly, the response per mg ingested was smaller than that of all-*trans* β-carotene (Figure 1). In a previous study the plasma response of α-carotene was more pronounced than that of β-carotene after four weeks of supplementation with palm oil carotenoids, resulting in similar final plasma concentrations for α- and β-carotene (Van het Hof et al, 1998). This difference may be due to the shorter period of supplementation in the present study. The kinetics of the plasma increase may differ between α- and β-carotene, with a slower rate of increase for α-carotene. As a new steady state may not have been reached in four days, the relative difference between α- and β-carotene may be affected by differences in kinetics between the two carotenoids.

The relative plasma responses were not only different for α- and β-carotene, they also varied between the isomers of β-carotene. No specific function has been reported for 13-*cis* β-carotene, whereas 9-*cis* β-carotene can be converted into 9-*cis* retinoic acid (Nagao & Olson, 1994; Wang et al, 1994), which is involved in the regulation of gene expression (Heyman et al, 1992; Levin et al, 1992). All-*trans* β-carotene is however the predominant isomer in plasma and the present study indicates that this is not entirely due to the fact that all-*trans* β-carotene is also the major isomer in the diet. As has been reported previously for 9-*cis* β-carotene (Stahl et al, 1993; Gaziano et al, 1995; Tamai et al, 1995; Ben-Amotz and Levy, 1996; Johnson et al, 1996; Von Laar et al, 1996; Yeum et al, 1996; You et al, 1996), the impact of increased intake of 9-*cis* and 13-*cis* β-carotene on their plasma levels was very low as compared to that of all-*trans* β-carotene. This may be due to inefficient intestinal uptake or degradation of the *cis* isomers, more extensive conversion to vitamin A, isomerisation to all-*trans* β-carotene or rapid uptake by tissue cells. You et al (1996) found evidence for isomerisation of 9-*cis* β-carotene to all-*trans* β-carotene. The present study does not support this hypothesis. Figure 1 shows that the increase in plasma level of all-*trans* β-carotene relative to the intake was similar for both carotenoid supplements. Based on the hypothesis of *cis-trans* isomerisation, a relatively larger increase would be expected after consumption of the palm oil carotenoids supplemented meal because of the higher content of *cis*-β-carotene isomers. However, the dosage used in this study may have been too large to detect such an effect.

We conclude that four days consumption of a meal supplemented with about 25 mg/d of either palm oil carotenoids (α- and β-carotene) or synthetic β-carotene improves plasma carotenoid status substantially. The presence of α-carotene does not affect the bioavailability of β-carotene from palm oil and may deliver additional benefits.

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9

General discussion and conclusions

INTRODUCTION

Carotenoids are thought to contribute to the inverse relationship between fruit and vegetable consumption and the risk of coronary heart disease and some types of cancer (Van Poppel, 1996). To increase our understanding of the potential benefits of carotenoids, it is important to obtain more insight in their bioavailability from foods and the factors that determine carotenoid bioavailability. This latter aspect may also lead to options for improvement of carotenoid bioavailability from foods and thus possibly their beneficial effects.

The absorption of carotenoids includes several steps, as described previously (Van het Hof et al, 1998) and shown in Figure 1. Factors that may interfere with the rate of each of these steps will impact on the overall bioavailability of the carotenoids ingested. The mnemonic "SLAMENGHI" describes these factors: Species of carotenoids, Linkages at molecular level, Amount of carotenoid, Matrix, Effectors, Nutrient status, Genetics, Host-related factors and Interactions between these variables (De Pee & West, 1996; Castenmiller & West, 1998). As this mnemonic has been proposed to determine the provitamin A value of carotenoids, the Species of carotenoids is of importance: two molecules of retinol may be formed from one molecule β-carotene, whereas other provitamin A carotenoids provide only one molecule of retinol. In addition, different carotenoids and carotenoid isomers may vary in absorbability as indicated by differences in responses of plasma concentrations to supplementation (Gaziano et al, 1995; Kostic et al, 1995; Ben-Amotz & Levy, 1996; Paetau et al, 1997; *Chapters 5 and 8*).

Linkages at a molecular level include esterification of carotenoids with fatty acids, such as lutein esters and β-cryptoxanthin esters, which are present in some fruits and vegetables (Khachik et al, 1991) and in marigold flowers from which lutein is extracted for use as food colourant or supplement (Granado et al, 1998).

The Amount of carotenoid ingested defines the maximum amount that may be absorbed. However, the efficiency of conversion to vitamin A or even of absorption may decrease with increasing amounts. This latter aspect seems to become important at very high doses only. A more or less proportional relation has been found between intake of β-carotene and its plasma response after supplementation with 12 to 90 mg β-carotene (Dimitrov et al, 1986; Brown et al, 1989; Micozzi et al, 1992). In contrast, 210 mg β-carotene supplemented in a single dose resulted in a similar increase in plasma β-carotene concentrations as 60 mg (Canfield et al, 1997). This suggests a plateauing of the dose-response curve beyond dosages of 60 to 90 mg β-carotene.

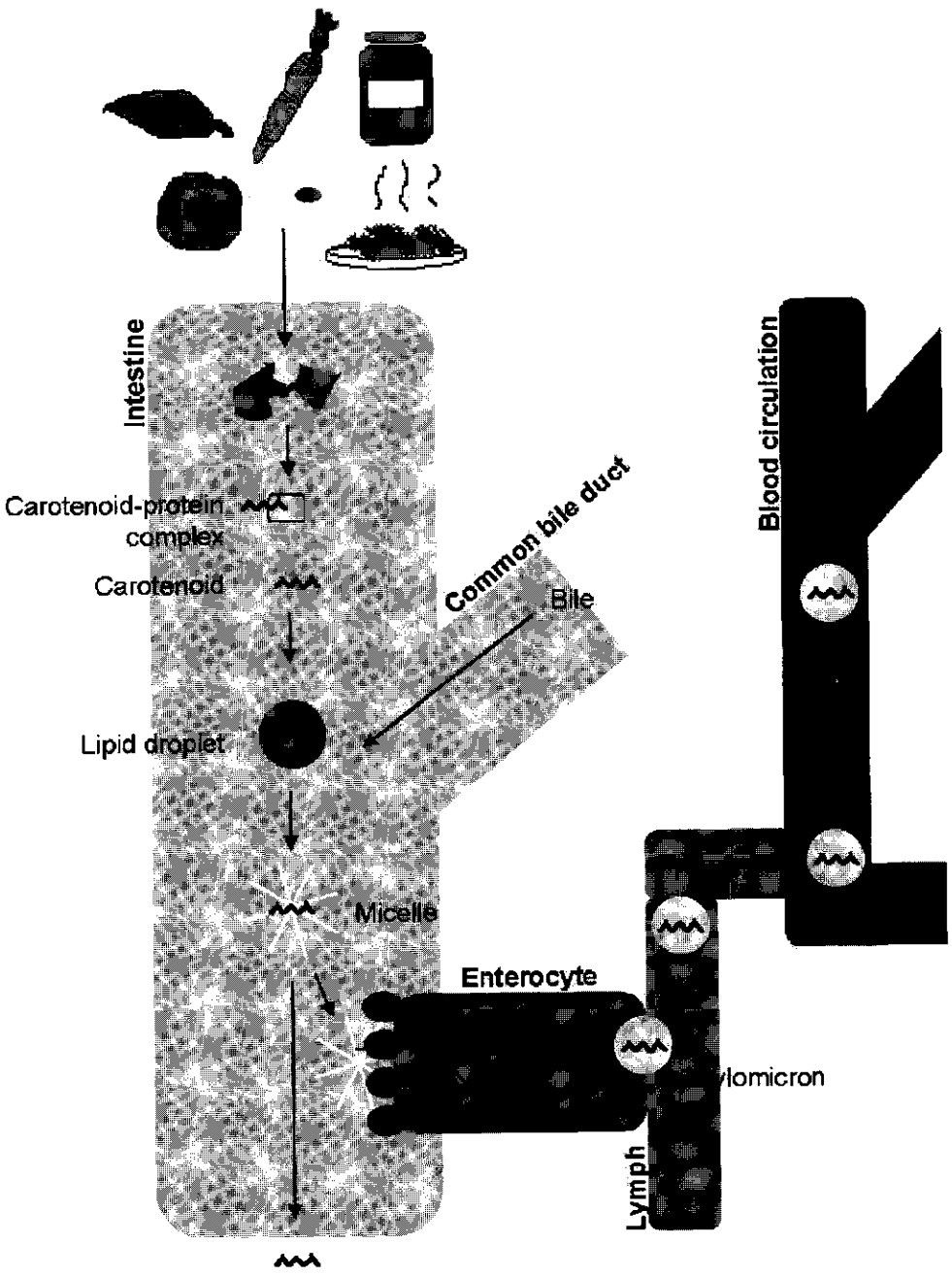

Figure 1 Steps of carotenoid absorption.

The Matrix in which the carotenoids ingested are incorporated is an important determinant of their bioavailability. This aspect of carotenoid bioavailability will be discussed in detail below.

Effectors include both bioavailability enhancing as well as inhibiting constituents in the diet. An enhancing agent is dietary fat, which is essential in the absorption process of carotenoids. Inhibitors may include other carotenoids. It has been speculated that interaction between carotenoids at the intestinal level may reduce the absorbability of some carotenoids. Such competition between carotenoids may also be considered as an effect of the Amount of carotenoids ingested.

Nutrient status, Genetics, Host-related factors are all related to the persons' characteristics. Interactions reflect the mathematical interactions among all factors of the SLAMENGHI-mnemonic.

The research described in this thesis focused on the effect of dietary factors, i.e. Matrix and Effectors, on the bioavailability of carotenoids. The following paragraphs will discuss our findings together with the current scientific literature in these areas.

IMPORTANCE OF FOOD MATRIX

Bioavailability of carotenoids from different food matrices

Disruption of the food matrix and release of carotenoids is the first step in carotenoid absorption. Several studies have indicated that the food matrix in which carotenoids are located is a major factor determining the bioavailability of carotenoids. Although consumption of carotenoid-rich foods (i.e. vegetables and fruits) is significantly associated with plasma concentrations of carotenoids in cross-sectional studies (e.g., Campbell et al, 1994; Yong et al, 1994; Scott et al, 1996; Drewnowski et al, 1997; Polsinelli et al, 1998), and increased vegetable and fruit consumption enhances plasma carotenoid concentrations (Yeum et al, 1996; Rock et al, 1997; Zino et al, 1997), the bioavailability of in particular β-carotene from vegetables has been shown to be low when compared to that of purified β-carotene added to a simple matrix (e.g. capsule, oil, sauce, salad dressing).

Table 1 summarises studies in humans in which the plasma response of carotenoids after supplementation with vegetables or fruits has been compared with the response to supplementation with pure carotenoids. As in some cases, the carotenoid intake differed among the different supplements, we divided the plasma responses by the daily intake of the carotenoids provided. Dividing the plasma responses, thus corrected for differences in intake, induced by vegetables or fruit consumption by those induced by pure carotenoid supplementation, provides a measure of "relative carotenoid bioavailability". This

calculation assumes a proportional linear relationship between the extent of carotenoid absorption and the plasma response. It should be noted, however, that the plasma response is the result of various processes, of which the extent of absorption at the intestinal level is only one. Tissue uptake and release, and metabolism of carotenoids, which may all be affected by the amount of a carotenoid entering the body, also interfere with the plasma response observed.

The relative bioavailability of β-carotene from vegetables as compared to purified β-carotene ranges between 3-6% for green leafy vegetables, 19-34% for carrots and 22-24% for broccoli (Brown et al, 1989; Micozzi et al, 1992; De Pee et al, 1995; Törrönen et al, 1996; Castenmiller et al, 1999; *Chapter 3*). β-Carotene from fruits was found to be 2.6-6 times more effective in increasing plasma concentrations of retinol and β-carotene than green leafy vegetables (De Pee et al, 1998). In fruits, such as mango and tomatoes, carotenoids are located in chromoplasts in their crystalline form (Gidley, personal communication). In this location, they are suggested to function primarily as colouring agent to attract insects and animals for reproductive reasons. In plant leaves, carotenoids are present in the chloroplasts where they play a role in light energy collection and photoprotection during photosynthesis (Britton, 1995). It was speculated that β-carotene is released more easily from the chromoplasts where they are located in fruits than from the chloroplasts in green leafy vegetables (De Pee et al, 1998). Chloroplasts may be less efficiently disrupted in the intestinal tract.

We found that broccoli and green peas induced a larger β-carotene response in plasma than whole leaf and chopped spinach, despite a ten times lower β-carotene content in the former vegetables (*Chapter 3*). The location of carotenoids in other parts of the plant than the leaf and fruit, such as the flower (e.g. broccoli) or seeds (e.g. green peas), has not yet been elucidated and probably they are located in chromoplasts.

The above mentioned studies indicate that the bioavailability of β-carotene may vary substantially, not only among vegetables, fruits and other foods, but also between different types of vegetables.

Few data are available on the relative bioavailability of carotenoids other than β-carotene from vegetables. We showed that the relative bioavailability of lutein from a diet supplemented with a variety of vegetables is much higher than that of β-carotene (i.e. 67% vs 14% respectively) (*Chapter 2*). The same was found for the relative bioavailability of lutein and β-carotene from spinach (i.e. 45% vs 5.1%, respectively) (Castenmiller et al, 1999). The release of lutein into an aqueous environment is probably higher than that of β-carotene, due to its lower lipophilicity compared to β-carotene. Also the bioavailability of

lutein seems lower from green leafy vegetables than from other vegetables, although the differences are less pronounced than those of β-carotene (*Chapter 3*)

The presence of dietary fibre in vegetables and fruits may partly explain the lower bioavailability of carotenoids from plant foods. It has been suggested that fibre interferes with micelle formation by partitioning bile salts and fat in the gel phase of dietary fibre. This may explain the effect of pectin on carotenoid bioavailability. A 60% reduction in plasma β-carotene response was observed after simultaneous ingestion of β-carotene with pectin as compared to the plasma response after β-carotene alone (Table 4) (Rock & Swenseid, 1992). A similar reduction was found in chicks, not only for pectin but also for other dietary fibres (Erdman et al, 1986).

In addition, the presence of carotenoids in protein complexes has been suggested as limiting for their bioavailability from e.g. vegetables (Erdman et al, 1993). These complexes have to be disrupted before carotenoids are dissolved in lipid droplets and incorporated into mixed micelles (Figure 1).

Effect of disruption of the food matrix

Not only the intracellular location, but also the intactness of the cellular matrix may be a determinant of carotenoid bioavailability from vegetables and fruits. Several data indicate that processing of vegetables (i.e. homogenisation and/or heat treatment), which disrupts the vegetable matrix, improves the bioavailability of carotenoids. Table 2 shows an overview of human and animal studies in which the effect of vegetable processing on carotenoid bioavailability has been assessed.

Effect of mechanical homogenisation

Van Zeben & Hendriks (1948) investigated the influence of homogenisation on the bioavailability of β-carotene from carrots in humans. After 3 weeks, the response in plasma β-carotene concentration was 4.9-fold greater in the group which received homogenised carrots than in the group which consumed whole carrots. Two later studies examined the effect of homogenisation of carrots to juice on carotene bioavailability as determined from the increase in plasma concentrations of retinol or β-carotene (Hussein & El-Tomahy, 1990; Törrönen et al, 1996). In vitamin A-depleted boys who consumed raw grated carrots or carrot juice for two weeks, plasma concentrations of retinol and β-carotene were slightly higher in the group consuming carrot juice (Hussein & El-Tomahy, 1990). However, no statistical evaluation was presented in this study and the β-carotene and retinol concentrations in plasma continued to decrease during the supplementation period in most of the volunteers. On the other hand, Törrönen et al (1996) showed no

significant difference in bioavailability of β-carotene from raw carrots or carrot juice when consumed by adult females for 6 wk, although the average increase in plasma β-carotene concentration was ca. 70% larger following consumption of carrot juice. However, they found a large variation in plasma carotenoid responses between individuals, which reduced the power to show significant effects.

There are also indications that disruption of the matrix affects the bioavailability of various carotenoids differentially. The plasma response of lutein was significantly increased by ca. 14% when spinach was consumed as chopped spinach in stead of as whole leaf spinach, whereas the plasma response of β-carotene was not affected (*Chapter 3*). There are several explanations for this finding. Firstly, the different lipophilic character of the two carotenoids, resulting in a greater release of lutein in response to homogenisation. Secondly, it may well be that homogenisation releases both carotenoids to the same extent but that lutein inhibits β-carotene absorption (Kostic et al, 1995; Van den Berg & Van Vliet, 1998). In contrast, however, Castenmiller et al (1999) found that disruption of the matrix of spinach by enzymatic treatment enhanced the plasma response of β-carotene (by 60-70%) but not of lutein. As the bioavailability of lutein from spinach, relative to a supplement, is higher than that of β-carotene (Castenmiller et al, 1999; *Chapter 2*), it can be speculated that the vegetable matrix is a less important determinant of the bioavailability of the less lipophilic lutein than that of β-carotene.

This difference in effectiveness of homogenisation to enhance carotenoid bioavailability between different carotenoids was also found for lycopene and β-carotene from tomatoes (*Chapter 4*). Homogenisation enhanced the plasma response of β-carotene only for tomatoes which had not received additional heat treatment (289%). This points to a maximum β-carotene bioavailability from tomatoes, which can be achieved by either homogenisation or heat treatment, whereas a combination of these treatments induces no further enhancement. This was not found for lycopene, for which the bioavailability was enhanced by homogenisation irrespective of the duration of heat treatment (21-62%). Possibly, due to the difference in lipophilic character, the maximum bioavailability of the less lipophilic β-carotene is achieved under milder processing conditions than that of lycopene. That would be in line with the differences between lutein and β-carotene observed by Castenmiller et al (1999).

Effect of heat treatment

Some studies have found that cooking enhances the carotenoid content measured in vegetables, possibly due to increased extractability of carotenoids from the vegetable matrix (Dietz et al, 1988; Granado et al, 1992; Khachik et al, 1992). This has led to the

suggestion that increased extractability due to heat treatment may be associated with improved bioavailability of carotenoids from the vegetable matrix (Erdman et al, 1988). This hypothesis has been tested in studies in which homogenised carrots, carrot juice and carrot chromoplasts were fed to preruminant calves and ferrets (Poor et al, 1993; Zhou et al, 1996). In these studies, however, heat treatment, involving steaming of the carrot products, did not result in increased levels of α- or β-carotene in serum, adrenals or liver. We found that heat treatment increased the bioavailability of β-carotene from whole tomatoes but not homogenised tomatoes (*Chapter 4*). As homogenised carrots were used as starting material in the previous studies, heat treatment may also be effective in increasing β-carotene bioavailabiltiy from unhomogenised carrots only.

Prolonged heating of tomatoes tended to improve the bioavailability of lycopene also, but the effects did not reach significance (*Chapter 4*). The effectiveness of heat treatment in enhancing lycopene bioavailability from tomato products may be enhanced if oil is added before the treatment. Stahl & Sies (1992) showed that consumption of tomato juice which had been heated in the presence of oil, resulted in a significantly larger increase in serum lycopene concentration than did consumption of the unheated mixture. Further, *in vitro* studies have indicated that addition of oil during processing enhances the partitioning of the lipophilic lycopene in the fat phase after release from the by heat treatment disrupted tomato matrix (Van het Hof et al, 1998).

Effect of a combination of homogenisation and heat treatment

Processing of tomatoes into tomato paste includes both mechanical homogenisation and heat treatment. There is evidence that this process is very effective in increasing lycopene bioavailability. The lycopene response in plasma or triglyceride-rich lipoproteins was 22-380% larger after consumption of tomato paste than when the same amount of lycopene was consumed as fresh tomatoes (Gärtner et al, 1997; Porrini et al, 1998). This supports the suggestion of Giovannucci et al (1995) that the association between consumption of various tomato products and risk of prostate cancer depends on the bioavailability of lycopene. An association was only found with consumption of tomato paste and not with consumption of unprocessed and minimally processed tomatoes. Our own data indicate that the disruption of the matrix caused by homogenisation and heat treatment, applied during the production of tomato paste from fresh tomatoes, may both contribute to this enhanced lycopene bioavailability (*Chapter 4*).

Rock et al (1998) found that the plasma response of β-carotene was enhanced after consumption of pureed, cooked carrots and spinach as compared to that after consumption of the vegetables in their raw, unhomogenised form (3-fold higher increase in plasma β-carotene).

In conclusion, carotenoids are less available from more complex matrices, such as vegetables and fruits than when ingested as pure compounds, dissolved in oil or added to a fat rich wafer or salad dressing. Disruption of the vegetable matrix, either by mechanical homogenisation or heat treatment enhances carotenoid bioavailability. However, as shown in particular for β-carotene, this will not result in similar bioavailability as observed for the pure compound.

IMPORTANCE OF AMOUNT AND TYPE OF DIETARY FAT

Effect of amount of dietary fat present

A second step in the absorption process of carotenoids which may affect their bioavailability involves the incorporation of released carotenoids into mixed micelles. Formation of these micelles is, among other factors, dependent on the presence of fat in the intestine. Therefore, ingestion of fat along with carotenoids is thought to be crucial. Table 3 shows studies in which the effect of dietary fat on the bioavailability of carotenoids (mainly β-carotene) has been investigated.

Various studies assessed the importance of dietary fat in comparison with its complete absence at the moment of ingestion of β-carotene (Jayarajan et al, 1980; Dimitrov et al, 1988; Prince & Frisoli, 1993). Under these circumstances absorption of β-carotene seems to be suboptimal, as the increases in plasma concentrations improved substantially when fat was added to the test meals. However, from the findings of Jayarajan et al (1980) it appears that 5 g fat in a meal is already sufficient to ensure carotenoid uptake. They found no difference in improvement of the vitamin A status when 5 g or 10 g of dietary fat was added to spinach, whereas 0 g fat resulted in less improvement. A recently reported study of Jalal et al (1998) indicates that the cut-off point lays between 3 and 5 g of fat. They observed a significantly smaller increase in serum retinol if 3 g fat was added to a sweet potato snack than if 18 g fat was added.

Under normal circumstances, in Western countries, the presence of dietary fat seems not to be limiting. The average fat content of a hot main meal in, for instance, the Netherlands is about 35 g (Voorlichtingsbureau voor de Voeding, 1993). In case of fruit or vegetable drinks (e.g. tomato juice), consumed between meals, without snacks, the amount of fat may however be too low. In addition, very low fat products which are designed to replace full-fat products may reduce the bioavailability of carotenoids present either in the meal or in the product itself. We investigated the bioavailability of carotenoids from a very low fat spread enriched with carotenoids and compared that with the effectiveness of a full-fat carotenoid enriched spread (*Chapter 6*). In a previous study we

had shown that a full-fat spread (80% fat) fortified with α-carotene and β-carotene was highly effective in enhancing plasma concentrations of these carotenoids (*Chapter 5*). The amount of fat present in the very low fat spread (3 g/meal) was sufficient to ensure uptake of α-carotene and β-carotene. However, with respect to lutein, which was added as lutein esters, the plasma response was ca. 100% higher following consumption of the full-fat spread (35 g/meal) than following the very low fat spread (*Chapter 6*). We speculated that the low amount of fat may have limited the solubilisation of lutein esters in the fat phase and/or the release and activity of esterases and lipase. These enzymes are crucial for the hydrolysis of lutein esters, required before absorption.

Effect of type of fat and digestibility of fat-soluble components present in the diet

It has been shown that if subjects consume fat-soluble components that are not or only to a limited extent absorbable, their plasma carotenoid concentrations may decrease substantially. Table 4 provides an overview of studies on this subject.

Sucrose polyester, a non-absorbable fat replacer, decreased plasma levels of carotenoids by 20-120%, depending on the amount of sucrose polyester and the type of carotenoid (Koonvitsky et al, 1997; Schlagheck et al, 1997; *Chapter 7*). The largest decreases were found for the most lipophilic carotenoids (i.e. lycopene and β-carotene). Apparently, carotenoids released from the food matrix were incorporated into the non-absorbable sucrose polyester rather than into the micelles that were formed from dietary fat. This is supported by the fact that the effect was less pronounced if participants were allowed to consume snacks containing sucrose polyester at their own will (Koonvitsky et al, 1997) rather than together with the major dietary sources of carotenoids, i.e. during the main meal (Schlagheck et al, 1997; *Chapter 7*).

Plasma carotenoid concentrations also reduced during consumption of dietary phytosterols (Weststrate & Meijer, 1998). Phytosterols are largely unabsorbable plant components which are used as cholesterol lowering agents. Currently, the mechanism by which phytosterols decrease carotenoid status has not been elucidated. Phytosterols could perhaps hinder the incorporation of carotenoids into mixed micelles by aspecific binding or solubilisation of carotenoids.

Borel et al (1998) recently reported that the type of fat present in the diet also influences carotenoid bioavailability. This could not be explained by a reduced absorbability of the fat itself. Medium chain triglycerides are absorbed primarily via the portal vein and thus the chylomicron formation is low after a meal containing only these type of triglycerides. Borel et al (1998) showed that if β-carotene was added to such a meal, the incorporation of β-carotene in chylomicrons was also low, compared to when β-carotene was added to a meal containing long chain triglycerides.

In conclusion, the amount of dietary fat needed to be present simultaneously for complete absorption of carotenoids is low (ca. 3 g). This is thus not a limiting factor in Western diets in which, on average, fat is present in abundance. However, in case carotenoids are ingested as carotenoid esters, the amount of dietary fat needed for absorption is higher. The presence of non-absorbable fat replacers or other fat-soluble compounds with reduced absorbability may also interfere with carotenoid absorption and reduce their bioavailability.

INTERACTIONS BETWEEN CAROTENOIDS AND WITH OTHER FAT-SOLUBLE MICRONUTRIENTS

Interaction between carotenoids and other fat-soluble micronutrients at the intestinal level may reduce absorption of either the carotenoids or the other compounds. Competition for absorption may occur at the level of micellar incorporation, intestinal uptake, lymphatic transport or at more than one level. On the other hand, simultaneous ingestion of various carotenoids or carotenoids with other antioxidants may induce a sparing effect in the intestinal tract and thus result in increased levels of uptake of the protected carotenoids or antioxidants. A similar phenomenon may occur within the body, both with respect to sparing of other antioxidants as well as provitamin A carotenoids, and thus result in an enhanced status of carotenoids or other antioxidants.

Table 5 shows an overview of human studies in which the interaction between carotenoids and between carotenoids and vitamin E has been investigated. The majority of these studies have investigated the effect of supplementation with β-carotene on concentrations of other carotenoids and vitamin E in plasma or serum. Most animal studies have focused on the effects of canthaxanthin supplementation (Tang et al, 1993; White et al, 1993; Tang et al, 1995; Brown et al, 1997; Clark et al, 1998). This latter carotenoid is normally not present in the human diet in significant amounts and plasma levels of this carotenoid are generally low (Paetau et al, 1997).

The effect of β-carotene supplementation (12-300 mg/d) on plasma or serum concentrations of other carotenoids and vitamin E in humans is limited. Although some studies have shown that supplementation with β-carotene reduced plasma concentrations of other carotenoids or vitamin E (Prince et al, 1991; Micozzi et al, 1992; Morbarhan et al, 1994) and also an enhancing effects have been reported (Wahlqvist et al, 1994; Albanes et al, 1997), in most studies, no effect was found for the majority of the carotenoids investigated and six of the seven studies showed no effect on plasma or serum concentrations of vitamin E (Calzada et al, 1995; Fontham et al, 1995; Fotouhi et al, 1996; Albanes et al, 1997; Nierenberg et al, 1997; Mayne et al, 1998). Supplementation

with both β-carotene and lutein reduced plasma concentrations of lycopene in one study (*Chapter 2*), but not in another study (Castenmiller et al, 1999).

Single simultaneous ingestion of β-carotene with lycopene enhanced the lycopene response in serum (Johnson et al, 1997), whereas a reduced plasma or serum lutein and canthaxanthin response was found after single simultaneous ingestion of β-carotene with lutein or canthaxanthin (Kostic et al, 1995; White et al, 1994; Pateau et al, 1997).

β-Carotene was not affected by any other carotenoid (i.e. α-carotene, lycopene, lutein and canthaxanthin) or vitamin E in the majority of the studies (White et al, 1994; Kostic et al, 1995; Calzada et al, 1995; Johnson et al, 1997; Paetau et al, 1997; *Chapter 8*). Recently, Van den Berg & Van Vliet (1998) reported however a reduced α-carotene and β-carotene response in chylomicrons after simultaneous ingestion of these carotenoids with lutein or lycopene.

In conclusion, these data show that β-carotene supplementation has limited or no effect on plasma or serum concentrations of other carotenoids and vitamin E. However, the supplements may have been ingested at other times during the day than at which foods rich in carotenoids and vitamin E were consumed. Studies on simultaneous ingestion of carotenoids indicate that β-carotene may interfere with lutein (Kostic et al, 1995; Van den Berg & Van Vliet, 1998) and canthaxanthin (White et al, 1994; Paetau et al, 1997). Although the exact mechanism is unclear, the bioavailability of mainly the other carotenoids seems to be reduced as a result of this interaction.

CONCLUSION

Figure 2 summarises the dietary factors that affect carotenoid bioavailability and the steps during which they may interfere with the process of carotenoid absorption. Estimates of the quantitative impact of each factor, as far as possible, on the bioavailability (i.e. the responses in plasma, serum or triglyceride-rich lipoproteins) of carotenoids are presented in Table 6.

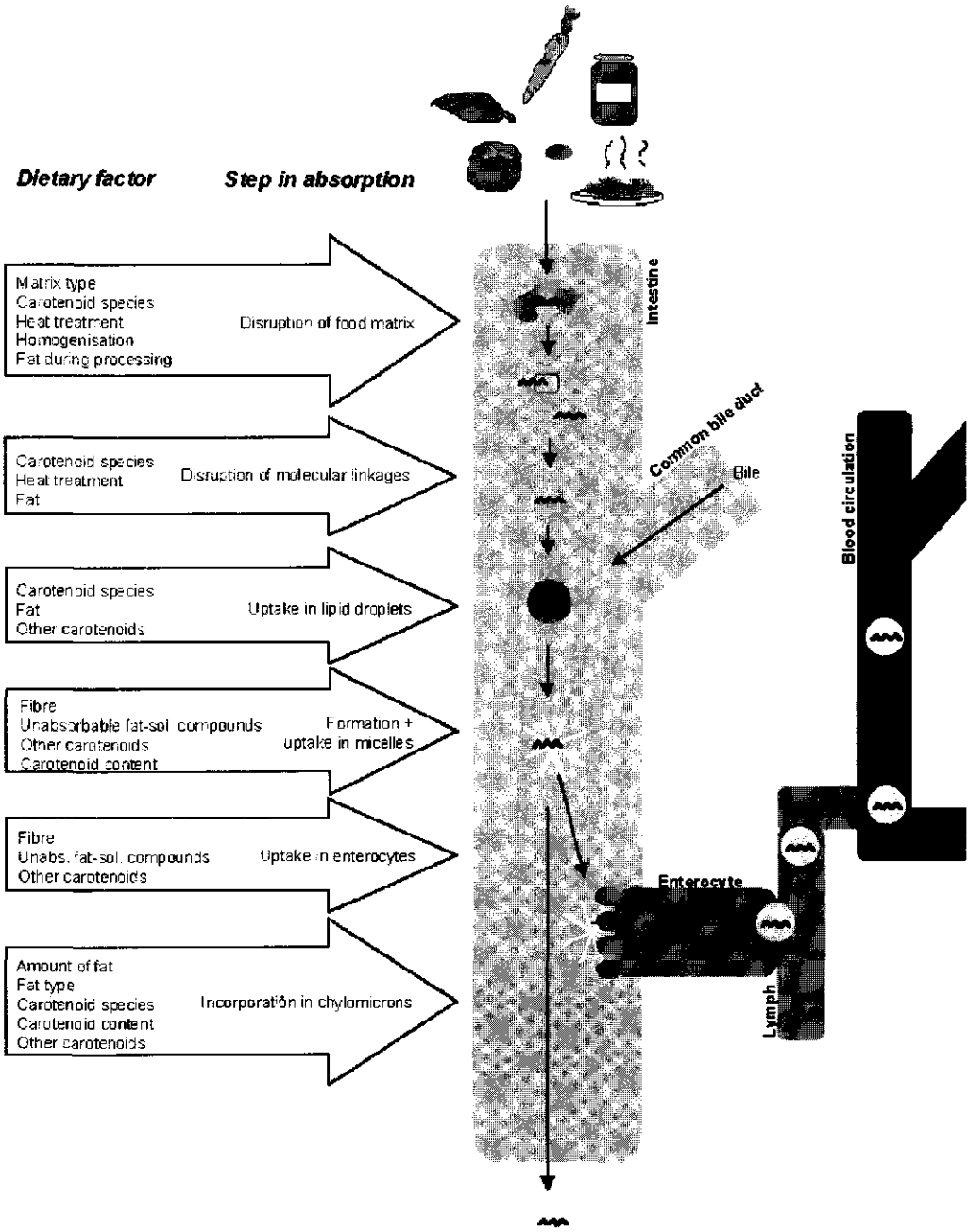

Figure 2 Dietary factors that affect carotenoid absorption.

In answer to the research questions stated in the introduction of this thesis, we conclude the following:

Does the bioavailability of carotenoids vary among different food matrices and does disruption of the matrix enhance the bioavailability of carotenoids from vegetables?

Goodman and his colleagues and Blomstrand & Werner showed in the 1960s that the absorption of carotenoids is far from complete (Goodman et al, 1966; Blomstrand & Werner, 1967). This thesis and research from others shows that the proportion absorbed is even smaller if carotenoids are located in complex food matrices, such as vegetables, than if they are present as pure compounds added to foods or capsules. Incomplete disruption of the vegetable matrix in the gastro-intestinal tract partly explains this observation, as disruption of this matrix prior to consumption enhances the carotenoid bioavailability. Disruption of the vegetable matrix does however not result in similar bioavailability shown for pure carotenoids. Other characteristics of vegetables, such as the presence of fibre, the physical state of carotenoids (e.g. crystalline form, carotenoid-protein complexes) or their intracellular location in the plant material (e.g. in chloroplasts or chromoplasts) may explain this gap.

Does the amount and digestibility of dietary fat affect the bioavailability of carotenoids?

Unless completely absent, the amount of dietary fat is not a limiting factor for carotenoid bioavailability as 3-5 g, simultaneously ingested with carotenoids, was already sufficient to ensure absorption. However, we also showed that if carotenoids are present as esters, solubilisation and/or hydrolysis of the esters prior to absorption may become a critical step if the amount of fat present is low. If fat-soluble, unabsorbable compounds are ingested along with carotenoids, as shown for sucrose polyester in the present thesis, they may solubilise part of the carotenoids, thereby preventing the absorption of these carotenoids and reducing their bioavailability.

Do individual carotenoids affect the bioavailability of other carotenoids?

With respect to the third factor investigated in this thesis and discussed in this chapter, i.e. interaction between carotenoids, the current data are less consistent. Simultaneous presence of the carotenoids in the diet seems crucial for their interaction and in these cases, competition rather than enhancement may occur. We showed that such an interaction was not present between α-carotene and β-carotene, but that supplementation

with β-carotene and lutein did affect the bioavailability of lycopene. The mechanism or steps in absorption where such an interaction takes place are yet unknown.

In conclusion, the type of food matrix in which carotenoids are located largely determines their bioavailability and unabsorbable, fat-soluble compounds reduce carotenoid absorption. Although some dietary fat needs to be present to ensure absorption of carotenoids, the amount seems to be very low (3-5 g per meal). The minimal amount needed depends however on the physico-chemical characteristics of the carotenoids ingested. Interaction among carotenoids may occur, depending on the type of carotenoids and on their simultaneous presence in the diet.

IMPLICATIONS

In future, more research will be needed to further increase our understanding of the bioavailability and function of carotenoids. The development and use of carotenoids labelled with stable isotopes incorporated into foods may contribute quantitative data on the extent to which carotenoids are absorbed from different food matrices. The studies presented in this thesis show that different types of vegetables may vary substantially with respect to the bioavailability of carotenoids. The underlying differences, e.g. cellular location of carotenoids, should be further explored. In addition, novel, non-invasive measurement techniques will provide more insight in the accumulation of carotenoids in tissues and allow to investigate this in healthy subjects (e.g. Stahl et al, 1998).

Research into the functional benefits of carotenoids should consider the data presented in this thesis, which show that the bioavailability of β-carotene in particular is one order of magnitude higher when provided as pure compound added to foods than when naturally present in foods. This should be taken into account when deciding on the amount of carotenoids to be provided either added to foods or to pharmaceutical preparations.

Processing, such as mechanical homogenisation or heat treatment, has the potential to enhance the bioavailability of carotenoids from vegetables. This may be applied in the development of foods with enhanced carotenoid bioavailability. A possible negative impact of such conditions on the content of other, more vulnerable, micronutrients should be taken into account. If novel food ingredients are developed, in particular if they are fat-soluble and absorbable only to a limited extent, attention should be paid to a possible negative impact on the bioavailability of carotenoids.

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Table 1 Comparison of bioavailability of carotenoids from vegetables and purified carotenoids, in humans[1].

Reference	Intervention	Study design	Results	Comments
Brown et al, 1989	Single dose: placebo vs 12 mg β-carotene vs 30 mg β-carotene vs carrots (272 g; 29 mg β-carotene, 9 mg α-carotene) vs broccoli (600 g; 6 mg β-carotene, 5 mg lutein) vs tomato juice (180 g; 12 mg lycopene); Complete low carotenoid diet supplied on days -3 to 11 (3.2 mg lutein/d).	Partly cross over design, n=7-15 per treatment, adult non-smoking males; Fasting blood samples drawn before and on days 1, 2, 3, 4, 7 and 11.	Sign. increase in plasma β-carotene conc. following 12 mg β-carotene, 30 mg β-carotene or carrots. No sign. increase after consumption of broccoli or tomato juice; Sign. increase in plasma conc. of α-carotene following carrots; no sign. increase in plasma conc. of lutein following broccoli or in lycopene following tomato juice.	Relative β-carotene bioavailability: carrots: 19%; 12 mg/d β-carotene induced a 110% increase and 30 mg/d β-carotene a 220% increase in plasma conc. of β-carotene; Plasma α-carotene-response/mg intake exceeds that of β-carotene from carrots; Plasma response to single dose (and number of volunteers) may not be sensitive enough to show sign. changes from baseline.
Castenmiller et al, 1999	3 wk: placebo vs supplement (10 mg/d β-carotene, 7 mg/d lutein) vs whole leaf spinach or minced spinach (~220 g/d; 9-10 mg β-carotene, 11-13 mg lutein).	Parallel design, n=10-12 per group, adult males and females; Fasting blood samples drawn before and after 3wk.	Sign. increases in serum β-carotene and lutein conc. following supplement or spinach consumption.	Relative β-carotene bioavailability: whole leaf spinach: 5.1% minced spinach: 6.4% Relative lutein bioavailability: whole leaf spinach: 45% minced spinach: 52%.
De Pee et al, 1995	12 wk: placebo vs enriched wafer (5.3 mg/d β-carotene) vs green leafy vegetables (100-150 g/d; 5.3 mg β-carotene).	Parallel design, n=56-62 per group, anaemic adult females; Fasting blood samples and breast milk collected before and after 12 wk.	Sign. increase in serum β-carotene conc., no increase in serum retinol and breast milk retinol concentrations in vegetables group; Sign. increase in serum β-carotene, serum retinol and breast milk retinol conc. in enriched wafer group.	Relative β-carotene bioavailability: green leafy vegetables: 4%; Subjects had a low vitamin A status.
Micozzi et al, 1992	6 wk: placebo vs 12 mg/d β-carotene vs 30 mg/d β-carotene vs carrots (272 g/d; 29 mg β-carotene, 9 mg α-carotene) vs broccoli (600 g/d; 3 mg β-carotene, 3 mg lutein) vs tomato juice (180 g/d; 12 mg lycopene); Complete diet supplied.	Parallel design, n=5 per group, adult non-smoking males; Fasting blood samples drawn before and twice per week during 6 wk.	Sign. increase in plasma β-carotene conc. (max change from baseline, as compared to placebo) following 12 mg β-carotene, 30 mg β-carotene or carrots. No sign. increase after consumption of broccoli or tomato juice; Sign. increase in plasma conc. of α-	Relative β-carotene bioavailability: carrots: 19% broccoli: 22-24%; 12 mg/d β-carotene induced a 1178% increase and 30 mg/d β-carotene a 2600% increase in plasma conc. of β-carotene;

			carotene following carrots, in lutein following broccoli and in lycopene following tomato juice.	Plasma α-carotene-response/mg intake exceeds that of β-carotene from carrots and lutein increase/mg intake exceeds that of β-carotene from broccoli.
Törrönen et al, 1996	6 wk: capsules (12 mg/d β-carotene) vs raw carrots (120 g/d, 12 mg β-carotene) vs carrot juice (100 ml/d, 12 mg β-carotene).	Parallel design, n=12-13 per group, adult females; Fasting blood samples drawn before, after 3 wk and 6 wk.	Sign. larger increase in serum β-carotene conc. following capsules than following carrots.	Relative β-carotene bioavailability: raw carrots, 3 wk: 34%, 6 wk: 26% carrot juice, 3 wk: 30%, 6 wk: 45%.
Chapter 2	4 wk: placebo vs supplement (5.7 mg/d β-carotene, 9.3 mg/d lutein) vs mixed vegetables (360 g/d, 3.6 mg β-carotene, 8 mg lutein) for 4 wk; Complete diet supplied.	Parallel design, n=10-22 per group, adult males and females; Fasting blood samples drawn before and after 4 wk.	Sign. increases in plasma β-carotene and lutein conc. following supplement or vegetables.	Relative β-carotene bioavailability: mixed vegetables: 14%; Relative lutein bioavailability: mixed vegetables 67%.
Chapter 3	4 d: placebo vs supplement (33 mg/d β-carotene) vs spinach (leaf or chopped, 300 g/d; 24 mg β-carotene, 25 mg lutein) vs broccoli (300 g/d; 2.4 mg β-carotene, 3.8 mg lutein) vs green peas (300 g/d; 1.7 mg β-carotene, 4.2 mg lutein).	Incomplete cross over design, n=26-67 per treatment, adult males and females; Fasting blood samples drawn after 4 d.	Sign. increase in plasma β-carotene conc. following supplement, broccoli or green peas, but not following spinach; Sign. increase in plasma lutein following all vegetables.	Relative β-carotene bioavailability: leaf spinach: 3% chopped spinach: 4% broccoli: 74% green peas: 96%; Short supplementation period may have limited sensitivity to find sign. increases.

[1]Relative bioavailability represents the plasma or serum carotenoid response induced by vegetable consumption expressed as proportion of the plasma or serum carotenoid response induced by supplementation with the pure compound, corrected for differences in carotenoid intake

Note: Sign. denotes significant(ly) ($P<0.05$); conc. denotes concentration(s). All studies were well conducted, unless otherwise stated.

Table 2. The effect of processing on the bioavailability of carotenoids from vegetables in humans and animals

Reference	Intervention	Study design	Results	Comments
Human studies				
Castenmiller et al, 1999	3 wk: whole leaf spinach vs minced spinach vs enzymatically liquefied spinach vs enzymatically liquefied spinach with added fibre (ca. 220 g/d; 9-10 mg β-carotene, 11-13 mg lutein).	Parallel design, n=12 per group, adult males and females; Fasting blood samples drawn before and after 3wk.	β-Carotene response in plasma sign. larger with liquefied spinach than with whole leaf spinach (69%); No sign. difference in lutein responses among spinach groups.	Improved bioavailability of β-carotene by enzymatic disruption of cell walls (pectinase, hemicellulase and cellulase activity); Plasma β-carotene response induced by liquefied spinach was only 9.5% of that induced by β-carotene added to oil.
Gärtner et al, 1997	Single dose: raw tomatoes vs tomato paste (400 g tomatoes vs 40 g tomato paste, 23 mg lycopene).	Within-person design, n=5, adult males and females; Blood samples drawn before and after 2, 4, 5, 6, 7, 9 and 12 h.	Lycopene response in TRLs larger with tomato paste than with raw tomatoes (3.8-fold higher area under the curve).	Improved bioavailability of lycopene by mechanical homogenisation and heat treatment; Period effects may have interfered with treatment effect (cross over design was not applied).
Hussein & El-Tohamy, 1990	2 wk: grated carrots vs carrot juice (30-150 g/d carrots, 1.7-8.4 mg/d carotene vs 30-60 ml/d carrot juice; 1.2-2.4 mg/d carotene); Supplementation preceded by 2 wk vitamin A depletion.	Parallel design, n=12 vs n=8 per group, boys aged 11-13 yr; Fasting blood samples drawn before and after 2 wk.	Plasma retinol conc. reported to increase slightly more with carrot juice than with grated carrots.	Indication of improved bioavailability of provitamin A-carotenoids by mechanical homogenisation; Not possible to draw conclusions from the study design used, no statistical evaluation presented.
Porrini et al, 1998	Single dose or 1 wk: raw tomatoes vs tomato paste (300 g tomatoes vs 57 g tomato purée, 16.5 mg lycopene).	Single dose: cross over design, n=9, adult females, single consumption of product; Blood samples drawn before and every 2 h until 12 h and after 24, 32, 56, 80 and 104 h. 1 wk: parallel design, n=5 per group, adult females; Blood samples drawn daily during 5 d and after 7, 9 and 11 d.	Response of plasma lycopene sign. larger after both single or 7 d consumption of tomato purée than after consumption of raw tomatoes (22% difference after single dose; 51% difference after 7 d).	Improved bioavailability of lycopene by mechanical homogenisation and heat treatment; Baseline plasma lycopene conc. were not subtracted from the area under the plasma lycopene concentration curves. Although not sign., mean baseline conc. was slightly higher before consumption of tomato purée than before consumption of raw tomatoes.

Rock et al, 1998	4 wk: raw carrots & spinach vs cooked homogenized carrots & spinach (55 g/d raw carrots & 39 g/d raw spinach vs 113 g/d processed carrots & processed spinach; 9.3 mg/d β-carotene).	Cross over design, n=8, adult females; Fasting blood samples drawn before and after 4 wk.	Response of plasma β-carotene almost sign. larger with pureed and heated vegetables than with raw vegetables (213% difference, P=0.09).	Indication for improved bioavailability of β-carotene by mechanical homogenisation and heat treatment; Number of volunteers insufficient to show sign. difference.
Stahl & Sies, 1992	Single dose: raw tomato juice vs tomato juice boiled for 60 min with 1% corn oil (~700 ml tomato juice; 2.5 µmol lycopene/kg body weight).	Parallel design, n=2 vs n=3 per group, adult males and females; Blood samples drawn before and after 1, 2, 3, 5, and 7 h.	Serum all-*trans* and *cis*-lycopene conc. increased with boiled tomato juice but not with untreated tomato juice.	Indication for improved bioavailability of lycopene by heat treamtent in presence of oil; Number of volunteers does not allow statistical evaluation of the results.
Törrönen et al, 1996	6 wk: Raw carrots vs carrot juice (120 g/d carrots vs 100 ml/d; 12 mg/d β-carotene); Supplementation preceded by 10 d low carotenoid diet.	Parallel design, n=13 per group, adult females; Fasting blood samples drawn before and after 3 and 6 wk.	Response of serum β-carotene conc. not sign. different between groups (70% difference).	No improved bioavailability of β-carotene by mechanical homogenisation.
Van Zeben & Hendriks, 1948	3 wk: cooked unhomogenised carrots vs cooked homogenised carrots (100 g/d carrots, 6.5 mg/d carotene).	Parallel design, n=10 vs n=7 per group, adult females; Blood samples drawn before and after 10 d and 3 wk.	Response of serum carotene conc. larger in homogenised carrot group than in unhomogenised carrot group (488%).	Improved bioavailability of carotene by mechanical homogenisation.
Chapter 3	4 d: cooked whole leaf spinach vs cooked chopped spinach (300 g/d spinach; 24 mg/d β-carotene, 25 mg/d lutein).	Incomplete cross over design, n=26 per treatment, adult males and females; Fasting blood samples drawn after 4 d.	Response of plasma lutein conc. sign. larger after chopped spinach than after unchopped spinach group (14% difference); No sign. difference between treatments in response of plasma β-carotene conc.	Improved bioavailability of lutein but not β-carotene by mechanical homogenisation; Short supplementation period may have limited sensistivity to find sign. differences.

TABLE 2 - *continued*

Reference	Intervention	Study design	Results	Comments
Chapter 4	Single dose or 4 d: whole canned tomatoes vs homogenised canned tomatoes vs under high pressure homogenised tomatoes; minimal additional heat treatment vs extensive (1 h) additional heat treatment (300 g; 21-23 mg lycopene, 1 mg β-carotene).	Split plot design: homogenisation in cross over design, heat treatment in parallel design, n=36 in 4 d, n=12 in single dose study, adult males and females; Single dose: blood samples before and 2, 3, 4.5, 6 and 8 h after consumption (TRL response); 4 d: fasting blood samples before and after 4 d (plasma response).	Lycopene: response sign. larger with homogenised tomatoes than whole tomatoes, both in TRLs (21%) and in plasma (62%), no sign. effect of heating (44% in TRLs, 10% in plasma); β-Carotene: TRL response sign. larger with homogenised tomatoes than whole tomatoes (816%), no sign. effect of heating; Sign. larger 4 d plasma response by homogenisation of minimally heated tomatoes (289%) and sign. larger 4 d plasma response of β-carotene by heating whole tomatoes (378%).	Improved bioavailability of lycopene and β-carotene by mechanical homogenisation, homogenisation under high pressure is more effective than under "normal" pressure; Sign. interaction between homogenisation and heating in effect on plasma β-carotene response indicates maximum bioavailability achieved by either of these treatments; Effect of heat treatment was tested with less power because of parallel comparison in split plot design; Percent differences for β-carotene are less reliable because of small amount ingested.
Animal studies				
Poor et al, 1993	1 wk: homogenised raw carrots vs homogenised cooked carrots (ca. 15 mg/d β-carotene, ca. 8 mg/d α-carotene).	Parallel design, n=6 per group, preruminant calves; Animals killed after 1 wk.	Serum, adrenal and liver conc. of α- and β-carotene at end of intervention not sign. different between groups.	No improved bioavailability of α- or β-carotene by heat treatment.
Zhou et al, 1996	3 d: unheated carrot juice vs heated carrot juice; unheated carrot chromoplasts vs heated carrot chromoplasts (10 mg/d β-carotene).	Parallel design, n=6 per group, ferrets; Animals killed 2 wk after the 3 d supplementation.	Serum, adrenal and liver conc. of α- and β-carotene at end of intervention not sign. different between heated and unheated carrot product groups.	No improved bioavailability of α- or β-carotene by heat treatment.

Note: Sign. denotes significant(ly) ($P<0.05$); conc. denotes concentration(s); TRL(s) denotes triglyceride-rich lipoprotein(s). All studies were well conducted, unless otherwise stated.

Table 3. The effect of dietary fat on the bioavailability of carotenoids in humans

Reference	Intervention	Study design	Results	Comments
Dimitrov et al, 1988	5 d: 45 mg/d β-carotene ingested at breakfast; 5 d low fat diet (0 g at breakfast, 6 g at lunch) or high fat diet (18-24 g at breakfast, 45 g at lunch), followed by 3 wk normal fat diet.	Parallel design, n= 5 vs n=4, adult males and females; Blood samples drawn daily.	Plasma β-carotene response on 5 d high fat diet was sign. larger than on low fat diet.	No average values presented; Difference between two groups in plasma β-carotene conc. remained after switch to normal fat diets.
Henderson et al, 1989	Single dose: 15 mg β-carotene ingested without any food until 16 h later or with food (500 kcal, 18 g fat).	Parallel design, n=1 vs n=9, adult males and females; Blood samples drawn before and at 24 h.	No increase in serum β-carotene conc. in case of no simultaneous food intake vs sign. increase.	Results do not indicate the importance of fat, but foods in general; Only 1 volunteer in "no foods" group; Unexplained differences between tables.
Jalal et al, 1998	3 wk: 750 RE/d β-carotene from sweet potato snacks with low fat meal (3 g fat) or high fat meal (18 g fat).	Parallel design, n=41 vs n=43, boys and girls (3-6 y); Non-fasting blood samples before and after 3 wk.	Increase in serum retinol conc. was higher on high fat diet than on low fat diet (64%).	Amount of fat in meals and background diet not clearly stated.
Jayarajan et al, 1980	4 wk: 1.2 mg/d β-carotene from spinach, consumed after addition of 0 g, 5 g or 10 g fat (background diet contains ca. 5 g/d fat).	Parallel design, n=26 vs n=22 vs n=22 vitamin A deficient children (2-6 y); Blood samples drawn before and after 4 wk.	Consumption of spinach with 5 g or 10 g fat resulted in larger serum response of retinol than spinach without fat (110%); No difference between 5 g and 10 g fat.	Study in vitamin A deficient children.
Prince & Frisoli, 1993	Single dose: 51 mg β-carotene ingested with no fat intake until 6 h later vs with 200 g fat.	Cross over design, n=3, adult males and females (2 mo wash-out); Blood samples drawn during the day and until 168 h later.	No increase in serum β-carotene conc. following ingestion of β-carotene without fat vs 4-fold increase with high fat breakfast.	It is not clear whether any breakfast was consumed when β-carotene was ingested without fat.

TABLE 3 - *continued*

Reference	Intervention	Study design	Results	Comments
Shiau et al, 1994	Single dose: 15 mg β-carotene, ingested after total gut wash out, without breakfast or with breakfast containing 20 en% vs 40 en% (500 kcal, 11.2 g vs 22.2 g fat; 1000 kcal, 22.1 g vs 44.3 g fat).	Cross over design, n=11 males, 18-30 y and n=8 males, 65-80 y (4 wk wash-out); Faecal output during 2 d, finished by another total gut wash out; Blood samples drawn before, hourly until 8 h and at 24 and 48 h.	83% β-carotene excreted, no increase in serum β-carotene conc. in case of no simultaneous food intake; 49% - 71% β-carotene excreted, no significant effect of fat content; Young males: 40 en% resulted in *sign. larger serum β-carotene response at 8 h and 24 h than 20 en%, no sign. difference in 8 h area under the serum response curve; Older males: no sign. effect of fat content on serum β-carotene response.	Complicated, but balanced, experimental design: many variables tested; Not clear which results to base conclusions on: faecal output, increase in serum β-carotene conc. at 8 h and 24 h or 8 h area under the serum response curve; Information on composition of diet following breakfast is limited.
Chapter 6	7 d: control spread vs palm oil carotenoids enriched spread (2 mg/d α-carotene, 4 mg/d β-carotene) vs lutein enriched spread (8 mg/d lutein) ingested with a low fat spread (3 g fat in meal) or full-fat spread (35 g fat in meal).	Split plot design: type of supplement in parallel design, amount of fat in cross over design, n=15 per carotenoid group, adult males and females (5 wk wash-out); Fasting blood samples drawn before and after 7 d.	Sign. increase in plasma α- and β-carotene conc. following palm oil carotenoids enriched spread, no sign. effect of amount of fat present; Sign. increase in plasma lutein conc. following lutein enriched spread, sign. larger response following full-fat spread (ca. 100%).	Lutein were present as lutein esters, whereas α- and β-carotene were free carotenoids. We speculate that hydrolysis of the lutein esters was reduced when ingested with the low fat meal.

Note: Sign. denotes significant(ly) ($P<0.05$); conc. denotes concentration(s). All studies were well conducted, unless otherwise stated.

Table 4. The effect of non or less absorbable fat-soluble components, type of dietary fat and dietary fibre on the bioavailability of carotenoids in humans

Reference	Intervention	Study design	Results	Comments
Sucrose polyester				
Koonvitsky et al, 1997	16 wk: Olestra-containing cookies and frozen dessert vs regular products, consumed at times chosen by volunteers, 18 g/d Olestra.	Parallel design, n=65-67 per group, adult males and females; Fasting blood samples drawn before and every 2 wk, until 16 wk.	Sign. decrease in serum conc. of carotenoids: β-carotene: -27%, lycopene: -23%, α-carotene: -24%; lutein/zeaxanthin: -19%.	
Schlagheck et al, 1997	8 wk: Olestra containing snacks vs regular snacks eaten with all meals, 8 g/d or 20 g/d or 32 g/d Olestra; Complete diet provided.	Parallel design, n=21-24 per group, adult males and females; Fasting blood samples drawn before and every 2 wk, until 8 wk.	Sign. decrease in serum conc. of carotenoids: β-carotene: -98%, -117% and -117% on 8, 20, 32 g Olestra/d, respectively; effect on lycopene and α-carotene was similar; effect on lutein and zeaxanthin was smaller.	Baseline values differed between groups and the control group showed an increase in serum conc. of carotenoids. Therefore, decreases in Olestra group corrected for changes in control group exceed 100%; To obtain a maximum effect, no foods were consumed withouth simultaneous ingestion of Olestra containing products.
Chapter 7	4 wk: SPE containing margarine vs regular margarine (no carotenoids added as colourant) added to the main meal, 3 g/d or 12.4 g/d SPE.	3 g SPE/d: parallel design, n=26-27 per group, adult males and females; 12.4 g SPE/d: cross over design, n=21 adult males and females; Fasting blood samples drawn before and after 4 wk.	Sign. decrease in plasma conc. of carotenoids: 3 g SPE/d: lycopene: -38%, (α+β)-carotene: -20%; 12.4 g SPE/d: lycopene: -52%, (α+β)-carotene: -34%, β-cryptoxanthin: -23%, lutein: -20%, zeaxanthin: -18%.	Decreases were most pronounced for more lipophilic carotenoids (lycopene and β-carotene); Magnitude of effect of 3 g SPE/d >25% of effect of 12.4 g SPE/d: apparently no linear dose-response relation.
Phytosterols				
Gylling et al, 1996	1 y: Phytosterols containing margarine (3 g/d sitostanol) vs regular margarine.	Parallel design, n=51/group; Blood samples drawn before and after 1 y.	Non-sign. decrease in plasma conc. (lipid standardized) of β-carotene as compared to control group; No sign. effect on plasma conc. of α-carotene.	Study published as abstract only.

TABLE 4 - *continued*

Reference	Intervention	Study design	Results	Comments
Weststrate & Meijer, 1998	3.5 wk: Phytosterols containing margarines vs regular margarine; added to lunch and main meal, A: 2.7 g/d Benecol sterols, B: 3.2 g/d soybean oil sterols, C: 1.7 g/d ricebran sterols, D: 3.0 g/d sheanut oil sterols.	Incomplete Latin square design, n=75-77 per treatment, adult males and females; Fasting blood samples drawn after each period (3.5 wk).	Sign. decrease in plasma conc. of carotenoids (lipid standardized). (α+β)-carotene: A: -19%, B: -19%, C: -8.6%; D: -43%; lycopene: A: -19%, D: -40%.	A sign. period effect on plasma carotenoid conc. was found. This may indicate that a duration of 3.5 wk was insufficient to estimate the complete effect of sterols on plasma carotenoid conc.
Type of dietary fat				
Borel et al, 1998	Single dose: 120 mg β-Carotene with 40 g medium chain triglycerides (C8:0 and C10:0) vs 40 g long-chain triglycerides (C18:1 and C18:2).	Cross over design, n=16 adult males; Blood samples drawn before and hourly until 12 h.	No increase in chylomicron triglycerides and β-carotene conc. following consumption of the medium chain triglycerides; Sign. increase in chylomicron triglycerides and β-carotene after ingestion of a second meal containing long chain triglycerides at t=6 hr. This riglyceride response was larger, but the β-carotene response was smaller than if the first meal also contained long chain triglycerides.	Medium chain triglycerides are mainly absorbed via the portal vein and thus chylomicron formation is low. The authors speculate that β-carotene may have remained in the enterocytes and thus been excreted with chylomicrons formed after the second long chain triglycerides containing meal; Data on plasma responses would have been valuable as perhaps part of β-carotene may also have been absorbed by the portal vein.
Dietary fibre				
Erdman et al, 1986	4 wk: 18.2 µg/g diet β-carotene without fibre vs with 7% fibre: A: hemicellulose; B: lignin; C: polygalacturonic acid; D: citrus pectin.	Parallel design, n=9 chicks/group; Animals killed after 4 wk.	Sign. decreased utilisation of β-carotene (i.e. determined from liver retinol conc.) from diet with pectin (52-60%), lignin (34%) and hemicellulose (31%); No sign. differences in serum conc. of retinol.	Well conducted study; Methoxyl content of pectin was crucial: low methoxyl pectin had no effect on β-carotene utilisation; Pectin has been shown to reduce serum cholesterol conc. as well, probably via increased faecal excretion of bile acids and total fat.

Rock & Swenseid, 1992	Single dose: 25 mg β-carotene with meal without vs with added fibre (12 g citrus pectin).	Cross over design (3 wk wash out), n=7, adult females; Blood samples drawn before and after 8, 30, 48 h and 8 d.	Sign. reduction in plasma response of β-carotene (60%) by pectin.	Authors speculate that effects of pectin may be due to delay of gastric emptying or interference with micelle formation due to uptake of bile salts in gel phase.

Note: Sign. denotes significant(ly) ($P<0.05$); conc. denotes concentration(s). All studies were well conducted, unless otherwise stated.

Table 5. Effects on carotenoid bioavailability of interactions between carotenoids and between carotenoids and vitamin E in humans.

Reference	Intervention	Study design	Results	Comments
Albanes et al, 1997	6.7 y: placebo (± 50 mg/d vitamin E) vs 20 mg/d β-carotene (± 50 mg/d vitamin E).	Parallel design, n=237-254 per group, adult males; Non-fasting blood samples drawn at end of study.	Sign. higher plasma conc. of β-carotene (1483%), α-carotene (145%) and β-cryptoxanthin (67%); Sign. lower plasma conc. of lutein (-11%); No effect on lycopene, zaxanthin, α-tocopherol.	Effect of vitamin E may have interfered (half of placebo and β-carotene supplemented group received vitamin E).
Calzada et al, 1995	8 wk: placebo vs 15 mg/d β-carotene vs 300 mg/d vitamin E.	Parallel design, n=9-10 per group, adult males and females; Blood samples drawn before and after 8 wk.	Sign. increased plasma conc. of β-carotene (2.6-fold increase); No sign. effect of β-carotene supplementation on plasma conc. of α-tocopherol; No sign. effect of α-tocopherol supplementation on plasma conc. of β-carotene	
Fontham et al, 1995	1 y: placebo vs 30 mg/d β-carotene.	Parallel design, n=36-38 per group, adult males and females with premalignant lesions of the stomach; Non-fasting blood samples drawn before and after 1 y.	Increase in plasma conc. of β-carotene not presented; No sign. difference in changes of plasma conc. of α-tocopherol.	Subjects had stomach lesions which may have affected the outcome of the study.
Fotouhi et al, 1996	12 y: placebo vs 50 mg/d β-carotene on every other day.	Parallel design, n=29-30 per group, adult males; Fasting blood samples drawn at end of study.	Sign. higher plasma conc. of β-carotene (220%); No sign. differences in plasma conc. of α-carotene, lutein, β-cryptoxanthin, lycopene, α-tocopherol.	
Johnson et al, 1997	Single dose: 60 mg β-carotene or 60 mg lycopene vs combination of both (60 mg β-carotene + 60 mg lycopene).	Cross over design, n=10, adult males; Blood samples drawnbefore and after 1, 3, 5, 7, 9, 12 and 24 h.	Sign. larger serum response for lycopene at simultaneous intake with β-carotene (312%); No sign. difference in serum response of β-carotene.	Authors speculate that enhanced lycopene bioavailability is due to solubilisation of lycopene in β-carotene solution; Serum response to lycopene was not related to serum response to β-carotene.
Kostic et al, 1995	Single dose: 0.5 µmol/kg body weight β-carotene (14-24 mg) or 0.5 µmol/kg body weight lutein (15-26 mg) vs combination of both (0.5 µmol/kg β-carotene + 0.5 µmol/kg lutein).	Cross over design, n=8 adult males and females; Blood samples drawn before and after 2, 4, 6, 8, 12, 16, 24, 32, 60 h, 5, 18 and 35 d.	Sign. smaller serum response of lutein at simultaneous intake with β-carotene (-46%); No sign. difference in serum response of β-carotene, although effect varied among volunteers (5-fold enhancement to 69% reduction).	Wide variation in results for β-carotene; Serum lutein response and serum β-carotene response were almost sign. correlated (r=0.57, P=0.066); Mean area under the curve for β-carotene was only 44% of that of lutein, despite ingestion of similar dose.

Mayne et al, 1998	5 y: placebo vs 50 mg/d β-carotene and 1 mg/d α-carotene.	Parallel design, n=7-200 per group (depending on time point) adult cancer patients; Blood samples drawn before and after 3 mo and yearly until 5 y.	Sign. increase in plasma conc. of β-carotene (10-fold) and α-carotene (2-fold); No sign. differences in plasma conc. of lycopene, lutein/zeaxanthin, α-tocopherol.	
Micozzi et al, 1992	6 wk: placebo vs 12 mg/d β-carotene or 30 mg/d β-carotene.	Parallel design, n=5 adult males per group; Blood samples before and after 6 wk.	Sign. increase in serum conc. of β-carotene (12-fold after 12 mg/d, 26-fold after 30 mg/d); Sign. decrease in serum conc. of lutein (62% after 12 mg/d, 94% after 30 mg/d); No sign. effect on serum conc. of lycopene.	
Mobarhan et al, 1994	3 mo: placebo vs 30 mg/d β-carotene.	Parallel design, n=16-18 per group, adult males and females, colonic polyp patients; n=12 per group adult males and females, colon cancer patients; Blood samples and colonic mucosa tissue samples taken before and after 3 mo.	Sign. increase in serum conc. of β-carotene (10.5-12.5 fold in polyp and cancer group); Sign. decrease in serum conc. of α-tocopherol in cancer group (-20%) and sign. decrease in tissue conc. of α-tocopherol in polyp group (-64%).	Subjects had colonic lesions which may have affected the outcome of the study.
Nierenberg et al, 1997	4 y: placebo vs 25 mg/d β-carotene.	Parallel design, n=49-53 per group, adult males and females with at least one adenoma removed from colon; Blood samples taken before and after 4 y	Sign. increase in serum conc. of β-carotene (158%); No sign. differences in plasma conc. of α-carotene, lutein/zeaxanthin, lycopene, cryptoxanthin and α-tocopherol.	Not clear whether serum or plasma samples were analysed; Subjects had colonic lesions which may have affected the outcome of the study.
Paetau et al, 1997	*Single dose: 25 mg β-carotene* or 25 mg canthaxanthin vs 25 mg β-carotene and 25 mg canthaxanthin.	Cross over design (10 wk wash-out), n=9, adult non-smoking females; Blood samples taken before, hourly until 12 h after ingestion and after 1, 2, 3, 4, 8, 15, and 22 d.	Sign. reduction of canthaxanthin response in plasma and TRLs after simultaneous ingestion of β-carotene; No sign. effect of canthaxanthin on β-carotene responses (large between person variation).	Response of canthaxanthin was larger than that of β-carotene both in plasma and TRLs despite ingestion of similar dose.
Prince et al, 1991	3 wk: 300 mg/d β-carotene.	n=5, adult males and females; Blood samples taken before and every 2 days during study.	Sign. increase in serum conc. of β-carotene (7.8-fold) after 3 wk; Sign. decrease in serum conc. of lycopene.	Study was not placebo-controlled; Plateauing of increased serum β-carotene conc. started at 3 wk.

TABLE 5 - *continued*

Reference	Intervention	Study design	Results	Comments
Van den Berg & Van Vliet, 1998	Single dose: 5 mg α-carotene + 10 mg β-carotene vs 5 mg α-carotene + 10 mg β-carotene and 15 mg lutein vs 5 mg α-carotene + 10 mg β-carotene and 15 mg lycopene.	Cross over design (3 wk wash-out), n=12, adult nons-smoking males; Blood samples taken before and hourly until 10 h.	Sign. reduction of both α-carotene (-48%) and β-carotene (-34%) response in triglyceride-rich lipoproteins after simultaneous ingestion with lutein; Non-sign. reduction of response after simultaneous ingestion with lycopene (-30% α-carotene and -48% β-carotene).	Responses of lutein and lycopene in triglyceride-rich lipoproteins were smaller than those of α-carotene and β-carotene, despite the larger amounts consumed.
Wahlqvist et al, 1994	24 mo: placebo vs 20 mg/d β-carotene.	Parallel design, n=135 males and 85 females, adult patients with colorectal adenomas; Blood samples taken before and after 24 mo.	Sign. increase in serum conc. of β-carotene (10.7-fold in males, 8.4-fold in females); Sign. increase in serum conc. of lycopene (176% in males) and α-carotene (211% in males, 166% in females).	Subjects had colorectal lesions which may have affected the outcome of the study.
White et al, 1994	Single dose: 25 mg β-carotene or 25 mg canthaxanthin vs 25 mg β-carotene and 25 mg canthaxanthin.	Within person design, (10-33 wk wash-out), n=2, adult non-smoking females; Blood samples before and hourly until 10-11 h and after 24, 48 and 72 h.	Reduced serum response of canthaxanthin (-39%) after ingestion with β-carotene; No effect of canthaxanthin on serum response of β-carotene.	Number of volunteers does not allow statistical evaluation; Serum response of canthaxanthin exceeded that of β-carotene despite ingestion of similar dose.
Chapter 2	4 wk: placebo vs 5.7 mg/d β-carotene + 9.7 mg/d lutein.	Parallel design, n=10-22 per group, adult males and females; Fasting blood samples before and after 4 wk.	Sign. increase in plasma conc. of β-carotene (680%) and lutein (301%); Sign. decrease in plasma conc. of lycopene (-39%).	Complete diet supplied.
Chapter 8	4 d: 24 mg/d synthetic β-carotene vs 12 mg/d β-carotene + 8 mg/d α-carotene (palm oil carotenoids).	Incomplete cross over design, n=26-67 per treatment, adult males and females; Fasting blood samples drawn after 4 d.	Sign. increase in plasma conc. of all-*trans* β-carotene following both synthetic (686%) and palm oil carotene (345%), supplements and sign. increase in plasma conc. of α-carotene after palm oil carotenoids (716%); Response per mg all-*trans* β-carotene ingested was similar for both supplements.	No increase in plasma conc. of 9-*cis* β-carotene found, despite presence in supplement.

Note: Sign. denotes significant(ly) ($P<0.05$); conc. denotes concentration(s); TRL(s) denotes triglyceride-rich lipoprotein(s). All studies were well conducted, unless otherwise stated

Table 6. Estimation of the quantitative effects of various dietary factors on the bioavailability of carotenoids[a]

Dietary factor	Carotenoid			
	α-Carotene	β-Carotene	Lutein	Lycopene
Matrix type (carotenoids in oil=1.0)				
Mixed vegetables (*Chapter 2*)	na	0.14 (0.011)	0.67 (0.08)	na
Whole leaf spinach (*Chapter 3*)	na	0.03 (0.51)	na	na
Broccoli (*Chapter 3*)	na	0.74 (0.64)	na	na
Green peas (*Chapter 3*)	na	0.96 (0.71)	na	na
Green leafy vegetables[1]	na	0.04	na	na
Whole leaf spinach[2]	na	0.04	0.45	na
Broccoli[3,4]	na	0.19-0.22	na	na
Carrots[3,4,5]	na	0.19-0.26	na	na
Matrix disruption by homogenisation (unhomogenised vegetables=1.0)				
Chopped spinach (*Chapter 3*)	na	1.0	1.18	na
Under high pressure homogenised tomatoes (*Chapter 4*)	na	3.9 (4.5) 9.2 (24)[d],	na	1.23 (0.07), 1.62 (0.38)[d]
Liquified spinach[2]	na	1.69	1.0	na
Homogenised carrots[5,6]	na	[1.7], 5.9	na	na
Matrix disruption by heat treatment (unheated vegetables=1.0)				
1 h additionally heated tomatoes (*Chapter 4*)	na	3.0 (2.0), [2.7 (3.4)[d]]	na	[1.1 (0.14)], [1.4 (0.49)[d]]
Matrix disruption by homogenisation and heat treatment (unhomogenised and unheated vegetables=1.0)				
Tomato paste vs raw tomatoes[7,8]	na	na	na	1.2, 1.5, 4.8[d]
Homogenised and heated carrots and spinach vs raw carrots and spinach[9]	na	3.1	na	na
Amount of dietary fat (high amount of fat=1.0)				
36 g fat vs 3 g fat present in carotenoid supplemented meal (*Chapter 6*)	1.0	1.0	0.43 (0.062)[b]	na
5 g fat vs 0 g fat present in carotenoid supplemented meal[10]	na	0.48[c]	na	na
10 g fat vs 0 g fat present in carotenoid supplemented meal[10]	na	0.48[c]	na	na
10 g fat vs 5 g fat present in carotenoid supplemented meal[10]	na	1.0	na	na
3 g fat vs 18 g fat present in sweet potatoes containing meal[11]	na	0.63[c]	na	na

TABLE 6 - *continued*

Dietary factor	α-Carotene	β-Carotene	Lutein	Lycopene
Indigestible fat-soluble compounds (regular dietary fat=1.0)				
3 g/d sucrose polyester vs regular dietary fat with main meal (*Chapter 7*)	na	0.80 (03)	na	0.62 (0.05)
12.4 g/d sucrose polyester vs regular dietary fat with main meal (*Chapter 7*)	na	0.66 (0.02)	0.80 (0.04)	0.48 (0.05)
18 g/d sucrose polyester vs regular dietary fat at various times during days[12]	na	0.73	0.81	0.77
Dietary fibre (no dietary fibre=1.0)				
12 g/d citrus pectin added to carotenoid supplemented meal[13]	na	0.42	na	na
Ingestion of other carotenoids (no other carotenoids=1.0)				
β-Carotene and lutein supplemented (*Chapter 2*)	1.0			0.61 (0.20)
α-Carotene simultaneously present with β-carotene (*Chapter 8*)	na	1.0	na	na

[a] Values are presented as mean (SEM) or as mean. The factors were calculated from changes in plasma or serum concentrations of carotenoids, unless otherwise stated. The plasma or serum carotenoid response following the treatment stated was divided by the plasma or serum carotenoid response following the treatment which was taken as reference at 1.0 (identified between brackets for each dietary factor), if necessary corrected for differences in carotenoid intake. In case no change was expected from the reference treatment (e.g. in case of indigestible vs regular fat or ingestion of other carotenoids vs no other carotenoid present), the factors were calculated as the percent change from baseline, if necessary corrected the change in the control group. A factor smaller than 1.0 indicates that the bioavailability of carotenoids is reduced as compared to the reference chosen, a factor larger than 1.0 indicates an enhanced carotenoid bioavailability.

[b] Lutein was present as lutein esters

[c] Calculated from changes in serum concentrations of retinol

[d] Calculated from area under the curve of the carotenoid response in triglyceride-rich lipoproteins

[1]De Pee et al, 1995; [2]Castenmiller et al, 1999; [3]Brown et al, 1989; [4]Micozzi et al, 1992; [5]Törrönen et al, 1996; [6]Van Zeben & Hendriks, 1948; [7]Gärtner et al, 1997; [8]Porrini et al, 1998; [9]Rock et al, 1998; [10]Jayarajan et al, 1980; [11]Jalal et al, 1998; [12]Koonvitsky et al, 1997; [13]Rock & Swenseid, 1992

Note: 'na' denotes not assessed; values in parentheses, [ ], are not significantly different from 1.0 (α=0.05)

Summary

Carotenoids are thought to contribute to the relationship between a high fruit and vegetable consumption and a reduced risk of chronic diseases, such as some types of cancer and cardiovascular disease. To increase our understanding of the potential benefits of carotenoids, it is important to obtain more insight into their bioavailability from foods and the factors that influence carotenoid bioavailability. This latter aspect may lead to options for improving the bioavailability of carotenoids from foods and thus possibly enhancing their beneficial effects. The aim of this thesis was to determine the bioavailability of carotenoids from different food matrices and to identify dietary factors that affect carotenoid bioavailability. We primarily investigated the effect on carotenoid bioavailability of the type and intactness of the food matrix, of the digestibility and amount of dietary fat and of the interaction among carotenoids. Bioavailability was determined as the availability to the blood stream of carotenoids ingested. It may be assumed that an increase in blood carotenoid concentrations relates to an increase in availability of the carotenoids to tissues.

In the studies described in Chapters 2-4, we investigated whether the bioavailability of carotenoids varies among different food matrices and whether disruption of the matrix enhances the bioavailability of carotenoids from vegetables. In a 4-week intervention study (Chapter 2), we compared the changes in plasma carotenoid concentrations following consumption of a high vegetable diet with those following a low vegetable diet supplemented with pure β-carotene and lutein added to a salad dressing. The plasma carotenoid response induced by the high vegetable diet, expressed as the proportion of that induced by the pure carotenoids, corrected for differences in intake, was 14% for β-carotene and 67% for lutein. In another study (Chapter 3), we found that four days consumption of spinach did not significantly affect the plasma concentration of β-carotene. This was despite the fact that the β-carotene content of spinach was 10-fold greater than that of broccoli and green peas, both of which did induce statistically significant increases in plasma β-carotene concentrations. Compared to the increase resulting from supplementation with pure β-carotene, added to a mixed meal, the relative plasma responses observed for the vegetables were 74% for broccoli, 96% for green peas and only 3% for spinach. All three vegetables increased the plasma concentration of lutein. Broccoli and green peas were also more effective sources of lutein than spinach, when expressed per mg carotenoid ingested. Disruption of the spinach matrix increased the plasma response of lutein by 14%, whereas it had no significant effect on the bioavailability of β-carotene. The importance of the intactness of the vegetable matrix as a

determinant of carotenoid bioavailability from tomatoes was also demonstrated (Chapter 4). We investigated the effect of homogenisation and heating on the bioavailability of carotenoids from canned tomatoes. It appeared that both processes effectively enhanced the carotenoid bioavailability from tomatoes, although the effect of heating was not always statistically significant. The processing effects were apparent both in the carotenoid response in triglyceride-rich lipoproteins following single consumption and in fasting plasma after four days consumption of the tomato products. The plasma response of lycopene was increased by 21% and the area under the curve of the lycopene response in triglyceride-rich lipoproteins increased by 62% when the tomatoes were homogenised under high pressure (200 bar), compared to whole tomatoes. One hour of additional heat treatment (100°C) enhanced the lycopene responses by about 10% and 40% in plasma and triglyceride-rich lipoproteins respectively, but this effect was not significant ($P>0.05$).

The second question addressed in this thesis is related to the effects of the amount and digestibility of dietary fat on carotenoid bioavailability. Carotenoids are absorbed in association with dietary fat and therefore, the presence of dietary fat is thought crucial for carotenoid absorption. In the study described in Chapter 5, volunteers consumed 15 g/d of a full-fat margarine enriched with α- and β-carotene (2.7 mg/d α-carotene and 5.3 mg/d β-carotene) for four weeks. Compared to the group which consumed a non-enriched margarine, the concentrations of these carotenoids in plasma and low density lipoproteins increased substantially (15.5-fold and 4.3-fold in low density lipoproteins for α-carotene and β-carotene, respectively). In a subsequent study (Chapter 6), we compared the plasma responses of α-carotene, β-carotene and lutein following supplementation of these carotenoids together with a low-fat or high-fat meal (ca. 3 g vs 36 g fat). The carotenoids were added to a low-fat spread or a full-fat margarine (3% or 80% fat) which were provided with a standard hot meal. The plasma responses of α-carotene and β-carotene were not affected by the amount of fat present in the meal. In the case of lutein, however, the plasma response was significantly larger when lutein was added to the high-fat spread (2.3-fold larger increase in plasma lutein concentration). Lutein was present as lutein esters and we speculated that, in case of the low-fat treatment, the small amount of fat may have limited the solubilisation of lutein esters in the fat phase and/or the release and activity of esterases and lipases in the intestine. These enzymes are crucial for the hydrolysis of lutein esters, which is most probably a prerequisite for absorption. This study thus showed that a small amount of fat is sufficient to optimise the bioavailability of α-carotene and β-carotene, whereas the amount of fat required for the absorption of lutein esters is greater. In two other studies, we investigated the effect of an indigestible fat-replacer, sucrose polyester, on the plasma status of carotenoids (Chapter 7). Carotenoids

must be incorporated into mixed micelles before they are absorbed along with dietary fat. Sucrose polyester may intefere with the uptake of carotenoids into mixed micelles from dietary fat. Subsequently, carotenoids may be excreted together with intact sucrose polyester. In line with our hypothesis, we found that four weeks consumption of 3 g/d or 12.4 g/d of sucrose polyester, supplied in a spread and consumed with the main meal, reduced plasma concentrations of carotenoids. We found the largest decrease for the most lipophilic carotenoids, lycopene (48% and 62% with 3 g/d and 12.4 g/d sucrose polyester, respectively) and β-carotene (20% and 34%, respectively). It seems that a substantial proportion of the carotenoids ingested with the main meal was solubilised in the sucrose polyester, rather than in the dietary fat fraction.

Finally, we investigated whether individual carotenoids affect the bioavailability of other carotenoids. We compared the bioavailability of β-carotene from a palm oil carotenoid supplement, containing both α-carotene and β-carotene, with that of synthetic β-carotene (Chapter 8). Four days supplementation with the carotenoids, added to a mixed meal, resulted in significant increases in their plasma concentrations as compared with the consumption of a low-carotenoid meal. The relative plasma responses of β-carotene, per mg intake, were similar for the two supplements. We therefore concluded that α-carotene does not intefere with the bioavailability of β-carotene. The results of another study, however, suggest that carotenoids may interact with each other and affect bioavailability. In the study described in Chapter 2, we found that in the group which had received the low vegetable diet, supplemented with β-carotene (6 mg/d) and lutein (9 mg/d), plasma concentrations of lycopene were significantly reduced as compared to the control group receiving the low vegetable diet only. Apparently, β-carotene and/or lutein compete with lycopene for absorption and/or transport in plasma. Until now, however, data from other studies are conflicting.

In conclusion, the type of food matrix in which carotenoids are located largely determines their bioavailability and unabsorbable, fat-soluble compounds reduce carotenoid absorption. Although some dietary fat needs to be present to ensure absorption of carotenoids, the amount seems to be very low (3-5 g per meal). The minimal amount needed depends however on the physico-chemical characteristics of the carotenoids ingested. Interaction among carotenoids may occur, but the exact mechanism is unclear and data from ourselves and others are conflicting.

Research into the functional benefits of carotenoids should consider the data presented in this thesis, which show that the bioavailability of β-carotene in particular is one order of magnitude higher when provided as pure compound added to foods than when naturally present in foods. This should be taken into account when deciding on the

amount of carotenoids to be provided either added to foods or to pharmaceutical preparations.

Processing, such as mechanical homogenisation or heat treatment, has the potential to enhance the bioavailability of carotenoids from vegetables. This may be applied in the development of foods with enhanced carotenoid bioavailability. If novel food ingredients are developed, in particular if they are fat-soluble and absorbable only to a limited extent, attention should be paid to a possible negative impact on the bioavailability of carotenoids.

Samenvatting

De laatste jaren is uit grote bevolkingsonderzoeken gebleken dat een hoge consumptie van groenten en fruit samenhangt met een lager risico op het krijgen van ziekten die op latere leeftijd voorkomen, zoals kanker (vnl. longkanker en kanker aan het maagdarmkanaal) en mogelijk ook met hart- en vaatziekten en bepaalde oogziekten. Er wordt gedacht dat deze chronische ziekten ontstaan doordat het lichaam wordt blootgesteld aan zogenoemde vrije radicalen die het genetisch materiaal, het DNA, of andere moleculen, zoals de lipoproteïnen in het bloed, beschadigen. Deze vrije radicalen kunnen uit de omgeving komen, zoals luchtvervuiling, sigarettenrook of teveel zonlicht, maar ook van binnenuit tijdens het metabolisme. Stoffen in groenten en fruit die vrije radicalen onschadelijk kunnen maken, zoals bijvoorbeeld carotenoïden, zouden de gunstige effecten van een hoge groenten- en fruitconsumptie kunnen verklaren. Er bestaan heel veel verschillende typen carotenoïden. Er zijn zo'n 600 verschillende geïdentificeerd. In het menselijk lichaam zijn er echter maar 6 die in grotere hoeveelheden voorkomen. Dat zijn β-caroteen, α-caroteen, β-cryptoxanthine, α-cryptoxanthine, luteïne, zeaxanthine en lycopeen. De eerste drie carotenoïden kunnen in het lichaam worden omgezet in vitamine A.

Om de werking van carotenoïden te kunnen begrijpen is het belangrijk om te weten in welke mate ze uit de voeding worden opgenomen in het lichaam, met andere woorden, hun biobeschikbaarheid. Daarnaast is kennis van de factoren die de opname beïnvloeden van belang om na te gaan hoe de biobeschikbaarheid uit voedingsmiddelen kan worden verbeterd. We denken namelijk dat een grotere opname gunstig is voor de gezondheid. Het doel van dit proefschrift was daarom het bepalen van de biobeschikbaarheid van carotenoïden uit verschillende voedingsmiddelen en het identificeren van factoren in de voeding die de biobeschikbaarheid beïnvloeden. We hebben gekeken naar de invloed van het type en de "intactheid" van de voedingsmatrix, de verteerbaarheid en hoeveelheid vet in de voeding en de aanwezigheid van andere carotenoïden. De biobeschikbaarheid werd bepaald aan de hand van veranderingen in de gehaltes van carotenoïden in het bloed van gezonde vrijwilligers. Vaak wordt biobeschikbaarheid omschreven als de mate waarin een voedingscomponent beschikbaar is voor het weefsel waar het zijn functie uitoefent. Afgezien van het feit dat het niet bekend is welk weefsel dat voor de verschillende carotenoïden is, is dit met gezonde proefpersonen wat moeilijk. Je zou er echter vanuit kunnen gaan dat een verhoogde concentratie in het bloed samenhangt met een verhoogde beschikbaarheid van carotenoïden in het weefsel.

In de studies die in de hoofdstukken 2-4 beschreven zijn hebben we onderzocht of de biobeschikbaarheid van carotenoïden verschilt tussen verschillende voedingsmatrices en of

het kapot maken van de matrix de biobeschikbaarheid van carotenoïden uit groenten vergroot. In een vier weken durende voedingsproef hebben we de veranderingen in carotenoïd concentraties in plasma na consumptie van een dieet met veel groenten vergeleken met de veranderingen na consumptie van een dieet met weinig groenten, aangevuld met een supplement van eenzelfde hoeveelheid β-caroteen en luteïne als in het hoog-groentendieet. De plasma respons in de groep die het groentendieet had gekregen was, uitgedrukt als percentage van de plasma respons in de groep die het supplement had gekregen, 14% voor β-caroteen en 67% voor luteïne. Met name de stijging in het plasma gehalte van β-caroteen was dus veel kleiner in de groentengroep dan in de supplementgroep. In een andere studie, beschreven in hoofdstuk 3, vonden we dat de biobeschikbaarheid van carotenoïden niet alleen verschilt tussen groenten en een supplement, maar ook tussen groenten onderling. Vier dagen consumptie van extra spinazie gaf geen significante stijging van de plasma concentratie van β-caroteen, in tegenstelling tot vier dagen met extra broccoli of doperwten. Het frappante was dat de spinazie tien keer zoveel β-caroteen bevatte dan de broccoli en doperwten. In vergelijking met de stijgingen die een β-caroteen supplement gaf waren de relatieve stijgingen in plasma gehalten 74% voor broccoli, 96% voor doperwten en slechts 3% voor spinazie. Alle drie de groenten deden het plasma gehalte van luteïne stijgen. Echter, ook voor luteïne waren broccoli en doperwten effectiever, als rekening gehouden werd met de verschillen in hoeveelheid luteïne die de groenten leverden. Het malen van de bladspinazie vergrootte de plasma respons van luteïne met 14%, maar het had geen significant effect op de biobeschikbaarheid van β-caroteen. Het belang van de "intactheid" van de groentenmatrix voor de biobeschikbaarheid van carotenoïden werd ook gevonden voor tomaten (hoofdstuk 4). We onderzochten het effect van homogenisatie en verhitting op de biobeschikbaarheid van carotenoïden in tomaten. Het bleek dat beide processen effectief waren om de biobeschikbaarheid van carotenoïden uit tomaten te verbeteren, hoewel de effecten van verhitting niet altijd statistisch significant waren. De respons van lycopeen in het bloed was verhoogd met 62% direct na het eten van de tomaten en met 21% na vier dagen tomaten consumptie, als de tomaten niet als hele tomaten maar na homogenisatie onder hoge druk (200 bar) werden aangeboden. Een uur extra verhitting (100°C) verhoogde de respons van lycopeen met zo'n 40% direct na de maaltijd en 10% na vier dagen, maar deze effecten waren niet significant ($P>0.05$).

De tweede vraag die is beantwoord in dit proefschrift had betrekking op de invloed van de hoeveelheid en verteerbaarheid van voedingsvet op de biobeschikbaarheid van carotenoïden. Carotenoïden worden tegelijk met voedingsvet opgenomen in de dunne darm en daarom wordt gedacht dat de aanwezigheid van vet cruciaal is voor de opname van carotenoïden. In een proef beschreven in hoofdstuk 5, consumeerden vrijwilligers

gedurende vier weken dagelijks 15 gram van een margarine (80% vet) die verrijkt was met α- en β-caroteen (2.7 mg/dag α-caroteen en 5.3 mg/dag β-caroteen). Na vier weken waren de concentraties van deze carotenoïden sterk gestegen in het plasma. In vergelijking met de groep die een gewone margarine consumeerde, was er een 14.5-voudige stijging in α-caroteen en een 3.4-voudige stijging in β-caroteen gehaltes in het plasma. In een daarop volgende studie (hoofdstuk 6) vergeleken we de plasma responsen van α-caroteen, β-caroteen en luteïne na consumptie van deze carotenoïden met een maaltijd die weinig of veel vet bevatte (ca. 3 g vs 36 g vet). De carotenoïden waren toegevoegd aan een laag-vet spread of een volvette margarine (3% of 80% vet) en deze spread of margarine werd gegeten met een standaard warme maaltijd gedurende zeven dagen. De plasma responsen van α-caroteen en β-caroteen waren niet afhankelijk van de hoeveelheid vet in de maaltijd. De plasma respons van luteïne was echter significant groter als het luteïne was toegevoegd aan de volvette margarine (2.3-voud grotere stijging). Luteïne was toegevoegd als luteïne-esters en we denken dat de hoeveelheid vet die nodig is voor het oplossen van deze esters of voor de uitscheiding van esterases en lipases in de dunne darm groter is dan de hoeveelheid die aanwezig was in de laag-vet maaltijd. Esterases en lipases zijn nodig voor het splitsen van luteïne-esters en waarschijnlijk moet het luteïne eerst vrijgemaakt worden voor het kan worden opgenomen. In twee andere studies hebben we onderzocht wat de invloed was van een niet-verteerbare vetvervanger, nl. sucrose polyester, op de plasma gehaltes van carotenoïden (hoofdstuk 7). Dit sucrose polyester smaakt precies hetzelfde als gewoon vet, maar levert geen calorieën omdat het niet verteerbaar is. Een negatief bij-effect zou kunnen zijn dat het ook de opname van carotenoïden vermindert. In plaats van opname met het normale voedingsvet in de dunne darm zouden ze samen met het sucrose polyester uitgescheiden kunnen worden in de faeces. Deze hypothese werd bevestigd. We vonden namelijk dat consumptie van 3 g/dag of 12.4 g/dag sucrose polyester, toegevoegd aan een spread en met de warme maaltijd gegeten, de plasma concentraties van carotenoïden verlaagde. De grootste reductie werd gevonden voor de meest vetoplosbare carotenoïden, lycopeen (48% en 62% reductie met respectievelijk 3 g/dag en 12.4 g/dag sucrose polyester) en β-caroteen (20% en 34% reductie met respectievelijk 3 g/dag en 12.4 g/dag sucrose polyester). Het lijkt erop dat een deel van de carotenoïden uit de warme maaltijd beter oplost in het sucrose polyester dan in het normale vet van de warme maaltijd.

Tenslotte hebben we onderzocht of carotenoïden de biobeschikbaarheid van andere carotenoïden beïnvloeden. We vergeleken de biobeschikbaarheid van β-caroteen uit een palmolie-supplement dat zowel β-caroteen als α-caroteen bevatte, met de biobeschikbaarheid van synthetisch β-caroteen (hoofdstuk 8). Na vier dagen supplementatie waren de plasma gehaltes van de carotenoïden significant gestegen, in

vergelijking met de gehaltes die gevonden werden na vier dagen consumptie van een maaltijd zonder carotenoïden. Per miligram inname was de stijging in β-caroteen-gehalte vrijwel gelijk voor de twee typen supplementen. Daarom concludeerden we dat de aanwezigheid van α-caroteen geen invloed heeft op de biobeschikbaarheid van β-caroteen. In een andere studie, echter, vonden we wel dat carotenoïden elkaars biobeschikbaarheid kunnen beïnvloeden. In de studie die in hoofdstuk 2 beschreven staat, vonden we in de groep die het supplement van β-caroteen en luteïne had gekregen een significante daling in de plasma concentratie van lycopeen. Blijkbaar heeft er een competitie plaatsgevonden tussen β-caroteen en/of luteïne en lycopeen tijdens absorptie en/of voor transport in het plasma.

Op basis van deze gegevens concluderen we dat de biobeschikbaarheid van carotenoïden grotendeels bepaald wordt door het type voedingsmatrix waarin de carotenoïden zich bevinden en dat onverteerbare, vetoplosbare componenten in de voeding de absorptie van carotenoïden verminderen. Hoewel vet in de voeding aanwezig moet zijn voor de opname van carotenoïden, is de hoeveelheid die nodig is zeer klein (3-5 g in een maaltijd). De minimum hoeveelheid hangt echter af van de fysisch-chemische kenmerken van de carotenoïden. Interactie tussen carotenoïden kan plaatsvinden, maar het mechanisme dat daarvoor verantwoordelijk is, is niet bekend en de data beschreven in dit proefschrift en die van andere onderzoekers op dit gebied zijn niet eenduidig.

Toekomstig onderzoek naar de gezondheidseffecten van carotenoïden moet rekening houden met onze bevinding dat de biobeschikbaarheid van met name β-caroteen zo'n tien keer groter is als het als pure component wordt aangeboden dan als het in de natuurlijke voedselmatrix (nl. groenten) aanwezig is. Dat is met name van belang bij de beslissing over de hoeveelheid die wordt verstrekt, toegevoegd aan voedingsmiddelen of als farmaceutisch preparaat.

Bewerking van groenten, zoals homogenisatie of hittebehandeling, kan de biobeschikbaarheid van carotenoïden uit groenten vergroten. Dit zou toegepast kunnen worden bij de ontwikkeling van producten met een verhoogde biobeschikbaarheid van carotenoïden. Bij de ontwikkeling van nieuwe ingrediënten voor voedingsmiddelen moet rekening worden gehouden met een mogelijk negatief effect op de biobeschikbaarheid van carotenoïden. Dit geldt met name voor vetoplosbare ingrediënten met een verminderde opneembaarheid.

Dankwoord

Omgevingsfactoren die het succes van een promovendus bepalen

In dit proefschrift zijn factoren besproken die de biobeschikbaarheid van carotenoïden kunnen beïnvloeden. We denken dat voldoende opname van carotenoïden in het lichaam van belang is voor een goede gezondheid. Daarom streven we naar een zo optimaal mogelijke opneembaarheid van carotenoïden uit de voeding. De besproken factoren vertonen een interessante gelijkenis met de factoren die het succes van een promovendus bepalen. Deze gelijkenis wil ik in dit dankwoord bespreken.

Het soort carotenoïd

Dat is de basis, het allereerste begin. Uit welk hout is de promovendus of promovenda gesneden. Dat was mijn eerste geluk. Mama, dank je wel dat je mij (naast natuurlijk vele andere dingen) discipline en doorzettingsvermogen hebt bijgebracht. Zonder die eigenschappen lukt slechts weinig. Op deze plaats wil ik ook mijn vader noemen, die helaas niet meer is. De helft van mij bestaat uit hem. Het is zo jammer dat ik niet meer van hem mocht leren. Dank echter aan allen die wel een voorbeeld konden geven en mij stimuleerden om mezelf te ontplooien. Marian van Duren, bedankt dat je me bij de invulling daarvan hebt geholpen. Michiel Meijers en Merel Ritskes, bedankt voor jullie stimulans om een rode lijn in mijn onderzoek te ontdekken.

De matrix waarin een carotenoïd zich bevindt

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De hoeveelheid carotenoïd aanwezig

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Interactie met andere carotenoïden

Als we het over interactie tussen carotenoïden wat betreft biobeschikbaarheid praten, denken we in eerste instantie aan een remmend effect van de een op de ander. Het kan echter ook zo zijn dat die carotenoïden elkaar sparen of versterken en er sprake is van synergie. Dat laatste is ook voor een promotie en voor een optimaal functioneren in het algemeen, van belang. Mijn collega's van het UNC en in Colworth en gelijkgezinden in Wageningen en in Düsseldorf hebben daarom een belangrijke bijdrage geleverd aan mijn wetenschappelijke en werk-sociale vorming. Een paar noem ik bij naam: Lilian Tijburg, Sheila Wiseman, Annet Roodenburg en Rianne Leenen, dank jullie wel voor de samenwerking en voor de ruggespraak die ik met jullie kon houden. Lilian, fijn dat je mijn paranimf wilt zijn. Misschien kun je me nog wat "tactische" antwoorden toespelen. Ben de Boer, dank voor alles wat ik van jou over groenten en communicatie heb geleerd. And my colleagues in Colworth from the Veg Nutrition team: thanks for the good team work we have built over the years. Jacqueline Castenmiller, gelijkgezinde in Wageningen, dank voor de informatie en gegevens die we samen uitwisselden. Willi Stahl and Christine Gärtner from the University of Düsseldorf: thank you for the fruitful co-operations we have had.

De aanwezigheid van vet

Vet is essentiëel voor de vorming van micellen en de opname van carotenoïden. Zo is het ook met vrijwilligers die aan voedingsproeven deelnemen. Zonder hen geen studie en zonder hen geen proefschrift. Ik heb altijd weer bewondering gehad voor de flexibiliteit en bereidheid van "onze" vrijwilligers. Daarom bij deze: mijn dank aan hen. Daarnaast is een andere groep mensen van essentiëel belang: vrienden en familie die ervoor zorgden dat ik kon functioneren. Dank jullie voor de belangstelling voor mijn werk en de tips die ik van jullie kreeg. Lianne Pieters-van het Hof, mijn zus, er komt nu eindelijk een feestje, je hebt het verdient. En dan mijn speciale druppel olie: Albert Krikke, die mij stimuleert en steunt. Mijn lief, mijn dank, mijn liefde.

About the author

Karin van het Hof was born on June 12, 1968 in Zwanenburg, the Netherlands. After finishing high school, i.e. Atheneum, at the Scholengemeenschap 'Oost-Betuwe' in Bemmel in 1986, she started the study Human Nutrition at the Wageningen Agricultural University. As part of her training, she spent 5 months at the Human Nutrition Unit of the University of Sydney in Australia. She received a Master of Science in Human Nutrition in 1992. In the same year, she commenced working in the Unilever Nutrition Centre of Unilever Research Vlaardingen as manager clinical trials. In this job, she performed research on a variety of subjects, including the impact on health of foods containing modified or less fat and dietary antioxidants (e.g. tea flavonoids, carotenoids). Since 1995, her research has become more focused on dietary factors that may affect the bioavailability of carotenoids from vegetables and tomato products. In 1997, she became account manager Frozen Vegetables in the Unilever Nutrition Centre and one of her major tasks is translation of marketing questions into a research strategy in the area of vegetables and health and communication of nutrition research to the Unilever Vegetables business. Currently, she also participates in a project at VandenBergh Nederland, Rotterdam, as part of a business training programme. Karin participated in the European Nutrition Leadership Programme of 1998 in Luxembourg. She is invited to present the research described in this thesis at the US Food and Nutrition Board's workshop on Vitamins A and K in May 1999.

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