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Carotenoid availability – from dietary intake to factors governing their bioavailability as studied by *in vitro* digestion coupled to Caco-2 cellular uptake models

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Vielen Dank an meine Familie,

für die Unterstützung,

und an Martina,

für ihre Geduld

1 Zusammenfassung

Hintergrund

Carotinoide sind natürlich vorkommende, gelb-rote Farbstoffe. Sie werden von Pflanzen und Mikroorganismen synthetisiert und dienen als Elektronenakzeptoren im Phytosyntheseprozess. Menschen profitieren von ihren antioxidativen Eigenschaften, sind aber alleinig von deren Zufuhr über die Nahrung abhängig. Regelmäßige und reichliche Aufnahme von Carotinoiden, besonders in Form von carotinoidreichen Früchten und Gemüse, wird mit der Prävention von koronaren Herzerkrankungen, Tumoren, Makuladegeneration und möglicherweise auch Osteoporose in Verbindung gebracht. Weiterhin besitzen einige Carotinoidspezies, wie zum Beispiel Alpha- und Beta-Carotin, Pro-Vitamin-A-Aktivität. Vitamin A ist essentiell für die embryonale Entwicklung, Zellteilung, den Sehvorgang und die Immunabwehr.

Die Aufnahme einer Substanz ist nicht nur von der verzehrten Menge abhängig, sondern auch von deren Bioverfügbarkeit, d.h. dem Anteil der Gesamtmenge einer Substanz, der während des Verdauungsprozesses in den Körper aufgenommen werden kann, die systemische Zirkulation erreicht und für verschiedenste Prozesse zur Verfügung steht. Die Bioverfügbarkeit von Carotinoiden liegt - hauptsächlich aufgrund ihrer hauptsächlich lipophilen Eigenschaften - weit unter 100% und wird von einer Vielzahl von Faktoren beeinflusst: auf der einen Seite finden sich physiologische (wirtsabhängige) Faktoren, wie zum Beispiel die Menge an Verdauungsenzymen und Gallensalzen, die ausgeschüttet werden, den Passagezeiten der Nahrung durch den Gastro-Intestinaltrakt und möglichweise auch die Darmflora. Auf der anderen Seite ist die Bioverfügbarkeit von der Nahrungsmatrix, der Menge an Fett in der Mahlzeit, den unterschiedlichen Carotinoidspezies und einer Vielzahl von Inhibitoren/Verstärkern abhängig (matrixabhängige Faktoren). Vor allem im Bereich der Aufnahmemechanismen (aktiv, passiv oder erleichterte Diffusion) und im Bereich der Inhibitoren/Verstärker scheinen weitere Studien erforderlich zu sein.

Um ethisch fragwürdige Tier- und Humanstudien zur Bioverfügbarkeit von Carotinoiden zu vermeiden, wurden in den in vergangenen Jahren *in vitro* Methoden entwickelt, die eine Simulation der Verdauung mit anschließender Aufnahme in menschliche Darmzellen ermöglichen. Mit Hilfe dieser *screening*-Techniken können

Einflussfaktoren der Carotinoid-Bioverfügbarkeit bestimmt werden, von denen wiederum nur die vielversprechendsten in gezielten Tier- und Humanstudien getestet werden.

Zielsetzung

Ziel der vorliegenden Doktorarbeit war es, wirtsabhängige und matrixabhängige Faktoren zu testen, welche potentiell die Bioverfügbarkeit von Carotinoiden bzw. deren Abbauprodukte beeinflussen können. Ein besonderer Augenmerk sollte auf Mineralstoffe und Spurenelemente gelegt werden, da diese mit Fettsäuren und Gallensalzen interagieren können, welche wiederum für die Mizellarisierbarkeit von lipophilen Stoffen entscheidend sind. Weiterhin wurde eine Reihe von häufig verzehrten Lebensmitteln, vor allem Obst und Gemüse, auf ihren Carotinoid-Gehalt hin untersucht. Mit Hilfe von nationalen Verzehrsdaten wurde dann eine Abschätzung der Carotinoidaufnahme im Großherzogtum Luxemburg durchgeführt und mit den Daten anderer Länder verglichen.

Studienaufbau

Zunächst wurde der Carotinoidgehalt von 50 verschiedenen, häufig in Luxemburg konsumierten Nahrungsmitteln bestimmt. Dazu wurden Verzehrsdaten einer nationalen Herz-Kreislauf-Risikofaktoren-Studie (ORISCAV-LUX) mit den Carotinoidgehalten der Nahrungsmittel multipliziert, um die Carotinoidaufnahme der Luxemburger Bevölkerung abzuschätzen.

Ein *In-vitro*-Verdauungs-Modell und die humane Caco-2 Zelllinie wurden etabliert und auf Reproduzierbarkeit/Wiederholbarkeit im Hinblick auf Carotinoid-Konzentrationen untersucht. In weiteren Etappen wurden bereits publizierte Daten (Garrett *et al* 1999a) genutzt, um die Testsysteme zu validieren und eine geeignete Testmahlzeit (und deren Zusammensetzung) zu finden, sowie die Enzym- und Gallensalzkonzentrationen zu optimieren.

Nach der Validierung wurden potentielle Inhibitoren und Verstärker der Carotinoidverfügbarkeit getestet, wie zum Beispiel Cholesterin, Stigmasterol, α-Tocopherol, sowie verschiedene Mineralstoffe und Spurenelemente.

Ergebnisse

Karotten (18.0 ± 2.3 mg/100 g), Spinat (13.1 ± 1.4 mg/100 g) und Cremespinat (11.1 ± 0.5 mg/100 g) enthielten die höchsten Carotinoidkonzentrationen von allen untersuchten Lebensmitten. Die abgeschätzte Carotinoidaufnahme in Luxemburg war mit 12.9 mg/Tag etwas geringer als in anderen Ländern, mit der Ausnahme Deutschlands (5.3 mg/Tag) und Spaniens (8.1 mg/Tag).

Cholesterin in sehr hohen Konzentrationen (1.9 g/100 g Testmahlzeit) verringerte signifikant die Mizellarisierbarkeit von Gesamtcarotinoiden während der simulierten Verdauung auf 61.3 ± 2.9% ($p<0.001$, Bonferroni) der Kontrolle (= 100%), während Stigmasterol in gleichen Konzentrationen sowie α-Tocopherol (16.6 mg/100 g) keinen Einfluss hatten. Erhöhte NaCl-Konzentrationen (280 mM und 530 mM) während des simulierten Verdauungsprozesses steigerten die Mizellarisierbarkeit von Beta-Carotin auf 137.5 ± 24.3% bzw. 165.3 ± 29.2%, (beide $p<0.001$, T-Test), während die Mizellarisierbarkeit von Lutein (77.4 ± 13.0%, $p<0.05$ bzw. 85.7 ± 5.6%, $p<0.005$) im Vergleich zur Kontrolle (100 ± 7.9%, 150 mM NaCl) verringert wurde.

Sowohl die Mizellarisierbarkeit als auch die zelluläre Aufnahme von Gesamt-Carotinoiden wurde durch die Anwesenheit von divalenten Ionen (Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}; Konzentrationen: 3.8 mM – 25 mM) reduziert. Die geringste Mizellarisierbarkeit (12.5 ± 0.9%, $p<0.001$, Bonferroni) und Zellaufnahme (24.1 ± 5.1%, $p<0.001$, Bonferroni) im Vergleich zur Kontrolle wurde in Anwesenheit von Fe^{2+} (12.5 mM) bestimmt, während der Effekt von Mg^{2+} (25 mM) im Vergleich zur Kontrolle am geringsten war (96.8 ± 0.5% Mizellarisierbarkeit (nicht-signifikant) und Zellaufnahme 69.2 ± 17.8% ($p<0.001$, Bonferroni)). Wenn lediglich die prozentuale Zellaufnahme betrachtet wurde, war die Aufnahme von Beta-Carotin aus den Mizellen - in Anwesenheit von Fe^{2+}, Zn^{2+} und Ca^{2+} - 5-10fach im Vergleich zur Kontrolle (= 100%) erhöht während die Lutein-Aufnahme lediglich 1.3-1.6fach höher war ($p<0.001$, Bonferroni).

Die Umwandlung der beiden Epoxycarotinoide Neoxanthin und Violaxanthin zu Furanoidcarotinoiden sowie deren Mizellarisierbarkeit und Aufnahme in Caco-2-Zellen konnte nachgewiesen werde. Neochrom, ein Umwandlungsprodukt von Neoxanthin, wurde von den Zellen signifikant besser aufgenommen (6.6 ± 0.9%) als

Lutein (3.3 ± 0.8%) und Beta-Carotin (0.2 ± 0.04%) ($p<0.001$, Bonferroni). Die Umwandlungsprodukte von Violaxanthin, Luteoxanthin und Auroxanthin, wurden ähnlich gut aufgenommen wie Lutein (kombinierte Aufnahme beider Spezies: 3.1 ± 0.4%). Carotinoidvorstufen, wie z.B. Phytoen und Phytofluen, konnten in der Testmahlzeit nicht nachgwiesen werden.

Schlussfolgerungen

1) Die Carotinoidaufnahme in Luxemburg scheint geringfügig unter dem Durchschnitt anderer europäischer und außer-europäischer Länder zu liegen. Um eine vollständigere Abschätzung zu erhalten, müsste jedoch der Carotinoidgehalt von weiteren Lebensmitteln, insbesondere der von kompletten Mahlzeiten, einschließlich Fertigmahlzeiten, bestimmt werden.

2) Die mit Hilfe des *in vitro* Models und der human Darmzelllinie Caco-2 gewonnen Ergebnisse stimmten zum großen Teil mit Daten von Tier- und Humanstudien überein und bestärken dadurch deren Verwendbarkeit als *screening*-Methoden.

3) Mineralstoffe und Spurenelemente, wie zum Beispiel Ca^{2+}, Zn^{2+}, Fe^{2+} und abgeschwächt Mg^{2+}, beeinflussten die Löslichkeit von Carotinoiden konzentrationsabhängig. So wurde mit steigender Mineralstoffkonzentration eine verminderte Mizellarisierbarkeit von Carotinoiden festgestellt. Obwohl die Beta-Carotin-Konzentration in den Mizellen mit steigenden Mineralkonzentrationen vermindert wurde (Ausnahme Mg^{2+}), war die Zellaufnahme, im Vergleich zur Kontrolle, weniger betroffen und somit stark erhöht im Vergleich zu Lutein. Die verstärkte, relative Aufnahme ist möglicherweise auf verminderte kompetititve Hemmung oder Wechselwirkung mit anderen Carotinoiden zurückzuführen. Weiterhin deuten die Ergebnisse auf erleichterte Diffusion als Transportmechanismus hin.

4) Epoxycarotinoide, wie zum Beispiel Neoxanthin und Violaxanthin, reagierten unter den gewählten Bedingungen vollständig zu Furanoid-Carotinoiden. Diese scheinen gut mizellarisiert und in Zellen aufgenommen zu werden. Vermutlich kann durch die Epoxid-Furanoid-Umwandlung teilweise die geringe Bioverfügbarkeit der Epoxymoleküle erklärt werden, welche in Humanstudien beschrieben wurde.

Summary

Background

Carotenoids are naturally abundant yellow-to-red pigments. They are synthesized by all plants and some microorganisms and function as electron acceptors in the process of photosynthesis. Humans benefit from their antioxidant properties but are exclusively dependent on dietary uptake. Regular consumption of carotenoids, especially in form of fruits and vegetables, has been associated with the prevention of cardio-vascular disease, cancer, macular degeneration and perhaps osteoporosis. Some carotenoid species, such as alpha- and beta-carotene show provitamin A activity. Vitamin A is essential for embryonic development, cell division, the vision process and the immune defense.

In general, the uptake of a substance is not only dependent on the amount ingested, but also on its bioavailability, that is the fraction of a substance taken up by the human body during digestion, reaching the systemic circulation and being available for various functions. The bioavailability of carotenoids, usually far below 100 %, is influenced by a multitude of factors: on the one hand, there are physiological (host-dependent) factors, such as the amount of digestive enzymes and bile salts that are secreted, passage time through the gastro-intestinal tract and perhaps the intestinal flora. On the other hand, bioavailability is influenced by the food matrix, the amount of fat ingested, different carotenoid species present and a variety of inhibitors/enhancers. Especially the different uptake mechanisms (active, passive or facilitated diffusion) and the dietary inhibitors/enhancers are controversially discussed.

In order to avoid ethically disputable animal and human studies on the bioavailability of carotenoids, *in vitro* methods have been developed that allow simulating gastro-intestinal passage coupled to cellular uptake in human intestinal cells. Employing these techniques, factors influencing bioavailability can be screened. Out of these factors, only the most relevant may then be further investigated in well-directed animal and human studies.

Aims

The objective of this PhD programme was to evaluate host- and matrix dependent factors potentially impacting carotenoid bioavailability including their digestion products. A special emphasis was placed on minerals, which may interact with fatty acids and bile salts, which in turn are essential for the micellarisation of lipophilic compounds. Furthermore, carotenoid content of a variety of commonly consumed foods, especially fruits and vegetables, was investigated. Together with national food consumption data, carotenoid intake of the Luxembourgish population was assessed and compared to data from other countries.

Study design

Carotenoid content of 50 different, commonly consumed food items was analysed. Consumption data derived from a locally conducted cardio-vascular risk factor study (ORISCAV-LUX) was then multiplied with the carotenoid content of the consumed foods and the carotenoid intake of the population was estimated.

An *in vitro* digestion model and the human intestinal Caco-2 cell line were both established and the reproducibility/repeatability with respect to carotenoid recovery was investigated. In further steps, published data (Garrett *et al* 1999a) were used to validate the test systems and to find a suitable test meal (and composition) as well as to optimise enzyme and bile salt concentrations.

After validation, potential inhibitors and enhancers of carotenoid bioavailability were investigated, such as cholesterol, stigmasterol, α-tocopherol, and different minerals.

Results

Carrots (18.0 ± 2.3 mg/100 g), spinach (13.1 ± 1.4 mg/100 g) and cream spinach (11.1 ± 0.5 mg/100 g) contained highest carotenoid concentrations of all foods investigated. Estimated carotenoid consumption in Luxembourg (12.9 mg/day) was slightly lower than in other countries compared, with the exception of Germany (5.3 mg/day) and Spain (8.1 mg/day).

Cholesterol at very high concentrations (1.9 g/100 g test meal) diminished carotenoid micellarisation during *in vitro* digestion (61.3 ± 2.9%, $p<0.001$, Bonferroni), in

contrast to stigmasterol at similar concentrations as well as α-tocopherol (16.6 mg/100 g).

Increased sodium chloride concentrations (280 mM und 530 mM, resp.) present during simulated digestion increased micellarisation of beta-carotene to 137.5 ± 24.3% and 165.3 ± 29.2% ($p<0.001$, t-test), respectively, while the micellarisation of lutein (77.4 ± 13.0%, $p<0.05$ and 85.7 ± 5.6%, $p<0.005$, Bonferroni, resp.) was decreased compared to the control (100 ± 7.9%, 150 mM NaCl).

Both solubility and cellular uptake of total carotenoids were diminished in the presence of divalent minerals (range: 3.8 mM – 25 mM). Lowest micellarisation (12.5 ± 0.9%, $p<0.001$, Bonferroni) and cellular uptake (24.1 ± 5.1%, $p<0.001$, Bonferroni) compared to the control was detected in the presence of Fe^{2+} (12.5 mM), while the impact of Mg^{2+} (25 mM) was lowest (96.8 ± 0.5% micellarisation (non-significant) and cellular uptake 69.2 ± 17.8% ($p<0.001$, Bonferroni)) compared to the control. Focussing on the fractional cellular carotenoid uptake from micelles, beta-carotene uptake was 5-10-times increased compared to the control, in the presence of Fe^{2+}, Zn^{2+} and Ca^{2+}, while lutein uptake was only 1.3-1.6-times augmented ($p<0.001$, Bonferroni).

The conversion of both epoxycarotenoids neoxanthin and violaxanthin to furanoid carotenoids, their micellarisation and uptake in Caco-2 cells was detected. Neochrome, a digestion product of neoxanthin, was significantly better taken up into the cells (6.6 ± 0.9%) compared to lutein (3.3 ± 0.8%) and beta-carotene (0.2 ± 0.04%) ($p<0.001$, Bonferroni). Cellular uptake of the conversion products of violaxanthin, such as luteoxanthin and auroxanthin, was similar to lutein (combined 3.1 ± 0.4%).

Conclusion

1) Carotenoid intake of the Luxembourgish population appears to be slightly lower than in other European countries, as well as Israel and Australia. Carotenoid contents of further foods, such as complete meals, including ready-to-eat-meals, have to be determined in order to achieve a more thorough estimation.

2) Results found with the *in vitro* model and the human intestinal Caco-2 cell line are in agreement with data from animal and human studies, hence confirming the usefulness of the screening methods.

3) Minerals and trace elements, such as Ca^{2+}, Zn^{2+}, Fe^{2+} and partly Mg^{2+} affect carotenoid micellarisation and cellular uptake in a concentration dependent manner. With increasing mineral concentrations, a diminished carotenoid solubility was detected. In the case of beta-carotene, cellular uptake was less affected in comparison to the control (exception Mg^{2+}), although the concentration in the micelles decreased with increasing mineral concentrations. This enhanced uptake might be due to a reduced competitive inhibition or interaction with other carotenoid species.

4) Epoxycarotenoids, such as neoxanthin und violaxanthin, react entirely to furanoid carotenoids under the conditions chosen. They were micellarised and taken up by the Caco-2 cells to a similar extent compared to xanthophylls. This might explain the low bioavailability of the parental compounds described in human studies. Carotenoid precursors, such as phytoene and phytofluene, were not detected in the test meal.

2 Introduction

2.1 General properties of carotenoids

2.1.1 Occurrence

Carotenoids are lipophilic pigments widespread in nature, with more than 700 species known today (Britton *et al* 2004). They can be synthesized by all plants, many bacteria and some fungi, with animals including humans relying solely on dietary uptake. Carotenoids are part of the light harvesting complexes of photosynthetic organisms, and additionally, in plants, they are stored in chromoplasts where they contribute to the coloration, such as in petals, e.g. of marigold and in roots, e.g. of carrots.

2.1.2 Formation

During synthesis, eight isoprene units are fused to a long chain of conjugated double bonds, forming a polyisoprenoid structure of 40 carbon atoms. Different carotenoid species are mainly derived by introduction of oxygen functions and by modifications of the hydrocarbon chain, such as by cyclisation at one or both ends. The oxygen-containing species are known as xanthophylls, while simple hydrocarbon species are termed carotenes (Britton 1995). In Figure 1, synthesis pathways from carotenoid precursors to major xanthophylls are presented.

2.1.3 Properties

Oxo-carotenoids can stabilise biological membranes, limit oxygen penetration and lipid peroxidation, crossing the lipid bilayer with the polyene chain, with their polar groups being anchored in the opposite polar membrane-aqueous interphases. In contrast, carotenes, such as lycopene and beta-carotene, are described to reside in the apolar core of lipid bilayers, potentially reacting only with radicals created in the membrane core (reviewed by Gruszecki *et al* 2005).

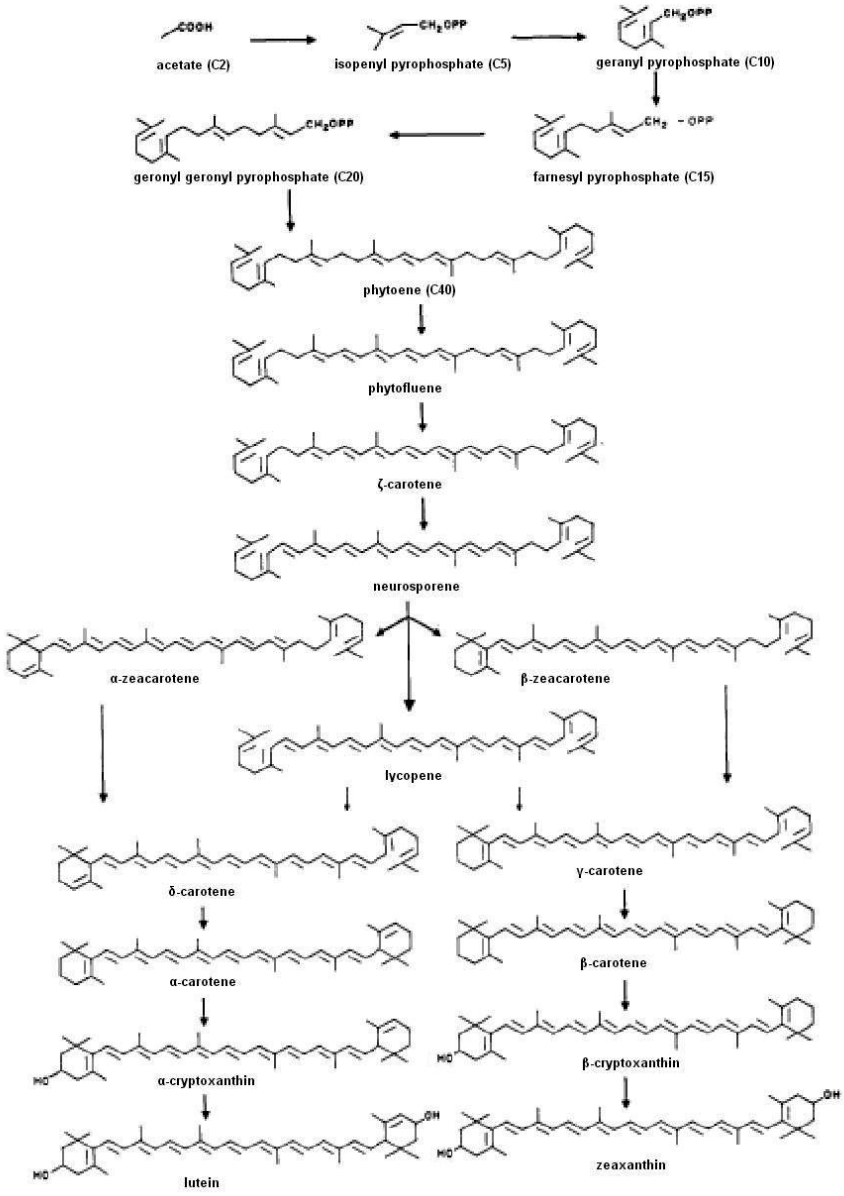

Figure 1: Biosynthetic scheme for major carotenoids (adapted from Bauernfeind 1972)

Although the introduction of oxygen has a significant impact on the chemical properties of the molecule, such as the potential to form esters, the pigment properties are mostly depending on the length of the conjugated Π-electron system, ranging in colour from flimsy yellow to dark red. Typically, carotenoids show a three peaked absorption spectrum, with the middle 'finger' yielding the absorption maximum (see Figure 2). The ratio (absorption peak III / absorption peak II) is, next to retention time in chromatography and intensity of absorption, a major criterion for

quantification/detection by UV-VIS (ultra violet – visible light) spectrometry (Rodriguez-Amaya *et al* 2004).

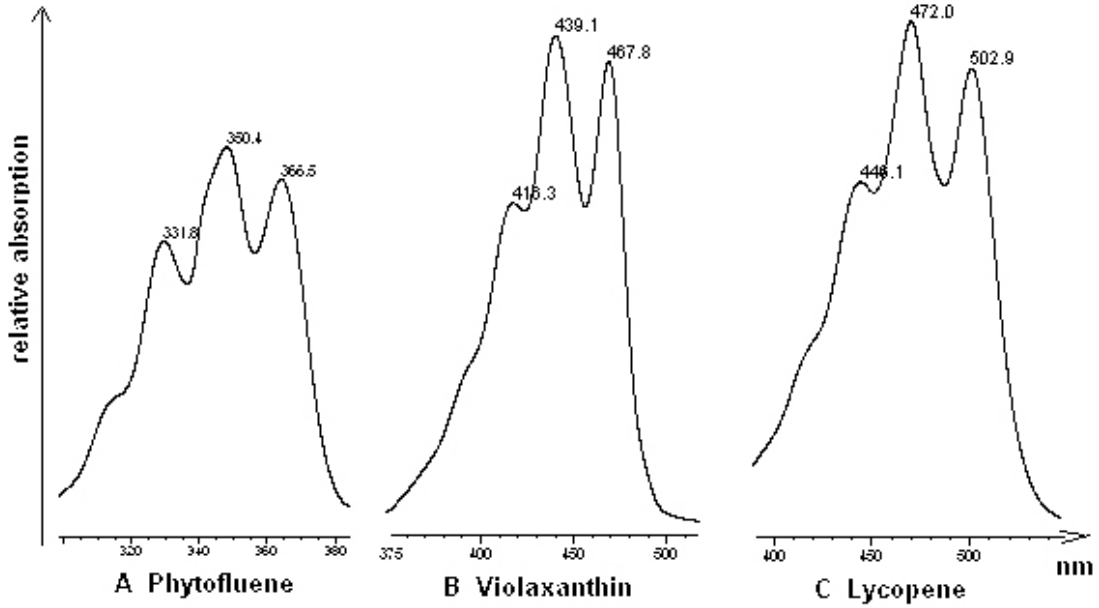

Figure 2: Typical three-peaked carotenoid absorption spectra as recorded by diode array detector, shown for A phytofluene (flimsy yellow, 5 conj. double bonds), B violaxanthin (yellow, 9 conj. double bonds) and C lycopene (dark red, 11 conj. double bonds). The abscissae display the wavelength.

2.1.4 Natural functions

The highly delocalised electron system is not only responsible for the colour, but is further efficient in stabilising reactive molecules, such as radicals, acting either as singlet oxygen ($^1O_2$) quencher or free radical, e.g. peroxyl- (ROO°) or hydroxyl- (OH°) radical scavenger (Britton 1995). In photosynthetic organisms, carotenoids such as lutein, zeaxanthin and violaxanthin are located in the light harvesting complexes, partially filling the 'green gap' of chlorophyll absorption (450 – 550 nm), efficiently transferring singlet energy to chlorophyll and protecting the complex by removing triplet energy from chlorophyll a (van Amerongen *et al* 2003), with direct radical interaction also being possible. However, as only a minor fraction of light energy captured by carotenoids is transferred to the chlorophylls, the protection from direct or indirect light damage is likely to be the main feature (Scheer 2003).

2.1.5 Importance for humans

For humans, an additional function is of high importance: the provitamin A activity of some carotenoids. These species, containing one or two beta-ionone rings, such as beta-carotene (2 rings, Figure 3), alpha-carotene (1 ring) and beta-cryptoxanthin (1 ring) can be cleaved into retinol, which is then metabolised via retinaldehyde to retinoic acid. The latter three metabolites show vitamin A activity in humans and exert many functions during embryonic development (Morris-Kay *et al* 1999), participate in the immune response (Hughes 1999) and in the vision cycle (Bendich *et al.*, 1989, Simpson *et al.*, 1981). Although beta-carotene could theoretically yield two molecules and beta-cryptoxanthin/alpha-carotene one molecule of retinol, the conversion ratio *in vivo* appears to be much lower, yielding only 1 molecule of retinol per 12 molecules of dietary beta-carotene (24 molecules of dietary alpha-carotene) (Institute of Medicine 2001).

While research had mostly focused on vitamin A precursors, non-provitamin species, such as lycopene, lutein and zeaxanthin, have recently moved in the focus of attention. There is growing evidence for their preventive role in a number of chronic or age-related diseases, partly but not exclusively due to their antioxidant properties. For example, lycopene has been proposed to impede the onset and proliferation of prostate cancer (Giovannucci *et al* 2002, Kucuk *et al* 2001) and increase gap-junction intercellular communication (Livny *et al* 2002, Stahl *et al* 2000), while lutein and zeaxanthin seem to be of further importance for the proper functioning of the human retina of the eye (Bernstein *et al* 2001, Landrum *et al* 1997a). It has been suggested that some carotenoids, especially when consumed in form of fruits and vegetables, prevent or alleviate cardiovascular disease (Hozawa *et al* 2009, Voutilainen *et al* 2006) and may play a beneficial role for bone health and density (Sahni *et al* 2009, Wattanapenpaiboon *et al* 2003).

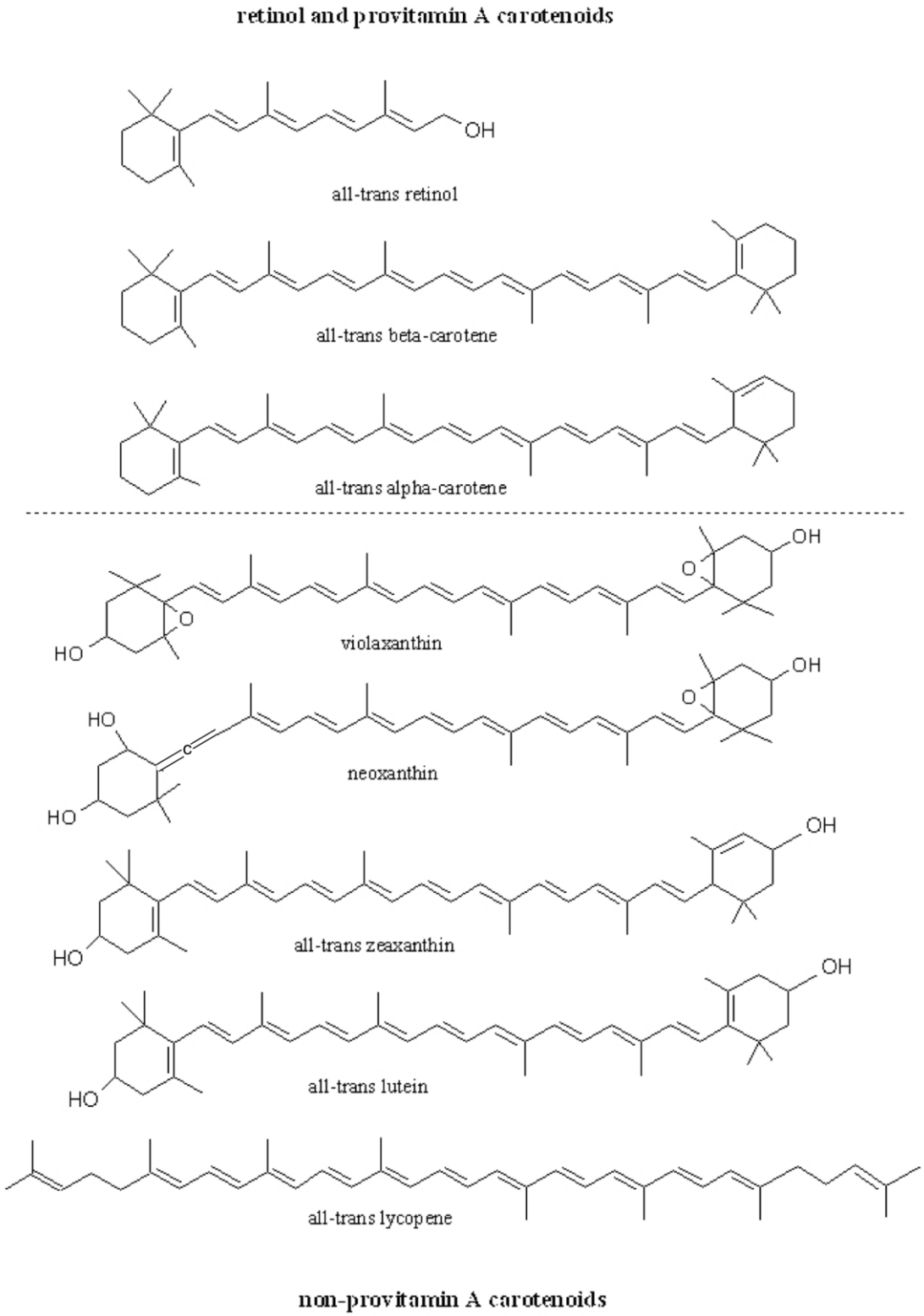

Figure 3: Chemical structures of carotenoids commonly found in the human diet and retinol, a vitamin A active compound.

2.2 Dietary sources and intake

2.2.1 Sources

Forming a major class of natural pigments, dietary carotenoids are mainly found in coloured fruits and vegetables, where they are either located in chloroplasts, bound to proteins and in combination with chlorophyll as part of the light harvesting complexes, such as in green-leafy vegetables, or in chromoplasts, in form of crystalline agglomerations, such as in carrots and tomatoes (Köhn *et al* 2008). There are also secondary carotenoid sources, including animal products, such as some fish, seafood, eggs and further processed foods, such as beverages, margarine or butter (O'Neill *et al* 2001, Souci *et al* 2000), the latter being partly fortified with carotenoids, especially with beta-carotene (Table 1).

Table 1: Carotenoid contents in food items typically included in the human diet (modified from O'Neill *et al* 2001).

	carotenoid content in mg/100 g edible portion				
Food items	**α-carotene**	**β-carotene**	**lutein**	**lycopene**	**total content**
Apple		0.022	0.051		0.073
Apricot	0.037	0.953	0.066		1.056
Beans	0.048	0.406	0.654		1.108
Broccoli		0.944	1.596		2.540
Brussels' sprouts		0.324	0.669		0.993
Butter		0.410			0.401
Carrots	2.186	7.975	0.271		10.432
Celery		0.570	0.860		1.430
Cheese		0.119			0.119
Grapefruit		1.310		3.362	4.672
Lettuce		0.890	1.250		2.140
Marrow		0.041	0.152		0.193
Orange	0.016	0.025	0.092		0.133
Pepper		0.480	0.290		0.770
Pizza		0.340	0.230	4.300	4.870
Potatoes			0.060		0.060
Pumpkin		0.490	0.630		1.120
Spinach		4.489	6.265		10.754
Sweet corn	0.033	0.024	0.819		0.876
Tomatoes		0.608	0.077	2.718	3.403
Watermelon		0.154	0.027	3.477	3.658

Given the accumulating evidence for beneficial health effects, dietary intake of several carotenoid species, such as lycopene, lutein and zeaxanthin and the vitamin A precursor beta-carotene, has been studied in nutritional surveys in several European countries (Lucarini *et al* 2006, O'Neill *et al* 2001, Pelz *et al* 1998). Additionally, the

occurrence of carotenoids in food items has also been compiled in (national) food ingredient databases in Europe and the US (Chaiter *et al* 2007, Mangels *et al* 1993, O'Neill *et al* 2001).

It appears that not only the carotenoid food content, but also the form of dietary intake can have an impact on carotenoid action: for example, high carotenoid consumption in natural form, such in fruits and vegetables (Michaud *et al* 2000, Wright *et al* 2003), was found to be protective against lung cancer (for both a smoking and non-smoking population, in a prospective and retrospective study, respectively). In contrast, very high doses of isolated carotenoids, such as beta-carotene in form of supplements, also showed adverse effects, especially for subjects exposed to higher levels of oxidative stress, such as smokers (Albanes *et al* 1995, Omenn *et al* 1996), potentially due to pro-oxidant actions (Bouayed *et al* 2010, Wright *et al* 2003).

In general, excess intake of carotenoid and vitamin A supplements can only be regarded as a minor problem in comparison to mal- and undernutrition. Playing minor roles in developed countries due to high intake of dairy and meat products, vitamin A deficiencies in developing countries are still a major health threat, causing increased susceptibility for infections, growth deficiencies, skin disorders and night blindness (Olson 1989, Rodriguez *et al* 1972). Crop fortification with pro-vitamin A carotenoids, such as beta-carotene in rice endosperm, i.e. golden rice (Welch *et al* 2004), may be useful in these countries.

Taken together, there is substantial interest for national and international health care boards to have access to detailed nutritional databases and consumption data. In contrast to neighbouring Belgium, from where results and data of any kind are usually simply transferred to Luxembourg, the Grand-Duchy is highly divers in regard to population, with more than 40% of the inhabitants originating from South Europe (STATEC 2010). Therefore, it seems appropriate to investigate Luxembourgish food consumption pattern separately.

2.2.2 Determination of carotenoid intake

For the determination of carotenoid intake in a country or population, two main factors need to be considered: dietary consumption patterns (quantity and frequency of food consumption) on the one hand and the carotenoid content (and patterns) of the consumed food items on the other. For the determination of the consumption patterns in a population, two methods are commonly employed:

One method is based on assessing food consumption by the means of food disappearance data (or food retail data), which can be defined as the 'amount of food that leaves the shelf'. This amount of food can be divided by the population, yielding an average consumption per person. If detailed disappearance data are available, this method allows for obtaining a crude estimation of actual food consumption. For example, this method was employed to estimate carotenoid consumption in Switzerland, as published within the Fünfter Schweizerischer Ernährungsbericht (Gremaud *et al* 2005). However, estimating nutritional behaviour via disappearance data tends to overestimate food consumption since not all purchased goods are consumed, e.g. due to microbial spoilage and due to animal feeding, etc.

The second method to evaluate food intake is to determine individual consumption is based on studies employing a) dietary history methods, covering eating behaviour over a certain period of time in the recent past, such as the last three months (Alkerwi *et al* 2010) or even one year (Chaiter *et al* 2007) and on b) dietary (24 hour-)recall protocols, focusing on detailed food intake during the last 24 hours (Resnicow *et al* 2000). Food surveys deal with individuals and deliver real consumption data. However, to obtain a valuable estimate for a whole population or country, several hundreds up to thousands of individuals have to be included in such a study, making a sound budget a prerequisite. Furthermore, there is the problem of underreporting, e.g. because people forget what they have eaten, especially small snacks, and additionally, there is the need for trained staff in sufficient number to conduct such a broad cohort study.

Determination and quantification of carotenoids in foods is, compared to the consumption data collection, relatively straightforward, when relying on appropriate carotenoid standards, instruments and protocols for the different matrices (Rodriguez-

Amaya *et al* 2004) and a sufficient large 'food basket' covering typical local food items consumed. Carotenoid contents in foods, especially in fruits and vegetables, however, vary due to different cultivars, climate, maturity, time of harvest, presence of fertilisers, etc., leading to potential differences compared to published data.

In Luxembourg, neither information on food carotenoid content nor on carotenoid dietary intake has been available. There is no national food database and no information on nutrients and non-nutrients in foods. Recently, first efforts have been made to determine national food consumption (Alkerwi *et al* 2010), which is likely to be different from other countries, especially as the Luxembourgish population is comparably inhomogeneous, consisting of 43.1% foreigners, with about half of the foreigners being of Southern European origin (STATEC 2010). However, further efforts are needed, as the European Union is trying to encourage its member states to conduct detailed nutritional surveys including 24hour-dietary recall protocols until the year 2017 (EU 2005).

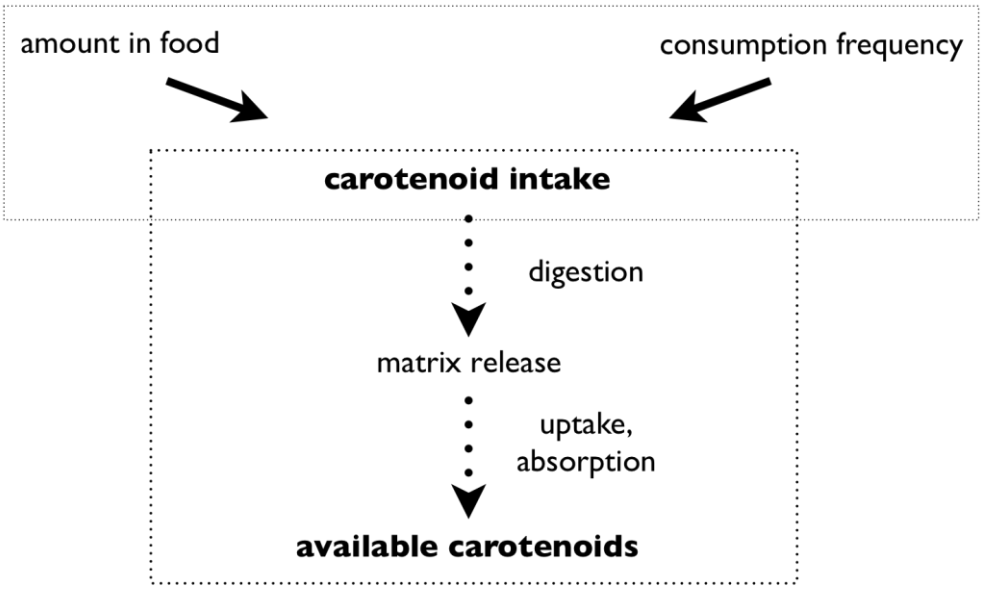

Figure 4: Aspects of carotenoid intake and uptake treated in the presented thesis.

2.3 Carotenoid uptake, transport and storage

2.3.1 Release from the food matrix

Determining carotenoid consumption is only a first step when aiming to estimate the amount of carotenoids that is taken up by the human body and is available for various physiological functions. It is further necessary to understand how efficient these species can be released from the food matrix, solubilised, and taken up by the intestine, how they are metabolised, transported and stored. Starting in the mouth, carotenoids are released from the food matrix during mechanic and enzymatic breakdown. Thereafter, proteins are hydrolysed by pepsin at low pH conditions in the stomach and about 10 to 30% of the ingested triacylglyerols are cleaved by gastric lipase, yielding diacylglycerol and free fatty acids (Pafumi *et al* 2002). While most carotenoids are still matrix-bound at this stage, free carotenoids are dissolved during the formation of lipid droplets, mostly consisting of di- and triacylglycerols, free fatty acids and cholesterol (Failla *et al* 2005).

The partially digested food is then stepwise released from the stomach via the pylorus into the duodenum, where the secretion of sodium bicarbonate from the bile increases the pH. The lipid droplets are then further processed under the influence of pancreatic lipase and the release of other lipid soluble compounds from the food matrix such as cholesterol, resulting in the formation of mixed micelles of ca. 8 nm diameter (El-Gorab 1973, Parker 1996) containing additional phospholipids, monoglycerides, fat-soluble vitamins (A, D, E, K), and bile salts required for emulsification. Considering the aqueous environment in the digestive tract, carotenoid micellarisation, which includes the release from the food matrix, transfer to lipid droplets and incorporation into mixed micelles, can be considered as a major factor determining bioavailability (Figures 5 and 6).

Differences between various carotenoids exist during micellarisation. Rather unpolar carotenoids such as the carotenes or remaining xanthophyll esters move preferably into the core of the micelles, while comparable more polar carotenoids may rest at the surface (Borel *et al* 1996). However, some carotenoid esters are cleaved by cholesterol esterase (Breithaupt *et al* 2002), acting at the interphase of the micelles

and the aqueous phase.

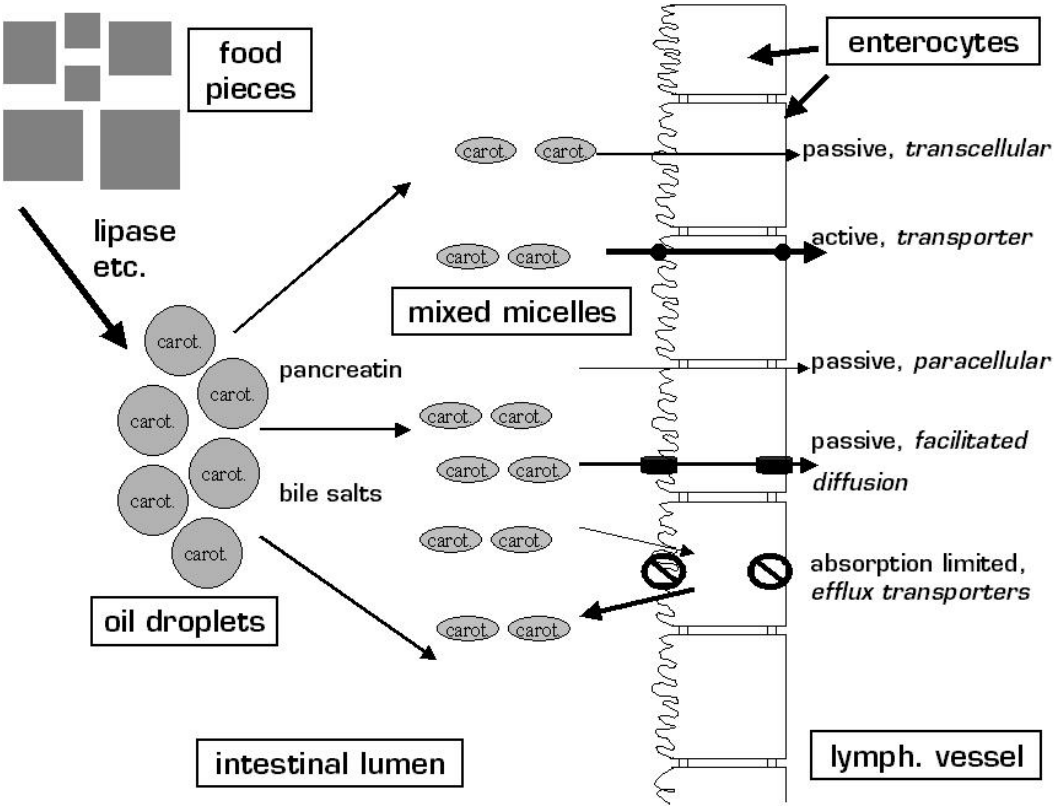

Figure 5: Schematic illustration of carotenoid transfer from the food matrix through the enterocytes to the lymphatic vessels via different, potential mechanisms (passive diffusion (trans- and paracellular), active transport, facilitated diffusion and limited absorption). Carot. = carotenoids.

2.3.2 Uptake

Earlier studies suggested carotenoid uptake by the enterocytes through passive diffusion via the stirred aqueous layer and cell membrane (El-Gorab *et al* 1975, Hollander *et al* 1978, Sugawara *et al* 2001), while recent data have favoured a facilitated uptake via membrane proteins, such as scavenger receptor class B, type 1 (SR-B1) (During *et al* 2005, Kiefer *et al* 2002, Moussa *et al* 2008, Reboul *et al* 2005, van Bennekum *et al* 2005), at least for some carotenoids. These processes have been suggested to occur mainly in the small intestine, with carotenoid uptake in the large intestine assumed to be low, even though carotenoids can reach the large intestine intact and may be available for absorption (Goni *et al* 2006, Serrano *et al* 2005). During intestinal passage, carotenoids can be additionally degraded, such as by bacteria in the large intestine, perhaps by as much as 80% (Goni *et al* 2006), further

decreasing bioavailability. In fact, carotenoid absorption is usually low, ranging for beta-carotene from 3% (O'Neill *et al* 1998) and 22% (Novotny *et al* 1995) up to 90% in ileostomic patients (Faulks *et al* 1997), while, on average, more polar xanthophylls seem to be slightly better available (Bohn 2008), with exception of epoxyxanthophylls such as neoxanhtin, fucoxanthin and violaxanthin, which seem to be of lower bioavailability and are likely to be converted to their furanoid forms due to the low pH during gastric passage (Asai *et al* 2004a, Asai *et al* 2004b, Barua *et al* 2001, Hashimoto *et al* 2009).

During the subsequent transfer through the enterocytes, provitamin A carotenoids are partially cleaved by 15-15'-monooxygenase (Goodman *et al* 1966, Olson *et al* 1965), converted to retinal by retinal reductase, reduced to retinol, and esterified, preferably into retinyl palmitate such as by lecithin:retinol-acyltransferase (Batten *et al* 2004). In addition to this central cleavage, asymmetric cleavage, such as through the 9'-10'-monooxygenase (Barua *et al* 2001, Hashimoto *et al* 2009, Lindqvist *et al* 2005) can occur, leading to the formation of beta-apo-10'-carotenal and beta-ionone. A fraction of the carotenoids might also be secreted or released back into the gut, such as by cell sloth.

2.3.3 Absorption and transport

Intact carotenoids, together with retinyl esters, can then be secreted from the Golgi apparatus of the enterocytes into the lymph in form of triacylglyerol-rich chylomicrons, reaching the blood stream via the thoracic duct (Parker 1996).

Passing the liver, carotenoids are either stored in the membrane fraction and lipid droplets of the hepatocytes (Glise *et al* 1998, Teodoro *et al* 2009, Yadav *et al* 2002), or re-enter the bloodstream within low-density lipoprotein (LDL) and high density lipoprotein (HDL) particles, with hydrocarbon species preferably transported by LDL and VLDL (very low density lipoproteins) (Goulinet *et al* 1997), and xanthophylls about equally by HDL and LDL (Furr *et al* 2005).

Carotenoids can then be transported to different tissues, especially to those with a high expression of LDL-receptors (Yeum *et al* 2002), such as adipose (Chung *et al* 2009), prostate (Clinton 1998) or adrenal tissue (Stahl *et al* 1992). Some carotenoids,

especially lutein and zeaxanthin, accumulate in the macula of the retina of the eye at almost even concentration (Landrum *et al* 1997b). However, it seems that carotenoids are rather generally and evenly distributed and stored throughout the entire human body and that there is no primary target tissue. It appears that the tissue distribution is not associated with the polarity of the carotenoid, but unpolar compounds seem to be slower depleted (Yonekura *et al* 2010). The further fate of carotenoids is less well understood. Excretion of the carotenoids or their degradation products (Khachik *et al* 1997) occurs predominantly via bile and pancreas into the faeces, urine excretion being negligible (Bowen *et al* 1993), with exception of retinol, which may be increased up to 1000 fold during infection, up to 10 μmol/d in urine (Stephensen *et al* 1994).

2.4 Carotenoid Detection

2.4.1 Spectrophotometry

Most carotenoids, with exception of some precursors, such as phytoene, absorb visible light between 400 and 500 nm and possess high molar absorption coefficients, around 125.000-155.000 $l*mol^{-1}*cm^{-1}$ (Britton *et al* 2004, Budavari 1989, Lide 2004), allowing for rapid and relatively sensitive quantification via VIS spectrometry (Rodriguez-Amaya *et al* 2004). Without prior separation, only the total carotenoid concentration can be determined, being often impeded due to the presence of other chromophores such as chlorophylls in plant extracts, which have to be subtracted mathematically (Lichtenthaler 1987) or removed via saponification (Larsen *et al* 2005, Lietz *et al* 1997). Nonetheless, different spectrophotometric protocols have been proposed for carotenoid determination (Hornero-Méndez *et al* 2001, Rodriguez-Amaya *et al* 2004, Schon 1935).

2.4.2 HPLC

Being more time and cost intensive compared to spectrophotometry, reverse-phase high-performance liquid chromatography (RP-HPLC) coupled to UV/VIS photodiode array detection (DAD) is widely used, as it allows for the simultaneous separation, detection and quantification of individual carotenoids (Gorocica-Buenfil *et al* 2007, Khachik *et al* 1986). Due to the chemical properties of the carotenoids, C-18, and,

more recently, C-30 stationary phases have been frequently employed, as they allow for the discrimination between geometrical isomers (Hadley *et al* 2003, Moussa *et al* 2008) and separation of very similar carotenoids, especially lutein and zeaxanthin, which is difficult to achieve on a C-18 phase. HPLC-DAD techniques typically show detection limits around 10-200 ng/ml (Barba *et al* 2006, Lee *et al* 2009), depending, among other, on the matrix. As carotenoid determination by fluorescence is rather of poor sensitivity and selectivity (Frank *et al* 2008) this technique has not been used for carotenoid detection. Alternatively, coulometric electrochemical detection can be employed, decreasing the detection limit of conventional liquid chromatography coupled to UV-Vis detectors by the factor >100 (Ferruzzi *et al* 1998), however, specialized equipment is needed. For additional information on the molecule mass and detection of very low abundant carotenoids, liquid chromatography can be coupled to mass spectrometry. Especially atmospheric pressure chemical ionization employing methods have been developed, both for HPLC-MS (Tian *et al* 2003, van Breemen 1997) and tandem HPLC-MS-MS, the latter allowing quantification of levels down to 0.1 ng/ml in human plasma (Gundersen *et al* 2007). However, for the latter methods, highly sophisticated instrumentation is needed, drastically increasing costs of analysis.

2.5 Methods to assess carotenoid bioavailability

2.5.1 General Aspects

Carotenoid bioavailability, which is defined the fraction of carotenoids that is absorbable and can be used for specific physiological functions, is influenced by a variety of factors often termed as SLAMENGHI (Bohn 2008, West *et al* 1998), including the a) species of carotenoid, b) molecular or chemical linkage, c) amount of carotenoids ingested, d) effects of food-matrix, e) effectors of absorption and bioconversion (i.e. presence of enhancers/ inhibitors), f) nutrient status of the host, g) genetic factors h) host-related factors and i) and the interaction of the above factors.

Large discrepancies can be found if results from different carotenoid bioavailability studies are compared. These discrepancies are, at least partly, due to the use of different study designs (Figure 6) and also the lack of standardised methods and models to assess bioavailability or aspects of it, such as carotenoid release from the

matrix, solubility in the gut, uptake by the mucosa, sequestration into chylomicrons, distribution in the bloodstream and incorporation into other tissues. The most appropriate approach to investigate carotenoid bioavailability is naturally within the human body. However, as human studies can be very expensive, time consuming and ethically disputable, many investigations have focused on animal or simpler models, even if not all aspects of bioavailability can be sufficiently simulated, and results not directly transferred to humans.

2.5.2 Animal models

A variety of animal models, from isolated gut sacs (El-Gorab *et al* 1975) to whole animals, especially rats and mice (Breithaupt *et al* 2007, Lee *et al* 1999) have been used to assess carotenoid bioavailability, as they may allow for assessing also transportation in the bloodstream and tissue distribution (Figure 6). However, the physiology of many of these animal species differs considerably from humans, e.g. in the intestinal flora, conversion of provitamin A into retinol, and distribution between lipoprotein fractions in blood plasma, making it difficult to compare results across models and to infer to humans (Lee *et al* 1999). In addition, ethical concerns have been increasingly confined their usage.

2.5.1 *In vitro* models

As an alternative to human and animal models, *in vitro* models have been developed, that allow for studying solubility aspects of bioavailability, such as micellarisation of carotenoids (Chitchumroonchokchai *et al* 2004b, Garrett *et al* 1999a), following gastro-intestinal digestion. This so called bioaccessibility determines the amount of carotenoids that can be released from the food matrix and emulsified in an aqueous solution, which is deemed to be the most important step during digestion and perhaps absorption. These experiments are often coupled to cell culture studies, such as with Caco-2 cells, aiming to simulate the small intestinal environment (Ferruzzi *et al* 2006, Garrett *et al* 1999a), allowing for studying uptake and transport mechanisms. Rapidity and ease of use make these *in vitro* techniques a valuable screening tool in carotenoid utilisation. Both *in vitro* digestion and cell studies will be presented in the following chapters.

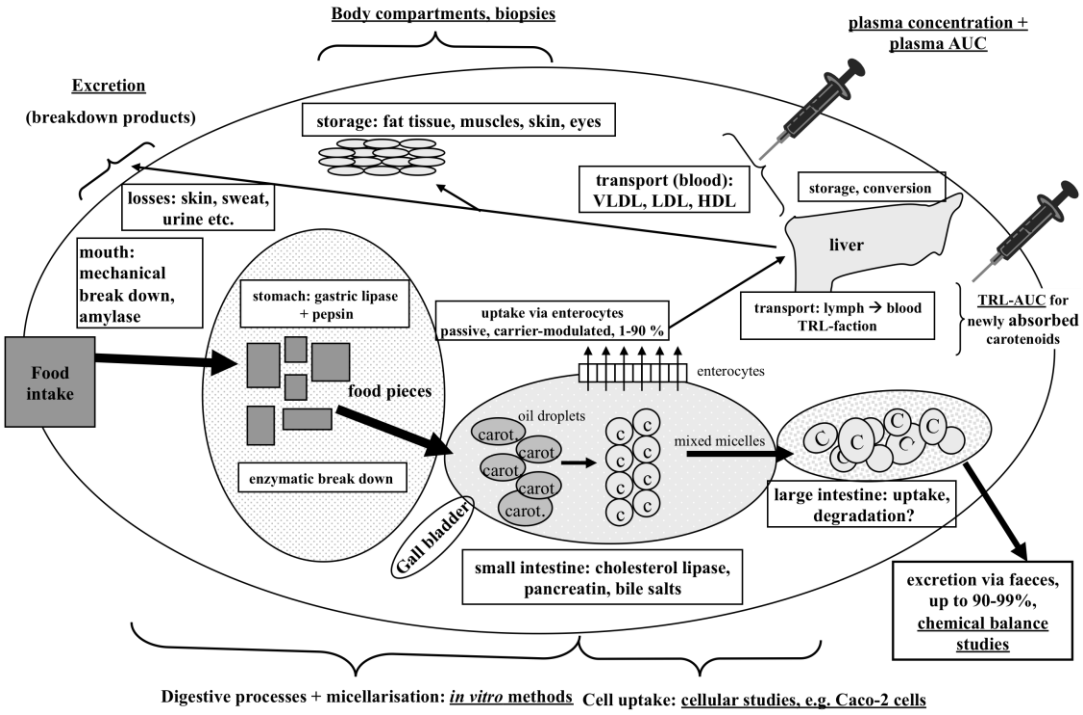

Figure 6: General overview on carotenoid digestion, uptake, and distribution, together with potential techniques to determine aspects of carotenoid bioavailability. Carot. and c = carotenoids; TRL = triacyl-rich lipoprotein fraction; AUC = area under curve. Chemical balance studies, TRL-AUC and biopsies can be carried out only in humans and animals, while *in vitro* digestion and cellular uptake studies can be performed *ex vivo*.

2.5.2 *In vitro* digestion to study micellarisation

2.5.2.1 General considerations

In 1981, Miller and colleagues (Miller *et al* 1981) described a method for estimating iron availability *in vitro*, providing a relatively rapid, simple, low cost approach for mimicking gastric and small intestinal stages of digestion in the upper parts of the gastro-intestinal tract. Typically, gastric digestion is simulated at 37°C with pepsin at pH 2-3 in a water bath, followed by pH adjustment to pH 7-7.5, and addition of a mixture of pancreatin and bile acids (Figure 7). Occasionally, an additional previous step simulating chewing and enzyme digestion during the oral phase with saliva containing α-amylase is included (Granado *et al* 2006). This *in vitro* technique has been used later for a variety of compounds, including various minerals and trace elements (Velasco-Reynold *et al* 2009), phytosterols (Bohn *et al* 2007), and also carotenoids (Failla *et al* 2005, Garrett *et al* 1999a, Hedren *et al* 2002). In order to

determine the amount of a compound that is released from the matrix and emulsified into the aqueous phase, i.e. the percentage of carotenoids present in mixed micelles compared to the total amount of ingested via the original matrix is quantified.

However, the percentage of micellarisation yields only relative values, depending, among other factors, on the concentration of carotenoids in the test meal and its composition, especially the amount and type of lipids present (Huo *et al* 2007), pH, enzyme concentration, bile acids added, and digestion time (Garrett *et al* 1999a), making it difficult to standardize experiments and to compare results across studies (Table 2). Another drawback of this model is being a static one, i.e. concentrations of food and enzymes being constant, reaching equilibrium during the gastric and small intestinal phase, which is not the case *in vivo*. Even though dynamic models have been developed (Blanquet-Diot *et al* 2009, Wolters *et al* 1993), these models have hardly been used due to their increased complexity.

Table 2: Various conditions of carotenoid micellarisation experiments of different studies.

study	**carotenoid studied***	**conc. carotenoids in final digesta (µg/ml) ♣**	**conc. pancreatin in final digesta (mg/ml)**	**conc. bile salts in final digesta (mg/ml)**	**pH during gastric; intestinal phase**	**digestion time gastric + intestine (min)**	**total micellarisation in the aqueous phase (%)**
(Miller *et al* 1981)	n.a.[+]	n.a.	ca. 0.3	ca. 2	2; 7.5	120+120	n.a.
(Garrett *et al* 1999a)	α-car, β-car, lutein, lycopene	0.9 1.9 0.8 2.0	0.4	2.4	2; 7.5	60+120	13 15 25 <1
(Hedren *et al* 2002)	β-car, α-car	ca 3.5 ca 1.0	0.6	3.8, 7.6	2; 7.5	60+30	3-39** 60% ***
(Chitchumroonchokchai *et al* 2004b)	lutein, zea, β-car	4.2 0.1 0.9	0.3	0.4 GDC[&]; 0.2 TDC; 0.4 TC;	2; 7.5	60+120	55-80 [§] 55-75 [§] 25
(Reboul *et al* 2006)	α-car, β-car, lutein, lycopene	n.d. *	0.3	9	4; 6	30+30	8.9 4.4 37.6 1.1
(Dhuique-Mayer *et al* 2007)	β-car	6.5 1.8	0.2	5	4; 6	30+30	26-33

Abbreviations used: α-car = α-carotene; β-car = β-carotene; zea = zeaxanthin; [+]: Dulbecco's minimum essential medium, ♣ all values approximations; [+]n.a.= not applicable; *: n.d. = no data; ** depending on processing steps; ***α-carotene micellarisation was found app. 60% lower than β-carotene; [&]GDC: glycodeoxycholate; TDC: taurodeoxycholate, TC: taurocholate,§ depending on the matrix (spinach vs. supplement)

Finally, even though micellarisation is the first important step for carotenoid absorption, a decrease in micellarisation may not necessarily result in lower uptake by the intestinal mucosa (see following chapter). It has been speculated that the positioning of carotenoids within the mixed micelles, that is at the surface vs. the core, which is typically not determined during micellarisation experiments, could impact the following transfer and uptake of carotenoids into the enterocytes (Borel *et al* 1996, Bowen *et al* 1993). In general, micellarisation of carotenoids varies from approx. 1-2% for all-trans lycopene (Failla *et al* 2008a, Huo *et al* 2007) to 80% for all-trans lutein (Chitchumroonchokchai *et al* 2004b) (Table 2).

Besides these drawbacks, the *in vitro* digestion method has frequently been used as a screening tool for studying the impact of several dietary factors on carotenoid release and solubility. It has been implemented for the investigation of various food matrices, including raw, mixed salads and vegetables (De Jesus Ornelas-Paz *et al* 2008, Ferruzzi *et al* 2001, Hedren *et al* 2002) citrus juice and apple sauce (Dhuique-Mayer *et al* 2007, Ferruzzi *et al* 2006), to complex, boiled meals based e.g. on potato and beef (Reboul *et al* 2006). It has further been used to study to effects of different triglycerides (Huo *et al* 2007), bile salts and enzymes (Garrett *et al.* , 1999a, Hedren *et al.* , 2002) and different carotenoid species present (Failla *et al* 2008b) on carotenoid micellarisation.

2.5.2.2 Host-related factors impacting carotenoid micellarisation

Which parameters do impact the micellarisation of carotenoids within this model? Usually, test meals are ground or blended in order to simulate homogenisation during the oral digestion phase (chewing). The following digestion stages are, however, briefly reviewed:

A) Stomach

In the publication of Miller and colleagues (1981), which described the original *in vitro* digestion method, the following gastric phase was set at two hours at pH 2, however, in more recent studies this incubation time has been reduced to one hour or 30 minutes (Garrett *et al* 1999a, Reboul *et al* 2006), even though the original time probably reflects more accurately the passage time through the stomach (Stenson 2006), and was altered to decrease time of analysis. As the pH of the stomach can

vary from pH 0.5 to ca. pH 7 in healthy subjects, with 3-6 being more typically following ingestion of a test meal (Gardner *et al* 2004, Tyssandier *et al* 2003), this parameter has also been varied, from pH 1.1 (Granado *et al* 2006), to 3 (Asai *et al* 2004b, Huo *et al* 2007) and even 4 (Reboul *et al* 2006). The pH optimum of pepsin is around pH 1.5-2 (Stenson 2006). pH could also impact carotenoid concentration directly, through the formation of breakdown products at more extreme pH. Asai and colleagues (2004b) e.g. found degradation of neoxanthin and violaxanthin into neochrome and luteoxanthin/ auroxanthin at a pH lower or equal 3. Pepsin concentrations have likewise been varied, ranging from 1.3 mg/ ml (Dhuique-Mayer *et al* 2007) to ca. 25 mg/ ml (Granado *et al* 2006) within samples to be digested. It appears, however, that the addition of pepsin does not play a crucial role for micellarisation, at least not for plant based test meals with low amounts of proteins in the matrix (Garrett *et al* 1999a, Hedren *et al* 2002).

B) Small intestine

To simulate the transition from the stomach to the duodenum, the pH of the predigested meal is, mostly stepwise increased by $NaHCO_3$. In a second step, lipase within pancreatin and bile extracts is added (Garrett *et al* 1999a, Miller *et al* 1981) for increasing efficiency of micellarisation (Chitchumroonchokchai *et al* 2004b, Failla *et al* 2008b). The following intestinal phase, originally lasting 2 hours (Miller *et al* 1981), has been retained in most studies. In some studies, though, it was shortened to 30 minutes (Dhuique-Mayer *et al* 2007), which might risk incomplete carotenoid micellarisation, although micellarisation of beta-carotene in the above study, using a very simple matrix (juice) was, with ca. 30% not lower compared to other studies (e.g. O'Connell *et al* 2008). On the other hand, prolonged incubation times risk carotenoid degradation, for alpha-carotene and lutein as shown by Garrett and coworkers (Garrett *et al* 1999a, Garrett *et al* 1999b) when increased to 6 hours and 24 hours respectively, however, no significant losses were detected for beta-carotene.

In contrast to the relative low impact of pepsin on the micellarisation efficiency of carotenoids, pancreatin and bile salts seem to play a predominant role for micellarisation. Without pancreatin, the digestion of the triglycerides is incomplete, resulting in lower concentrations of di-and especially monoglycerides and reduced

micelle formation, while the omission of bile salts or the complete small intestinal digestion phase almost completely inhibited micellarisation of beta-carotene (Garrett *et al* 1999a, Garrett *et al* 2000, Hedren *et al* 2002).

During the intestinal phase, pH could also impact micellarisation, as a too low pH (<4.5) was reported to result in decreased solubilisation of beta-carotene in mixed micelles, however, a slight acidity showed to improve micellarisation (El-Gorab 1973). The reasons remain speculative, but one might hypothesize that the protonisation of the bile salts at lower pH could improve the transfer of the apolar carotenoids during the formation of mixed micelles, while a too low pH does compromise the integrity of the micelles.

C) Separation of aqueous phase

Another underestimated step of importance is the separation of the micelle fraction from the digesta, achieved typically by ultracentrifugation (Garrett *et al* 1999a), even though shown to be replaceable by 45 min/ 5000*g (Failla *et al* 2008b) followed by filtration of the digesta for separation of aqueous phase (micelles) from oil droplets and sediments, typically achieved by 0.2 μm pore cellulose filters (Figure 7). While limited influence of filtration on the presence of xanthophylls and beta-carotene has been described, for lycopene, a drastically reduced presence from ca. 5 to 0.5% after filtration was shown, the reasons being unclear, emphasizing the difficulty of obtaining true lycopene rich micelles (Garrett *et al* 1999a). It is possible that filtration goes along with relative low absolute losses of carotenoids, more strongly affecting poorly micellarised carotenoids, which is especially true for lycopene.

2.5.2.3 Comparability to human studies

How do *in vitro* studies compare to cellular or human studies? In an investigation by Reboul (2006), micellarisation of alpha- and beta-carotene, lutein and lycopene *in vitro* was compared to micelles obtained from an earlier human study by the same group (Tyssandier *et al.*, 2003). Overall, a high and significant correlation ($R = 0.90$, $p<0.05$) was found, with very similar micellarisation efficiency, with exception for lutein, which was much better micellarised *in vitro* (37% vs. 7.7% *in vivo*). The authors suggested an underestimated bioavailability from spinach in the human study, due to analytical problems. This implicates that the *in vitro* digestion steps

reflect to a large extent the real *in vivo* situation. Reboul et al. (2006) further compared *in vitro* bioaccessibility ratios of lycopene, beta-carotene, and lutein from different test meals to absorption ratios obtained from blood plasma measurements in human studies investigating similar test meals. Although even though up to 8-fold differences were found between the bioavailability and bioaccessibility ratios, overall correlation was high and significant ($R = 0.98$, $p<0.001$), indicating that, on average, similar results can be obtained for micellarisation and absorption, being among the most important steps determining bioavailability. Discrepancies between *in vitro* studies and *in vivo* studies, however, can occur, especially when beta-carotene conversion into retinol is not taken into account (Granado *et al* 2006).

2.5.2.4 Applications

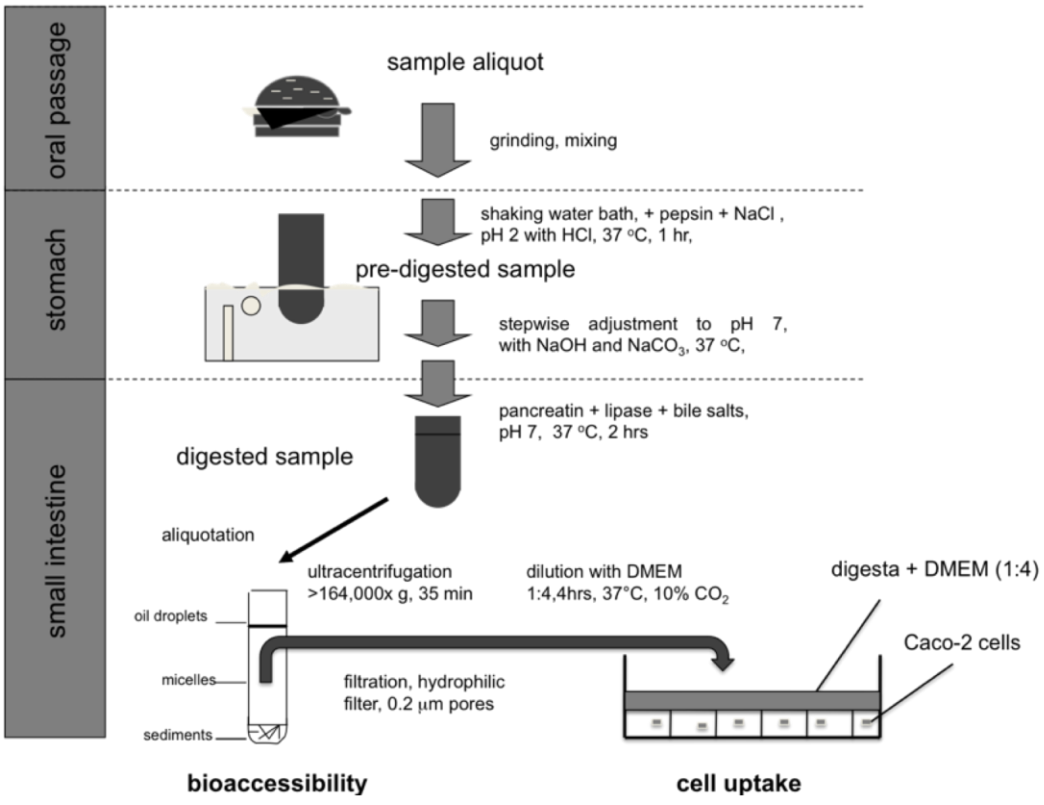

Figure 7: Simulated gastro-intestinal digestion to study micellarisation coupled to Caco-2 cellular uptake studies. Further details are described in the Material and Method section. DMEM = Dulbecco's Minimal Essential Medium.

Employing this *in vitro* technique, it has been possible to improve the understanding of processes involved in carotenoid release from the matrix and formation of mixed

micelles during digestion. For example, it appears that xanthophylls, due to their higher polarity, possess a higher tendency for incorporation into mixed micelles compared to carotenes, with lycopene showing lowest incorporation efficiency (Borel *et al* 1996, Dhuique-Mayer *et al* 2007, Garrett *et al* 1999a, Garrett *et al* 2000, Sugawara *et al* 2001). Micellarisation efficiency can be increased, especially for the carotenes, with the addition of various types of lipids (De Jesus Ornelas-Paz *et al* 2008, Hedren *et al* 2002, Hornero-Méndez *et al* 2007, Huo *et al* 2007, Pullakhandam *et al* 2007). On the other hand, micellarisation has been suggested to be lowered by the presence of higher amounts of dietary fibers from whole fruits and vegetables, probably impeding carotenoid release from the matrix or slowing down formation of mixed micelles and their transport to the mucosa, perhaps due to increased viscosity (De Jesus Ornelas-Paz *et al* 2008, O'Connell *et al* 2008, Yonekura *et al* 2009).

Taken together, the *in vitro* method can be used as a rapid tool for screening factors governing carotenoid release from the matrix, and their transfer and incorporation into mixed micelles, a prerequisite for their uptake from diet. Some of the limitations to date could be overcome if efforts are produced to increase comparability between various studies, i.e. standardisation.

2.6 Uptake studies employing cell models

2.6.1 General aspects

There are two main protocols employing cell models for studying carotenoid availability, cellular uptake and transport studies. The combination of a cellular uptake model and *in vitro* digestion, being far less expensive and time consuming than animal and human studies, has been widely used in pharmacologic studies for drug testing (Ingels *et al* 2003), but also for a number of other compounds, including carotenoids (Failla *et al* 2005, Moussa *et al* 2008, Sugawara *et al* 2009). While uptake models are cheaper and simpler, with only one compartment on the apical side of the cells, that allows studying cellular uptake, transport models with both basolateral and apical side compartment have also been developed. Transport models permit studying of transport fluxes and sequestration through the cell monolayer (During *et al* 2007, Hidalgo *et al* 1989, Sugawara *et al* 2009).

Table 3. Examples of studies employing Caco-2 cells for determination of carotenoid uptake or transport, and parameters of exposure.

Study	**carotenoid matrix and amount added**	**cell clone**	**type of well and surface**	**cell-post-con-flu-ency (d)**	**exposure time/ time of harvest (h)**	**carot-enoid species studied**	**amount carot. added to cells (μg)**	**bioavail-abilitiy (%)**
(Garrett *et al* 1999b)	digesta 1:3 diluted in DMEM, 1ml	n.d.	up, 12 well dishes, 4 cm^2	11-14	6	lutein, lycopene, α-car, β-car	0.28* 0.65 0.31 0.65	25 negligible 45 25
(Chitchumroonchokchai *et al* 2004a)	AM, 1:4 diluted in DMEM, 1.5 ml al + 2.5 mL DMEM bl)	HTB-37	TW, 6-well dishes, 9.6 cm^2	21-25	6 al, 20 bl	lutein	0.23 0.23	3 (bl); 37 (cells)
(During *et al* 2005)	AM in DMEM, 2 mL al + 2.5 ml DMEM bl)	TC-7	TW, 6-well dishes, 9.6 cm^2	21	16	β-car α-car, lutein lycopene	1.1 1.1 1.1 1.1	11 10 7 3 (cells)
(Reboul *et al* 2005)	AM: 1 mL al + 2 ml bl FBSF**)	TC-7	TW, 6 well dish, 9.6 cm^2	21	0.5 al, 0.5 bl	lutein	0.46	n.d.
(Failla *et al* 2008a)	digesta 1:4 diluted in DMEM, 12.5 ml	HTB-37	up, 75 cm^2 flask	11-14	4, har 20 h	α-car, β-car, lycopene	0.04 0.13 0.05	28 27 13

Abbreviations used: *all values are approximates, ** fetal bovine free serum medium, n.d. = no data, up = uptake; AM = artificial micelles; TW = transwell; har = harvest; al = apical; bl = basolateral; α-car = α-carotene; β-car = β-carotene, DMEM = Dulbecco's Modified Eagle Medium, d = day, h = hour

Several cell models for the investigation of carotenoid uptake have been proposed, i.e. rat hepatic stellate cells (HSC-T6) (During *et al* 2002), transfected renal COS-7 cells from monkeys (van Bennekum *et al* 2005), exfoliated human colonic epithelial cells (Kamra *et al* 2005), human retinal pigment epithelial cells (ARPE-19) (During *et al* 2008), human lung fibroblasts (WI-38) (Scita *et al* 1992) and the human Caco-2 cell line, which, once differentiated, mimics the enterocytes of the small intestine to a large extent, and has been, so far, the most frequently used cell line for studying carotenoid uptake. This cell line spontaneously differentiates into mature, epithelial cells following a proliferation stop due to contact inhibition. The differentiated cells show cellular polarisation, development of tight junctions and a brush border membrane (Fleet *et al* 2003) and the typical colonocyte/enterocyte hybrid phenotype after 2-3 weeks post confluence.

Differentiated Caco-2 cells express marker genes typical for differentiated enterocytes, e.g. alkaline phosphatase (Yuan *et al* 2009), sucrose isomaltase (Zweibaum *et al* 1983) and other metabolic enzymes including some cytochrome P450 isotypes (Ishida *et al* 2009). In addition, a number of typical membrane receptors and transporters are expressed, including SR-B1 (During *et al* 2005, Kiefer *et al* 2002, Moussa *et al* 2008, Reboul *et al* 2005), Niewmann-Pick disease type C1 gene-like (NPC1L1), and ATP-binding cassette transporter subclass member 1 (ABCA1), participating in retinol-efflux (During *et al* 2007), probably impacting carotenoid transport in these cells.

The human Caco-2 cell line was originally established in the 1970's from a colon carcinoma from a 72-year old white Caucasian patient (Fogh *et al* 1977) and subsequently cultured. In addition to the parental cell line, several subclones, such as the Caco-2/TC-7 (Chantret *et al* 1993) have been developed, showing altered gene expression, such as the different expression pattern of multi drug resistance transporters (Horie *et al* 2003).

2.6.2 Limitations

As it is the case for the micellarisation models, also the Caco-2 cellular models have been suffering from problems of comparability between different research studies. The use of different subclones such as TC-7 (Reboul *et al* 2005) and CRL-2102 for retinol (Yamaguchi *et al* 2009), different time points of post-confluency (2-21days), the way of carotenoid introduction to the cells, such as various concentrations, digesta or artificial micelles, as well as different culture conditions, e.g. percentage of added bovine serum, make it difficult to compare results across studies and laboratories (Chitchumroonchokchai *et al* 2004b, De Jesus Ornelas-Paz *et al* 2008, Dhuique-Mayer *et al* 2007) and standardisation procedures are highly desirable.

Another limitation of Caco-2 cells is that these cells do mimic only enterocyte-like cells, while other cells naturally present in the small intestine, such as mucus-producing cells and M(icrofold) cells, are not represented (Nollevaux *et al* 2006). Also, paracellular transport capacity in Caco-2 cells is known to be comparatively low (Artursson *et al* 2001), and could be increased by co-culturing of Caco-2 with the mucus producing HT29-5M21 cells (Nollevaux *et al* 2006), even though this transport

is probably of minor importance for carotenoid uptake, due to their lipophility and requirement for lipoprotein incorporation for further transport. As with micellarisation, there is also the lack of dynamic simulation. With the established stationary uptake model, the uptake from one meal during a certain time is usually investigated, while effects from subsequent meals such as altered sequestration of chylomicrons, can usually not be simulated (Failla *et al* 2005).

It has been shown that the uptake of carotenoids, such as lutein, does depend on the incubation time of Caco-2 cells with digesta, with increasing uptake over a time course between 4 and 20 hours. Similar results were earlier obtained by (Chitchumroonchokchai *et al* 2004b, Garrett *et al* 1999a) for alpha-and beta-carotene and lutein for up to 6 hours, with the majority of studies allowing 4 hours. Concentrations and ratios of micelle fraction to cells will also determine carotenoid uptake.

Smaller surfaces for cell experiments, such as on multi-well plates, have become more customary compared to cell flasks due to a higher number of parallel experiments possible, usually 6- to 12-well plates (Garrett *et al* 1999a). However, the comparatively large volume and amount of carotenoids needed for HPLC coupled to UV-VIS detection most commonly used for these experiments, currently precludes the miniaturisation below 12-well plates. Perhaps, the introduction of ultra pressure liquid chromatography with its lower flow rates and sampling volumes will improve this situation in the future.

2.6.3 Studies employing Caco-2 cell models to examine carotenoid uptake

Besides these apparent limitations, the Caco-2 cellular system has been a valuable model for screening trials of carotenoid uptake in many studies. These included either investigating transport of pure compounds solubilised in artificial micelles (During *et al* 2005, During *et al* 2007), or the uptake from predigested meals, i.e. resembling natural micelles (Garrett *et al* 2000, Huo *et al* 2007, Reboul *et al* 2006). While results from *in vitro* digestion alone, such as micellarisation, seem relatively consistent, at least in terms of general trends, results obtained from cellular uptake studies are less coherent, probably due to the larger number of varying parameters between different studies, such as increased complexity of the cell vs. the *in vitro* digestion alone. For

example, some studies have suggested a higher uptake of xanthophylls in Caco-2 cells compared to carotenes (Ferruzzi *et al* 2001, O'Sullivan *et al* 2007) while most studies suggested the exact opposite (During *et al* 2002, Garrett *et al* 1999b, Sugawara *et al* 2001).

Different uptake mechanisms between carotenes and xanthophylls into the cells have been proposed. During and colleagues (2005) found alpha- and beta-carotene uptake into Caco-2 cells inhibited by up to 50% when the cholesterol transporters SR-B1 (scavenger receptor, class B, type 1) were blocked, while xanthophyll uptake, e.g. for lutein and cryptoxanthin, was only slightly affected (7-20% decrease). While lycopene uptake was only reduced about 20% in the former study, Moussa and colleagues (2008), however, found the uptake to be decreased by 50-60%, using the same TC-7 clone. These data are in line with results of the same study employing mice overexpressing SR-B1, where lycopene absorption as detected in plasma was found to be 10-times higher concentration compared to control animals. This preference of SR-B1 for carotene uptake might, at least partly, counterbalance the lower micellarisation of carotenes or even result in higher cellular uptake compared to the xanthophylls. However, in ARPE-19 retinal cells, zeaxanthin and lutein uptake were stronger impaired than beta-carotene, when SR-B1 expression was knocked down (During *et al* 2008), indicating that different tissues may express different subtypes of receptors with different substrate specificities. Further studies investigating this tissue-specific expression are warranted.

When comparing *in vitro* micellarisation experiments and Caco-2 cellular uptake studies involving provitamin-A carotenoids is difficult, as within the cells, especially provitamin-A carotenoids are partly broken down by enzymatic cleavage into retinal or apo-carotenals. If these metabolites are not considered, there is a high potential for finding large discrepancies between micellarisation and uptake studies. However, differences exist between the parental cell line, showing no monooxygenase activity, and several subclones, such as the TC-7, yielding activity (During *et al* 2004).

In summary, the combination of *in vitro* digestion and cellular uptake studies yields an important screening tool, allowing for studying many processes that occur *in vivo*, and being suitable for high throughput screening in multi-well plates (Table 4). Unfortunately, the inter-laboratory differences are large and, up to date, perturbed the comparison of results to a large extent. However, given ethical concerns performing animal and human studies, cell studies have become increasingly popular.

Table 4: Summary of major pros vs. cons of *in vitro* digestion model coupled to cellular uptake compared to human/animal studies

Pros	**Cons**
Avoiding of animal/ humans studies	Only partially resembling aspects of absorption
No ethical limitations	Static, not adaptive
High throughput screening possible	Cell systems cannot fully mimic complex intestinal tissues
Low costs	Low comparability between studies

Aims of the thesis

2.6.4 Occurrence of carotenoids in food items and their consumption

In the first part of this thesis, existing gaps regarding the abundance of major carotenoid contents in food items produced or consumed in Luxembourg, such as alpha- and beta-carotene, lutein, zeaxanthin and lycopene were attempted to be filled. To that end, specific extraction protocols for different food matrices had to be developed, such as for fruits, vegetables, beverages and fat-containing matrices. Potentially interesting food items rich in carotenoids were screened for total carotenoid content with a newly developed, spectrophotometer-based method and promising items then analysed in detail with HPLC-DAD. Coupling these food carotenoid contents to food consumption data obtained from the first Luxembourgish population based cardio-vascular risk factor study (Alkerwi *et al* 2010), the average carotenoid uptake of the Luxembourgish population could be estimated. Since the risk-factor study's questionnaires only focus on food groups, such as 'green leafy vegetables' instead of single food items, such as 'spinach', 'endive" etc., these food groups had to be further refined by comparing results to food disappearance data generously provided from the major Luxembourgish supermarket chain CACTUS S.A. and additionally to the detailed Fünfter Schweizerischer Ernährungsbericht (Gremaud *et al* 2005). The estimated, average carotenoid intake in Luxembourg was then finally compared to studies from other European and non-European countries.

2.6.5 Factors impacting micellarisation and uptake

After having focussed on total carotenoid intake in general, the second part of this thesis aspired to investigate factors potentially influencing the bioavailability of carotenoids by means of an *in vitro* gastro-intestinal digestion model coupled to Caco-2/TC-7 cellular uptake model and detection via HPLC/DAD (Table 5). Carotenoid micellarisation and cellular uptake were described to depend on a variety of factors, such as the species and amount of carotenoid present in the test meal, the food matrix and the presence of enhancers/inhibitors (described below in detail). Additionally, there are host-related factors and interactions.

In order to set up and validate the *in vitro* model and the cell culture experiments in the first place, published results (i.e. on the impact of the food matrix and host-related factors, impact of carotenoid species) were re-investigated, and a spinach-based test meal equally rich in lutein and beta-carotene was finally employed to study various factors. In detail, these factors were:

2.6.5.1 Food matrix

a) Lipids: the presence/concentration of lipids was shown to influence (increase) carotenoid micellarisation (Hedren *et al* 2002, Huo *et al* 2007, O'Connell *et al* 2008, Yonekura *et al* 2006)

b) Matrix: complex vegetable matrices and the presence of dietary fiber lowered carotenoid bioavailability in earlier studies (De Jesus Ornelas-Paz *et al* 2008, Hedren *et al* 2002, Ryan *et al* 2008, van het Hof *et al* 1999b, Yonekura *et al* 2009)

c) Processing: processing of the food matrix was found to increase carotenoid availability (Gärtner *et al* 1997, van het Hof *et al* 1999b)

d) Phytosterol: carotenoid absorption in cell culture and *in vivo* was inhibited by phytosterols (Fahy *et al* 2004, Judd *et al* 2002, Mensink *et al* 2002, Plat *et al* 2005)

During preliminary studies, the impact of the disruption of the food matrix (processing) was addressed and the addition of complex ingredients, such as sausage, milk, soya milk were studied. Additionally, the impact of the presence of phytosterols, cholesterol and α-tocopherol on carotenoid micellarisation were tested.

2.6.5.2 Host-related factors

Carotenoid micellarisation and cellular uptake (Garrett *et al* 1999a, Hedren *et al* 2002), as well as carotenoid absorption *in vivo* (Rust *et al* 1998, Schupp *et al* 2004) were dependent on the presence and concentration of digestive enzymes and bile salts. Based on these published data, enzyme and bile salt concentrations in the present study were varied during simulated digestion in order to detect optimal conditions and to simulate incomplete digestion.

2.6.5.3 Impact of carotenoid species

Following aspects were investigated:

a) Xanthophylls were suggested to be better micellarised as compared to carotenes (Failla *et al* 2008b, Garrett *et al* 1999a).

b) Carotenes were described to be better taken up into Caco-2 intestinal cells as compared to xanthophylls, potentially due to facilitated diffusion as opposed to simple passive diffusion (During *et al* 2005, van Bennekum *et al* 2005).

c) Beta-carotene bioavailability *in vivo* was five times lower as opposed to lutein from a spinach based test meal (van het Hof *et al* 1999a).

d) Beta-carotene and lutein compete for absorption *in vivo* (Tyssandier *et al* 2002).

Employing again the spinach-based test meal, lutein and beta-carotene micellarisation and cellular uptake were studied and compared to the results from the above studies for comparison and validation.

2.6.5.4 Minerals affecting carotenoid uptake

After having conducted validation experiments, the focus of further experiments was set on investigating the impact of minerals and trace elements, such as calcium, magnesium, sodium, iron and zinc, on both carotenoid micellarisation and cellular uptake, as these factors have never been systematically studied. Minerals might, in theory, affect micelle formation or stability by influencing ion strength of the digesta, by forming insoluble complexes with food components, such as lipids (Graham *et al* 1983) and (conjugated) bile salts (Feroci *et al* 1995, Fini *et al* 1997, Graham *et al* 1982, Hofmann *et al* 1992). Additionally, cellular uptake was described to be impacted by the presence of trace elements, such as iron (Bengtsson *et al* 2009b).

2.6.6 Digestion/ conversion products

In a final series of experiments, conversion of carotenoid and the further fate of epoxycarotenoid degradation products, such as luteoxanthin/ auroxanthin (from violaxanthin) and neochrome (from neoxanthin) were investigated. Epoxycarotenoids were described to be only marginally taken up by mice and humans (Asai *et al* 2004a,

Asai *et al* 2004b, Asai *et al* 2008, Barua *et al* 2001), but seem to convert rapidly under gastric conditions to the respective furanoid products (Figure 19) (Asai *et al* 2004b). These furanoid carotenoids were shown to induce apoptosis and cell cycle arrest in prostate and colon cancer cell lines (Asai *et al* 2004a, Asai *et al* 2004b, Hosokawa *et al* 2004). Again employing the carotenoid-rich test meal, the conversion of neoxanthin and violaxanthin during digestion and their fate during micellarisation and cellular uptake was examined.

In summary, the here presented thesis covered carotenoid detection in local and locally consumed food items and estimated the carotenoid intake in Luxembourg. Furthermore, general factors influencing carotenoid micellarisation and cell uptake were examined, with a special focus on minerals and trace elements. Finally, the fate of carotenoid reaction products (metabolites) during simulated gastro-intestinal digestion followed by cellular uptake studies was investigated.

Table 5: Aims of the thesis with respect to factors impacting carotenoid micellarisation, cellular uptake and following digestion products

Factors employed	**Endpoints investigated**
food composition (matrix-related)	carotenoid micellarisation
enzymes, bile (host-related)	carotenoid micellarisation
selected phytochemicals (α-tocopherol, cholesterol, phytosterol)	carotenoid micellarisation
minerals (Ca^{2+}, Mg^{2+}, Na^{+}) and trace elements (Fe^{2+}, Zn^{2+})	carotenoid micellarisation and cellular uptake
impact of digestion on epoxycarotenoids	carotenoid degradation

3 Material and Methods

3.1 Enzymes, Chemicals and Standards

Chemicals were of analytical grade or superior, and only filtered water (0.2 µm; pore size, Millipore, Brussels, Belgium) was used for all aqueous solutions.

Chemicals	**Supplier**
Acetone	Merck (Darmstadt, Germany)
Alpha-tocopherol	Sigma-Aldrich (Bornem, Belgium)
Ammonium acetate	BioSolve (Valkerswaard, the Netherlands)
Beta-carotene	Sigma-Aldrich (Bornem, Belgium)
Bile salts (mixed, porcine)	Sigma-Aldrich (Bornem, Belgium)
Calcium carbonate	VWR (Haarode, Belgium)
Calcium chloride	VWR (Haarode, Belgium)
Cholesterol	Sigma-Aldrich (Bornem, Belgium)
Dulbecco's Minimal Essential Medium (DMEM) +GlutaMAX™	GIBCO (Merelbeke, Belgium)
Foetal bovine serum (FBS)	GIBCO (Merelbeke, Belgium)
Hexane	VWR (Haarode, Belgium)
Iron (II) chloride	Alfar Asar (Karlsruhe, Germany)
Lecithin (from chicken egg)	Sigma-Aldrich (Bornem, Belgium)
Lutein	Sigma-Aldrich (Bornem, Belgium)
Lycopene	Extrasynthèse (Lyon, France)

Chemicals	**Supplier**
Magnesium chloride	VWR (Haarode, Belgium)
Methanol	BioSolve (Valkerswaard, the Netherlands)
Monoolein	Sigma-Aldrich (Bornem, Belgium)
Neoxanthin	CaroteNature (Lupsingen, Switzerland)
Oleic acid	Sigma-Aldrich (Bornem, Belgium)
Pancreatin (porcine)	Sigma-Aldrich (Bornem, Belgium)
Penicillin (10,000 units), streptomycin (10mg/ ml) mixture	Sigma-Aldrich (Bornem, Belgium)
Pepsin (porcine)	Sigma-Aldrich (Bornem, Belgium)
Phosphate buffered saline (PBS)	GIBCO (Merelbeke, Belgium)
Potassium hydroxide	VWR (Haarode, Belgium)
Sodium chloride	VWR (Haarode, Belgium)
Sodium taurocholate	Sigma-Aldrich (Bornem, Belgium)
Stigmasterol	Sigma-Aldrich (Bornem, Belgium)
Methyl-tertbutyl-ether (MTBE)	Sigma-Aldrich (Bornem, Belgium)
Violaxanthin	CaroteNature (Lupsingen, Switzerland)
Zeaxanthin	Extrasynthèse (Lyon, France)
Zinc sulphate	BioSolve (Valkerswaard, the Netherlands)

3.2 Food items investigated

Food item	latin term
Apple (red)	*Pyrus malus*
Apricot	*Prunus armenica*
Arugula	*Eruca sativa*
Aubergine	*Solanum melongena*
Banana	*Musa paradisia*
Butter	
Bean (green)	*Phaseolus vulgaris*
Blackberry	*Rubus ulmifolius*
Broccoli	*Brassica oleracea*
Cabbage (green)	*Brassica oleracea*
Carrot	*Daucus carota*
Carrot (juice)	*Daucus carota*
Cheese (25% fat)	
Cheese (32% fat)	
Cherry	*Prunus avium*
Corn, sweet	*Zea mays*
Courgette	*Curcubita pepo*
Eggs (total)	
Endive (curly)	*Cichorium endivia*
Grapefruit (pink)	*Citrus paradisi*
Grapefruit (juice)	*Citrus paradisi*
Grapes (green)	*Vitis vinifera*
Lamb's lettuce	*Valerianella locusta*
Leek	*Allium ampeloprasum*
Lentil, green, dry	*Lens culinaris*
Lentil, white, dry	*Lens culinaris*
Lettuce	*Lactuca sativa*
Margerine (normal)	
Margerine (light)	
Melon (orange)	*Cucumis melo*
Orange	*Citrus reticulata*
Orange (juice)	*Citrus reticulata*
Orange (mandarin)	*Citrus sinensis*
Oil (olive)	*Olea europaea*
Peach	*Prunus persica*
Pear	*Pyrus communis*
Peas (garden)	*Pisum sativum*
Pepper (green)	*Capsicum annuum*
Pepper (orange)	*Capsicum annuum*
Pepper (red)	*Capsicum annuum*
Pepper (yellow)	*Capsicum annuum*
Plum	*Prunus domestica*
Potato (parisienne)	*Solanum tuberosum*
Potato (victoria)	*Solanum tuberosum*
Sour cream	
Spinach	*Spinacia oleracea*
Spinach (creamed)	
Tomato	*Solanum lycopersicum*
Tomato (juice)	
Tomato (ketchup)	
Watermelon	*Citrullus lanatus*

3.3 Selection of food items

Luxembourgish or locally consumed food items potentially containing high concentrations of carotenoids were purchased from the major Luxembourgish supermarket chain (CACTUS S.A., Windhof, Luxembourg). Food items were selected according to a minimum total carotenoid content, such as > 50-100 µg/100 g of edible portion (O'Neill *et al* 2001, Souci *et al* 2000) and according to their nutritional relevance for Luxembourg (Alkerwi *et al* 2010), i.e. based on estimated frequency of intake.

3.4 Carotenoid analysis of food items

Fresh fruits and vegetables were purchased at mature conditions. Different foods were aliquoted on the day of purchase. Solid items were rinsed with cold water, dried with paper towels and chopped with a kitchen knife into small cubes of approx. 0.5*0.5*0.5 cm^3 at room temperature and stored in aliquots of 10 - 30 g at -25°C for a maximum of 4 weeks until analysis, while juices, eggs and oils were stored in the original package in the cold chamber at 4°C in the dark and analysed within 2 - 3 weeks. Before analysis, juices and oils were shaken and aliquots of approx 10 ml were taken, while eggs were opened and aliquots were taken after homogenisation with a spatula at room temperature. Frozen spinach was allowed to thaw slightly, chopped with a kitchen knife at room temperature as described above and quickly re-frozen at - 25°C. On the day of analysis, food items were extracted as described in the following chapters.

3.4.1 Carotenoid extraction procedure from solid food matrices (low fat)

This extraction protocol was adapted from a method described earlier (Gorocica-Buenfil *et al* 2007). All procedures were carried out on ice and under dim light as much as possible.

3.4.1.1 Saponification

For screening purposes (spectrophotometer), a saponification step was included prior to quantification, in order to remove potential chlorophylls. Aliquoted food samples (approx. 10 - 30 g), an equal weight of quartz sand to facilitate homogenisation, plus

1 g of calcium carbonate to neutralize cytosolic acids were mixed in a mortar. Liquid nitrogen was added to improve homogenisation and to prevent pigment degradation. The mixture was homogenised for 3 minutes by grinding with a pestle. Aliquots of 2 g were weighed into 15 ml centrifuge tubes (BD Biosciences, San Jose, CA, USA) and 5 ml of methanol as well as 1 ml of 30% (w/v) methanolic potassium hydroxide were added. After vortexing for 1 min (Dual-press-to-mix apparatus from Snijders, Tilburg, The Netherlands), mixing and incubation for 15 min on ice, samples were centrifuged (Harrier 18/80 refrigerated centrifuge from MSEM, Chorley, UK) for 3 min at 2500*g at room temperature. A preliminary study indicated that a 15 min incubation time with potassium hydroxide was sufficient to remove chlorophylls from the organic phase by cleaving the hydrophobic phytyl-rest from the more polar porphyrin ring, the latter being responsible for the colour of the molecule, enabling separation from carotenoids due to increased polarity of the porphyrin ring. This incubation time was consequently chosen for sample preparation for further analyses. The supernatant was decanted into a 50 ml centrifuge tube (BD Biosciences, San Jose, CA, USA) and the pellet containing food, sand and calcium carbonate was re-extracted twice with 8 ml of a mixture of hexane: acetone (1:1, v/v) and the carotenoid-containing methanol-acetone-hexane fractions were combined in the 50 ml tube. 25 ml of saturated aqueous sodium chloride solution were added and vigorously shaken to ‘salt out’ remaining carotenoids present in the aqueous phase into the organic phase. The supernatant hexane phase was transferred to a third tube. 8 ml of hexane were used for re-extraction of the aqueous solution in the second tube and combined with the hexane extract, which was then weighed for exact volume determination. Depending on the intensity of colour, 5 - 12 ml aliquots were then taken from the combined extracts, evaporated to dryness under a stream of nitrogen in a TurboVapLV® apparatus (Caliper Life Sciences Benelux, Teralfene, Belgium), covered with a blanket of argon and stored under – 80°C or analysed directly. For spectrophotometric measurements, the residue was re-dissolved in acetone, sonicated and measured directly.

3.4.1.2 Simplified method without saponification

A similar extraction protocol was carried out for food samples not requiring saponification, i.e. for HPLC analysis. The procedure was identical except for the addition of methanolic KOH, which was replaced by 6 ml of pure methanol followed by centrifugation (Figure 8). For HPLC analysis, aliquots of 5 ml were taken from the final organic extracts, evaporated to dryness and stored under a blanket of argon at -80°C. On the day of analysis, residues were reconstituted in 0.5 - 1 ml (depending on the intensity of colour) of MTBE:methanol (3:7, v/v), filtered through an 0.22 µm membrane syringe filter (PALL Life Sciences, Ann Arbor, USA) into an amber glass HPLC tube and 25 µl were routinely injected.

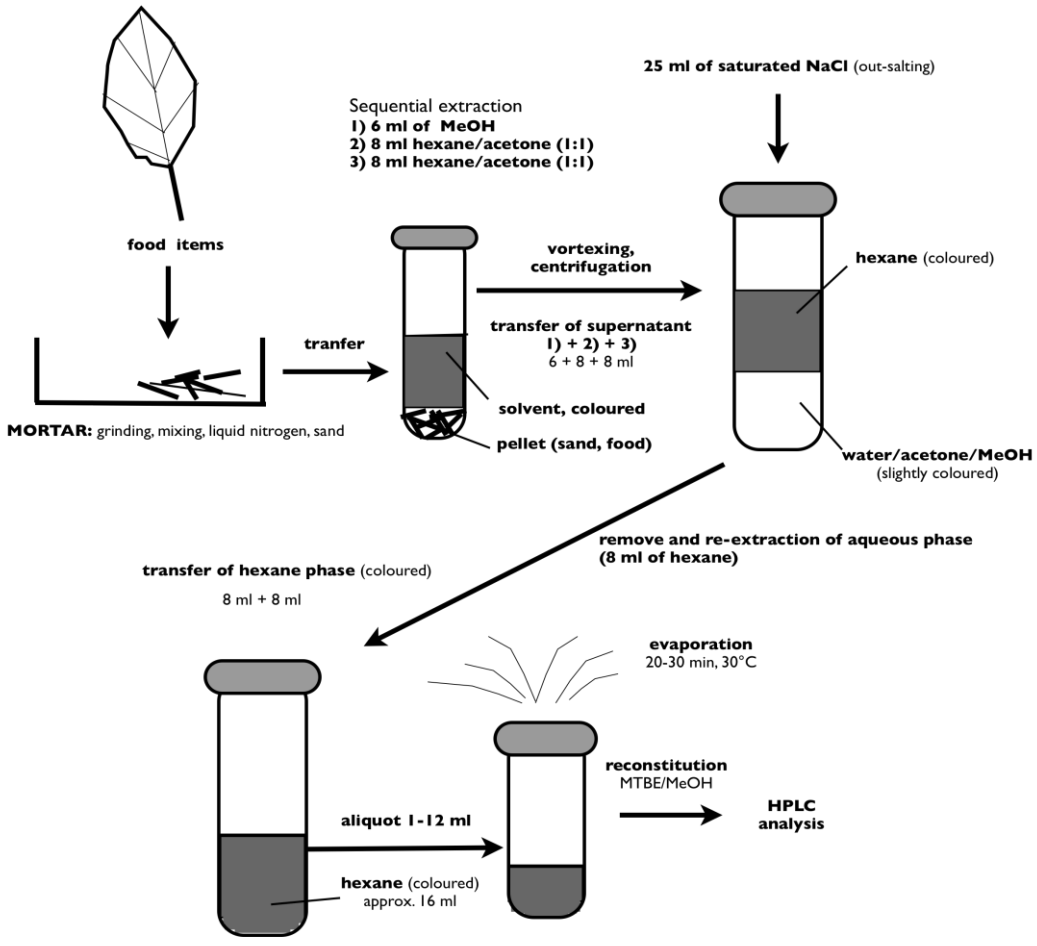

Figure 8: Schematic overview on the food extraction procedure for solid food items low in lipids. Details are described in the text.

3.4.1 Carotenoid extraction procedure from juices and ketchup

Depending on the intensity of the colour, 1 to 2 g of juice was weighed into a 50 ml centrifuge tube and 6 ml of methanol was added and the tube vortexed for 1 min. 8 ml of a mixture of hexane: acetone (1:1, v/v) were added, tubes were vortexed and the supernatant organic phase was transferred into a second tube. The extraction step with hexane was repeated and supernatant organic layers combined. The following steps, including the addition of sodium chloride solution and re-extraction with 8 ml of hexane and separation/quantification were performed as described in the chapter above. The final volume was reconstituted in 0.4 ml of methanol:MTBE (3:7, v/v) and injection volume was 25 µl.

3.4.2 Carotenoid extraction procedure from solid food matrices rich in lipids

High fat containing matrices, such as cheese, oils, butter and margarine were enzymatically treated before extraction, as lipids impede carotenoid separation from the matrix and also detection due to similar chemical properties. For extraction, 0.2 - 0.5 g of homogenised food matrix were weighed into a 50 ml centrifuge tube and 200 mg of monoolein, 10 mg of oleic acid, 120 mg of lecithin and 0.5 ml of canola oil (Colzol, Clemency, Luxembourg) were added to achieve emulsification of fats and carotenoids. The mixture was filled up to 50 ml with physiologic saline containing sodium taurocholate (1.1 mg/ml). After 20 min of sonication and shaking, an aliquot of 15 ml was taken and 9 ml of a bile salt (commercial porcine bile mixture; 24 mg/ml) and porcine pancreatin (commercial mixture of pancreatic digestive enzymes; 4 mg/ ml, both diluted in 0.1 M $NaHCO_3$) solution were added, filled up to 50 ml with physiological saline (final pH approx. 7) and incubated at 37°C for 4 hours (as a pilot study indicated that enzymatic lypolysis was sufficient at this time point). The following extraction and separation was performed as described above, omitting only the first methanol step (Figure 8). Residues were reconstituted in 150 µL MTBE:methanol (3:7, v/v) and 25 µl were injected for HPLC analysis.

3.5 Carotenoid detection and quantification methods

3.5.1 Screening method (Spectrophotometer)

Table 6: Specific molecular absorption coefficients (ε) of major carotenoids present in frequently consumed fruits and vegetables. Coefficients are given for their maximal absorbance wavelength (nm), and corrected by own spectrophotometric data for acetone if the solvent in literature was different.

Compound	Solvent [3]	λ_{max} [nm][4]	λ_{ad} [nm]	ε [l/mol*cm][4]	m [g/mol]
β-carotene	acetone	452	452	140663	537
β-cryptoxanthin[1]	petrol. ether	449	453	131915	553
lutein	ethanol	445	448	144900	545
lycopene[2]	acetone	448	448	120600	537
zeaxanthin	acetone	452	452	133118	569
	Mean	449	450.2	135310	548
	SD	2.7	1.9	7979	14
	RSD [%]	0.6	0.5	5.9	2.6

[1] β-cryptoxanthin was used for total cryptoxanthin due to high similarity between the α- and β- isomer

[2] absorption coefficient and wavelength taken from Budavari (1989)

[3] solvent described in literature

[4] Rodriguez-Amaya et al., 2004

λ_{max} = wavelength at maximum absorption

λ_{ad} = adapted wavelength for acetone

For the screening of food items potentially containing high amounts of carotenoids (Souci *et al* 2000), a rapid protocol was developed including spectrophotometric measurements at a single, fixed wavelength. This method was based on the assumption that up to 90% of the carotenoids in the human body and diet are represented by beta-carotene, alpha-carotene, lycopene, lutein and cryptoxanthin (Rao *et al* 2007). Taking into account solely these major abundant carotenoids and exchanging alpha-carotene detection by zeaxanthin for determining a broader range of carotenoids, an average molar absorption coefficient and absorption wavelength can be obtained (Table 6) for carotenoid quantification, especially given that absorption maxima do typically not vary more that 5-10 nm.

To calculate average carotenoid concentrations, the following equation was used:

$$c(mol/l) = \frac{A_{450} \times Fd}{135310} \quad \text{(d= 1 cm)} \qquad [1]$$

with A_{450} being the mean absorbance at a wavelength of 450 nm, Fd a dilution factor adjusting for extractions, drying and reconstitution processes and d the gauge auf the solvent in the cuvette (here: 1 cm). Using an average molar mass (g/mol), results were finally expressed as milligrams per hundred grams of edible portion (mg/100 g).

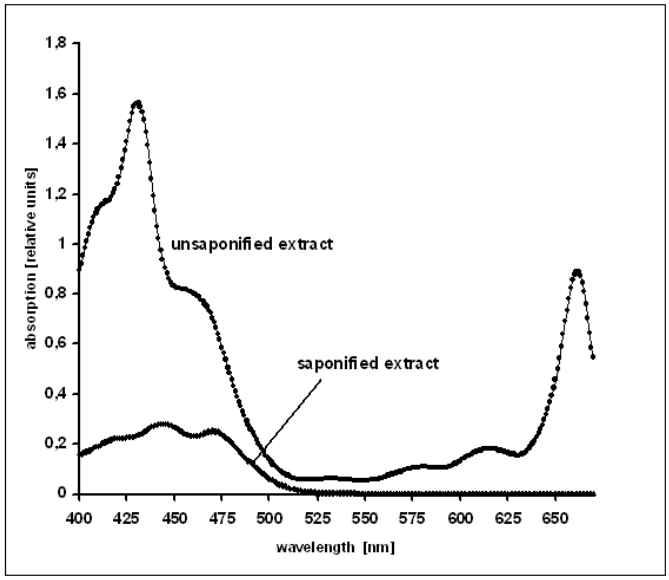

Figure 9: Effect of saponification on carotenoid extracts obtained from leek (*Alium ampeloprasum*). The typical chlorophyll interference between 400-500 nm and consequent overestimation of the carotenoid concentration was avoided due to treatment with 30% methanolic KOH for 15 minutes prior to pigment extraction.

Absorption spectra of extracts from fruits or vegetables, containing chlorophylls and carotenoids, were difficult to analyze as the wavelength of absorbance for both chlorophylls and carotenoids partly overlap. Depending slightly on the solvent, chlorophyll A and B possess two major absorbance peaks at 650 - 660 nm and between 400 nm and 440 nm, where carotenoids also show a characteristic absorption of typically three peaks or shoulders (Figure 9). Therefore, the absorbance (and hence concentration) of carotenoids is overestimated in a solution containing chlorophylls and an additional saponification step during extraction to remove chlorophylls from the organic phase or a mathematical subtraction is necessary (Lichtenthaler 1987).

Dried extracts were reconstituted in 1-10 ml of acetone, depending on the intensity of colour, and sonicated for 2 minutes. Visible spectra (340 to 700 nm, 1 nm interval) were collected using a 1 ml quartz cuvette (101-QS, Hellma GmbH, Müllheim, Germany) in a DU 800 UV/Visible spectrophotometer (Beckman Coulter, Palo Alto, CA, USA). For some samples (i.e. blackberries), a filtration step with 0.22 µm GHP membrane filter (Pall Life Science, Ann Arbor, MI, USA) had to be included to reduce turbidity.

3.5.2 HPLC

Carotenoids were separated and identified by a Dionex HPLC instrument including a P580 pump and Gina 50 autosampler in combination with a UVD340S photodiode array detector (Dionex Benelux B.V., Amsterdam, the Netherlands), by their retention times and spectral data as compared to pure, individual standards. For quantification, 10-point calibration curves based on external standard solutions were obtained and extinction coefficients from the suppliers were used for calculations. The detector was simultaneously set at 450 nm (detection of beta-carotene, lutein, beta-cryptoxathin and zeaxanthin) and 472 nm (detection of neoxanthin, violaxanthin and lycopene).
The separation protocol was adapted from a previous one (Gorocica-Buenfil *et al* 2007) in order to achieve optimal separation according to the theory of van Deemter and colleagues (1956). In detail, a guard column (C18, 3.9 x 20 mm, 3 µm particle size) protected a YMC C30 column (Waters Inc., Zellik, Belgium, 150*4.6 mm, 3 µm particle size, set at 28°C) was used in combination with a binary elution gradient consisting of solvent A) methanol/water/ammonium acetate/MTBE (88:5:2:5, v/v) and solvent B) MTBE/methanol/water/ammonium acetate (79:16:3:2, v/v). The gradient started at 100% A (0 to 5 minutes). Solvent B was increased to 65% (during minute 5 to 26) and further increased to 100% until minute 34, holding this constant for 5 minutes. The gradient changed back to 100% solvent A until minute 40, keeping this constant for 4 minutes. The injection volume was 25 µl and the flow rate was kept constant at 1.2 ml/minute.

3.6 Carotenoid intake – Estimation for Luxembourg

Carotenoid intake in Luxembourg was estimated based on food consumption and carotenoid concentrations in the frequently consumed food items. Food consumption was derived from on the first nationwide cardiovascular risk-factor study, conducted by the Centre de Recherche Public - Santé, Luxembourg (Alkerwi *et al* 2010). In this study, 1432 out of 4496 randomly chosen Luxembourgish inhabitants participated and answered self-administered questionnaires including dietary habits during the 3 months prior to participation. Details about recruitment, study design, data processing and the scope were described elsewhere (Alkerwi *et al* 2010).

Nutrition-related questionnaires contained 9 major food categories: starchy foods, fruits, cooked and raw vegetables, meat-poultry-fish-egg, prepared dishes, dairy products, fats, miscellaneous and drinks including sub-categories. The food consumption data were collected and stored in a newly created database. Access to a digital file containing detailed consumption data was generously granted.

In a first step, 8 out of 9 food categories, potentially containing food items with significant carotenoid contents were chosen (> 50-100 μg/100 g), while in a second step, potentially interesting subcategories (20 out of a total of 107 subcategories defined in the questionnaires) were selected. In order to simplify the calculations, different age categories and both sexes - originally differentiated in the raw data - were combined and an average carotenoid consumption for the entire study population was calculated.

Since the subcategories were often not detailed enough and contained pooled food items of different carotenoid content and patterns, a consumption frequency ranking for the most carotenoid contributing food items had to be created (see example on the following page). This was achieved by means of local food disappearance data. These data were generously provided by Mr Henri Jungels (CACTUS S.A., Windhof, Luxembourg), allowing for the estimation of the relative ‘consumption’ of each food item belonging to each individual subcategory. This consumption fraction was then multiplied with the carotenoid content detected for each food items. Summing up carotenoid content of each individual food items within a subgroup, an average, total carotenoid content for each group was determined. This average carotenoid content

was then multiplied by the dietary consumption data according to the ORISCAV-Lux study (Alkerwi *et al* 2010), yielding an average amount of carotenoids taken up due to consumption of this food group on one day.

Example: calculation of the relative composition of each food group ('consumption fraction') and carotenoid intake in the 'salad group' (other groups accordingly)

food items	ORISCAV [g/day]	Sales data [kg]*	consumption fraction	carotenoid content [mg/100g][$]	carotenoid intake [mg/day][§]
arugula	n.d.	11781	0.033	0.34	0.07
lamb's lettuce	n.d.	20823	0.058	0.45	0.09
lettuce	n.d.	326273	0.909	1.66	0.35
total 'salad group'	21.1	358877	1.0	2.45	0.51

[§] calculated via ORISCAV-LUX data (Alkerwi *et al* 2010); here: 21.1 g/day

[$] own carotenoid analysis (Table 8)

* obtained from CACTUS S.A. (Windhof, Luxembourg)

n.d. = no detailed data available

Calculation of consumption fractions was further validated by consumption data obtained from the Fünfter Schweizerischer Ernährungsbericht (Gremaud *et al* 2005), when Luxembourgish data was incomplete. These data were chosen, because this report is very detailed and it was estimated to resemble with its cuisine influenced by Germany and France to some extent Luxembourg, being also strongly impacted by these two countries. In a final step, carotenoid contents of locally grown and/or consumed foods obtained from own HPLC analyses were combined with the assessed frequency of consumption for each food item to obtain an estimation of total daily carotenoid intake.

3.7 In vitro digestion

3.7.1 Impact of different test meals

Table 7: Ingredients, description and composition of the test meals subjected to *in vitro* digestion.

Product/ Ingredient	Description/ composition[1]	Amount per meal[2]
spinach	chopped, frozen, protein 2.4%, dietary fibre 1.2%, carbohydrates 1.6%, fat 0.4%	4 g
cream spinach[3]	crude, frozen, protein 3.3%, dietary fibre 2%, carbohydrates 2.3%, fat 3.2%	4 g
1) condensed milk[4]	protein 3.4%, carbohydrates 2.0%, fat 4.0%	2 g
2) whole milk[5]	protein 3.5%, carbohydrates 4.8%, fat 3.5%	2 g
3) skimmed milk[5]	protein 3.8%, carbohydrates 5.8%, fat 1.5%	2 g
4) soya milk[5]	protein 2.9%, carbohydrates 4.8%, fat 2%	2 g
5) liver sausage[5]	protein 13.1%, carbohydrates 0%, fat 31.9%	0.5 g
6) Fresubin[5]	protein 3.8%, carbohydrates 13.8%, fat 3.4%	2 g
7) inhibitors[6]	α-tocopherol,	0.25 - 1 mg
	cholesterol	0.5 - 114 mg
	stigmasterol	108 mg
bile + enzymes	pepsin	110 mg
	pancreatin	18 – 36 mg
	bile	98 - 196 mg

[1] Food composition data were taken from food labels,[2] total volume 50 ml,[3] used instead of pure spinach,[4] condensed milk was used as an additive to the standard test meal,[5] tested as an additive to spinach during method development and validation,[6] added to standard meal to investigate micellarisation inhibiting potential

Food items were obtained from a local supermarket (CACTUS S.A., Esch-sur-Alzette, Luxembourg), aliquoted and frozen at -80°C (spinach) and -24° (all other ingredients) and stored for a maximum of 3 months. Prior to freezing, 1 kg of spinach was processed as one batch in a household blender for 5 min. The vanilla flavoured Fresubin® meal-replacement drink (Fresenius-Kabi, Bad Homburg, Germany) was

purchased in a local pharmacy (Esch-sur-Alzette, Luxembourg).

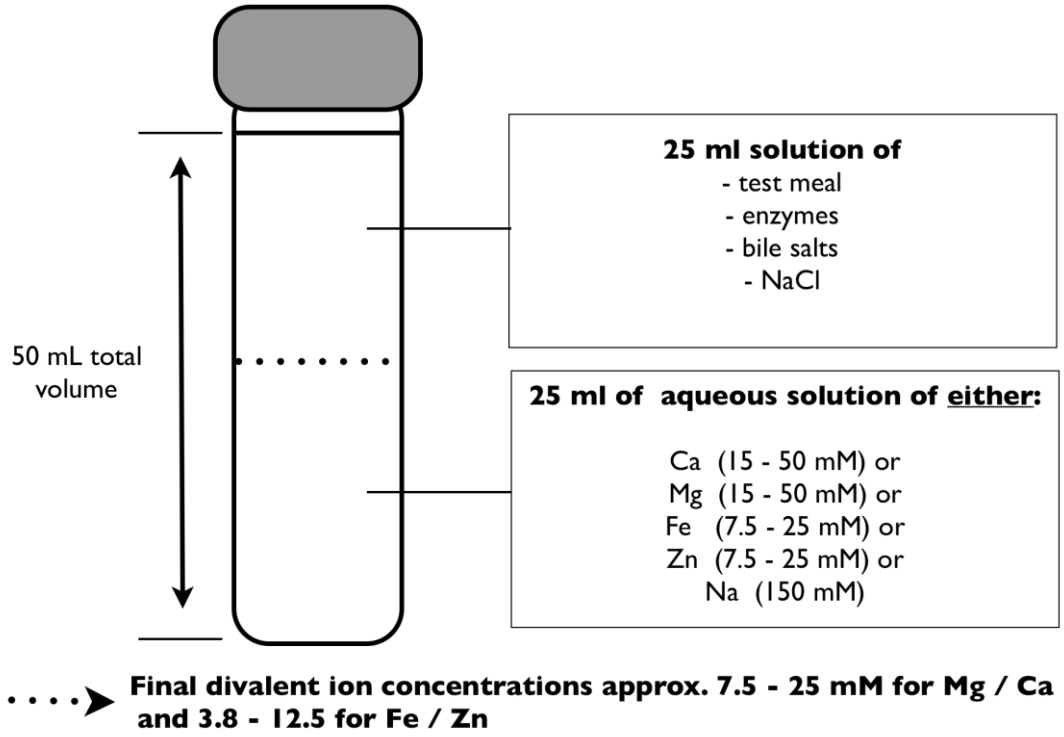

Figure 10: Final test meal composition for studies with divalent ions. Note that only one divalent ion is present during simulated digestion and the final concentration is approximately 50%. In the control sample, 150 mM NaCl is added.

3.7.2 Studying the impact of divalent ions on carotenoid micellarisation

To test the impact of divalent ions, spinach and milk (Table 7) were digested together with different concentrations of Ca, Fe, Mg and Zn added to the standard test meal in aqueous solutions instead of physiologic saline, following the same digestion protocol. Thus, 25 ml of a) calcium chloride at different concentrations (15, 20, 25, 30, 50 mM), b) magnesium chloride (15, 25, 50 mM), c) zinc sulphate (7.5, 15, 25 mM) and d) iron (II) chloride (7.5, 15, 25 mM) were added (Figure 10). Final divalent ion concentrations were, due to a final volume of 50 ml, half of these concentrations. Each sample, including controls with physiologic saline, contained in addition approximately 240 µM of calcium, 120 µM of magnesium, 2.1 µM of iron and 0.4 µM of zinc derived from the test meal (estimated from Souci *et al* 2000).

3.7.3 Simulation of the gastro-intestinal passage

The *in vitro* digestion protocol was adapted from Miller *et al.* (1981) and Garrett *et al.* (1999a). The following protocol represents the final version. For validation purposes and to investigate the effect of the food matrix on carotenoid solubilisation, different 'meals' were tested, e.g. 4 g of spinach were digested together with 2 g of milk containing different percentages of fat or even 0.5 g of sausage (Table 7). Additionally, single digestive enzymes and bile salts were omitted during digestion while all other parameters were kept constant. Further, the impact of the ratio '(enzymes + bile salts): (test meal)' was investigated and carotenoid micellarisation efficiency from spinach was tested by either omitting pepsin, pancreatin, bile salts or pancreatin and bile salts.

Potential inhibitors or enhancers of carotenoid micellarisation, such as the phytosterol stigmasterol, cholesterol and α-tocopherol (Table 7), were prepared as quantitative ethanolic parental solutions, mixed with the standard spinach-milk test meal in the centrifuge tube and treated with the standard protocol. For an overview on the entire *in vitro* digestion procedure, see Figure 6.

3.7.4 Gastric phase

Frozen spinach was thawed and approx. 4 g of spinach was weighed into a 50 ml screw-top polyethylene tube, combined with 2 g of semi-skimmed condensed milk (4% fat) and homogenized by gentle shaking and put on ice. 25 ml of 0.15 M NaCl were then added to the cooled test meal, which was then acidified with 1.3 – 1.5 ml of 1 M HCl to pH 2.0. Two ml of porcine pepsin (40 mg/ ml in 0.1 M HCl) were then added (either freshly prepared or stored for a max 1 week at –25°C). The spinach samples were additionally sealed with Parafilm® M (Brand GmbH + CO KG, Wertheim, Germany) and put into a closed plastic bag. The prepared tubes were then transferred to a shaking water bath (GFL 1083 from VEL®, Leuven, Belgium; 100 rpm, 37°C, 1 hour). The pH of the post-gastric meal was increased to 5.5 with 1 ml of 0.9 M $NaHCO_3$) to simulate the transition between stomach and duodenum.

3.7.5 Small intestinal phase

Enzyme and bile solutions were either freshly prepared or prepared and stored at -25°C until usage (max. 1 week). 9 ml of a mixture of pancreatin and porcine bile extract (4 mg/ml pancreatin and 24 mg/ml bile extract dissolved in 0.1 M $NaHCO_3$) were added to each digestion tube. Additionally, the pancreatin and bile concentration was reduced to 50% in one series of experiments, testing the impact of divalent ions at different conditions. The pH of the samples was finally increased to 7 - 7.5 with the addition of 700 - 800 µl of 1 M NaOH. The final volume of the samples was adjusted to 50 ml with 0.15 M NaCl. The 50 ml tubes were sealed with Parafilm® and incubated in a shaking water bath (100 rpm, 37°C) for two hours. The resulting solution is further referred to as 'digesta'.

3.8 Carotenoid extraction from gastro-intestinal digesta

12 ml aliquots of digesta were conveyed to Beckmann centrifuge tubes and centrifuged in a Ti-40 rotor at 164,000*g at 4°C for 35 min (Beckmann Optima^{TM} C-90U Ultracentrifuge, Beckmann Coulter, Palo Alto, CA) to separate the solid and oil contents from the aqueous micellar phase. Using a needle and syringe, 8 ml of the aqueous phase were taken and filtered through a 0.22 µm GHP membrane filter (Pall Life Science, Ann Arbor, MI, USA) into a 15 ml centrifuge tube. The following extraction was adapted from Ferruzzi *et al.* (2001): 2 ml of the aqueous/micellar phase were extracted by addition of 4 ml hexane: acetone (1:1, v/v), shaken for 1 min and centrifuged for 2 min at 4,000*g to hasten phase separation. The supernatant organic phase was collected and transferred into a second tube. The extraction process was repeated with 4 ml of hexane and the combined hexane phases dried under a stream of nitrogen. The residue was reconstituted in 400 µl MTBE/MeOH (1:1, v:v), filtered through a 0.22 µm GHP membrane syringe filter into an amber HPLC vial and stored at –80°C until analysis for no more than 1 week.

3.9 Carotenoid conversion during simulated gastro-intestinal digestion

Violaxanthin and neoxanthin conversion (Figures 20 + 21, Results) during simulated gastro-intestinal passage was assessed separately by an additional simulation experiment realized with isolated carotenoids and artificial micelles: 200 mg of monoolein, 10 mg of oleic acid and 120 mg of lecithin were added to 100 µg of each carotenoid standard, respectively, and also to both carotenoids combined (100 µg each). 0.5 ml of canola oil (Colzol, Clemency, Luxembourg) was added and the mixture was filled up to 50 ml with physiologic saline containing sodium taurocholate (1.1 mg/ml). After 20 min of sonication and shaking, an aliquot of 15 ml was taken and 2 ml of pepsin solution (40 mg/ml in 0.1 M HCl) were added (final pH ca. 2). After 1 hour in a shaking water bath, 9 ml of a bile salt (24 mg/ml) and pancreatin (4 mg/ml, both diluted in 0.1 M $NaHCO_3$) solution were added, brought to 50 ml with physiological saline (final pH app. 7) and further incubated at 37°C for 2 hours. Resulting digestion products were extracted and separated as described above. The final residues were dissolved in 150 µl MTBE:methanol (3:7, v/v) and 25 µl were injected.

3.10 Cell culture

The Caco-2/TC-7 cell line was cloned from the original Caco-2 cell line (ATTC® number HTB-37™) by Dr. Rousset and colleagues (Chantret *et al* 1993, Fogh *et al* 1977) and was obtained as a generous gift from University of Nancy, France.

Cells were routinely maintained in 75 cm^2 plastic flasks (nunclon™, Nunc, Denmark) in a moisture-saturated environment at 37°C and 10% CO_2 in Dulbecco's minimal essential medium (DMEM + GlutaMAX™), supplemented with 1% nonessential amino acids, 20% heat-inactivated foetal bovine serum (FBS), 1% penicillin (10,000 units)/ streptomycin (10 mg/ml) mixture and sub-cultured weekly after reaching confluence of 70-80%. The medium was replenished 3 times/week. For conservation, cell aliquots were stored under liquid nitrogen employing medium containing 30% FBS and 10% of glycerol as a protective additive. For mycoplasma testing prior to cell freezing, a polymerase chain reaction based kit was used according to the supplier's protocol (AppliChem, Darmstadt, Germany). During the first passage after thawing, cells were grown with DMEM containing 30% FBS

in a 25 cm^2 flask until reaching confluence, then cells were split into three 75 cm^2 plastic flasks, continuing with the routine protocol afterwards.

For uptake experiments, cells were seeded into 6-well plates (BD Falcon™, Franklin Lakes, NJ, USA) at a density of $5*10^4$ cells/cm^2, 10-13 days prior to exposure to allow for differentiation (Chitchumroonchokchai *et al* 2004b, Ferruzzi *et al* 2001).

3.11 Cell exposure

Cells were incubated with digesta (diluted in DMEM) for 4 hours at 37°C and 10% CO_2 to simulate a physiologic intestinal passage time. In a pilot study, cell viability was investigated with different ratios of digesta/DMEM. After 4 hours of incubation, cell appearance (microscope) as well as cell viability, as determined by the resazurin assay (O'Brian *et al* 2000), was not significantly compromised at a ratio of 1:4 (digesta:DMEM, v/v) compared to cells incubated with DMEM only. The 1:4 ratio was consequently chosen throughout the uptake experiments. A total volume of 2 ml medium/digesta mixture was given into each well.

3.12 Cellular carotenoid uptake studies

After 4 hours, plates were put on ice and medium was removed. Cells were washed with cold phosphate buffered saline (PBS) including 2 mg/ml bile salts to remove carotenoids attached to the cell membranes and once with pure PBS. Then, 4 ml of cold water was added and cells were lysed by osmotic stress during 20 min in the dark. No detergents were used to avoid interactions with carotenoids. Cells from 3 wells of each exposure were pooled in one 50 ml centrifuge tube in order to improve sensitivity of the assay and vortexed for 1 min. Total proteins per well were quantified following the assay protocol proposed by Bradford (1976), using bovine serum albumin as protein standard. 8 ml of hexane: acetone (1:1, v/v) were added and tubes were sonicated for 2 min to facilitate cell membrane rupture and additionally vortexed (2 x 20 sec). Phase separation was achieved by centrifugation (3 min at 4000*g, 4°C). The supernatant hexane phase was transferred into a second centrifuge tube. 25 ml of saturated sodium chloride solution were added to the first tube and the aqueous phase was re-extracted with 6 ml of hexane, vortexed (2 x 20 sec) and centrifuged (3 min at 4000*g, 4°C).

The hexane phases were combined, dried under a stream of N_2 at 30°C, over-layered with a blanket of argon and stored at – 80°C until further analysis (max. of one week). For HPLC analysis, carotenoids were dissolved in 150 µl MTBE:methanol (3:7, v/v) and 25 µl were injected.

3.13 Software and Statistics

Data were routinely sorted and analysed with Microsoft EXCEL (Redmond, WA, USA) and SigmaPlot 2001 (Systat Software Inc., San Jose, CA, USA), while structure formulae were created with ISIS Draw 2.4 (Accelrys Inc., San Diego, CA, USA). For statistical analysis, SPSS Versions 16.0 and 17 for Windows (SPSS Inc., Chicago, IL, USA) were employed. Normal distribution of data was verified by Q-Q-plots, Kolmogorov-Smirnoff tests and equality of variance by box-plots. Wherever possible, non-transformed data and numerical tests were performed. If needed, data were log transformed for statistical comparisons. Where indicated, Fisher-F-tests were followed by post hoc tests (Dunnett's for the inhibitors (α-tocopherol, stigmasterol cholesterol) and Bonferroni for all other comparisons).

The Bonferroni correction is a relatively straightforward approach employed when multiple pairwise comparisons are performed simultaneously. This correction lowers the statistical significance level alpha (α) for each single comparison, dividing α by the number of comparisons (n) being performed: α/n (Abdi 2007), in order to keep an overall α of 5%. Using the Bonferroni correction, each set of data can be compared to all other datasets, while with the Dunnett's correction, an alternative method to adjust the α error, multiple groups can only be compared to the same control (Dunnett 1955). The Dunnett's post hoc test is less versatile than the Bonferroni, however, it is also less conservative. Throughout all analyses, p-values <0.05 (2-sided) were considered as statistically significant; all presented values represent means ± standard deviation (SD), except for micellarisation inhibition curves and food consumption data (mean ± standard error).

For data presentation in the results section, statistical significance is indicate by the means of letters – bars or columns not sharing the same letter are significantly different.

3.13.1 Statistical tools and formulae employed for micellarisation experiments

Individual fractional carotenoid micellarisation was defined as:

$$fractional\ micellarisation\ [\%] = \frac{carotenoids\ in\ micelles\ [\mu g]}{extractable\ carotenoids\ in\ spinach\ meal\ [\mu g]} \times 100 \qquad [2]$$

Micellarisation experiments were based on n = 3-6 independent experiments. Preliminary studies showed that the results from the *in vitro* studies varied between different days of experiments. To minimize these day-to-day variances, all values were expressed as percent of the control, which was set to 100%.

The relative (fractional) micellarisation was defined as:

$$normalised\ fractional\ micellarisation\ [\%] = \frac{\frac{carotenoids\ in\ micelles\ \exp erimental\ group\ [\mu g]}{extractable\ carotenoids\ in\ spinach\ meal\ [\mu g]}}{\frac{carotenoids\ in\ micelles\ control\ group\ [\mu g]}{extractable\ carotenoids\ in\ spinach\ meal\ [\mu g]}} \times 100 \qquad [3]$$

Separate linear-mixed models were created to analyze the impact of enzyme concentrations, presence of bile salts and enzymes, presence and concentrations of inhibitors or enhancers, food matrix (or additives) and different salt concentrations on carotenoid micellarisation.

Micellarisation inhibition curves resulting from experiments with divalent ions were created, using a non-linear regression model, showing best fit in a sigmoid curve type for Fe^{2+}, Zn^{2+} and Mg^{2+} with 3 parameters and in a logistic curve with three parameters for Ca^{2+}.

3.13.2 Statistical tools and formulae employed for cellular uptake experiments

'Relative (fractional) cellular carotenoid uptake' from micelles in the digesta was defined as:

$$relative\ uptake\ [\%] = \frac{carotenoids\ in\ cells/well\ [ng]}{carotenoids\ in\ 1\ ml\ of\ digesta:DMEM\ mixture\ [ng]} \times \frac{4}{3}\ (Fd) \times 100 \qquad [4]$$

F_d = dilution factor (1 ml of digesta was diluted 1:4 with DMEM, v/v, and cells from three wells were pooled)

'Overall cellular carotenoid uptake' from test meal was determined as:

$$overall\ uptake\ [\%] = \frac{carotenoids\ in\ cells/well\ [ng]}{extractable\ carotenoids\ in\ spinach\ meal\ [ng]} \times \frac{4}{3}\ (F_D) \times 100 \qquad [5]$$

F_d = dilution factor (1 ml of digesta was diluted 1:4 with DMEM, v/v, and cells from three wells were pooled)

Cell studies were performed with n = 6-10, i.e. 18-30 wells, since cells from 3 wells were routinely combined to increase sensitivity of the assay. All values were normalized to the control to minimize day-to-day variances.

A linear-mixed model including appropriate post-hoc tests (Bonferroni) was employed, investigating the impact of divalent ions on the different carotenoid species (micellarisation and 'overall' cellular uptake), with percentage carotenoid micellarisation/overall cellular uptake as the observed variables and species carotenoid and minerals as the fixed factors. In a second linear-mixed model, additive effects of divalent ions on carotenoid uptake were investigated (fractional cellular uptake from micelles) with percentage carotenoid fractional uptake as the observed variable and species carotenoid and minerals as the fixed factors. In order to achieve normality of distribution, log-transformed data were used for the second model.

4 Results

4.1 Food composition

In order to assess precision and robustness of the methods applied, detection and quantification limits as well as comparability between experiments from one set of experiments and between different sets of experiments on different days were calculated.

4.1.1 LOD, Repeatability

4.1.1.1 Spectrophotometric method

Assessing the repeatability by measuring representative plant samples in octuplicate in parallel, the method (including extraction, preparation and measurement) showed a relative standard deviation (RSD) of 6.9%. The reproducibility was estimated at 7.0% (RSD), based on triplicate analysis conducted on three different days. Limit of detection (LOD) was assessed as 51 µg/100 g and 102 µg/100 g as limit of quantification (LOQ), respectively, based on total carotenoids in 7 independently measured spinach samples, following the method proposed by the US Environment Protective Agency (Zorn *et al* 1997), with 3 times SD yielding the LOD and 6 times SD the LOQ.

4.1.1.2 HPLC

Repeatability (including all steps from extraction to analysis) for total carotenoid content was assessed at 5.7% (RSD) based on seven independently measured spinach samples in parallel, while reproducibility was calculated at 7.8% (RSD), based on spinach analyses (n = 4) conducted on four different days. The limit of detection (LOD: 0.29 ng) and limit of quantification (LOQ: 0.59 ng) were determined following the method proposed by the US Environment Protective Agency (see above), investigating 7 independent beta-carotene standard injections of low concentrations (20 ng/ml).

The LOD/LOQ for beta-carotene in a typical extraction translates to approximately 12 µg/100 g and 24 µg/100 g of food matrix, respectively. For other carotenoid species, LOD were estimated by comparing slopes of calibration curves in relation to

that of beta-carotene, resulting in 28 µg/100 g for lutein, 16 µg/100 g for lycopene, 30 µg/100 g for neoxanthin 44 µg/100 g for violaxanthin, and 22 µg/100 g for zeaxanthin.

4.1.1.3 Comparison of HPLC and spectrophotometry

Using a preliminary dataset of 28 different food items, results from the spectrophotometric method (including a saponification step during extraction) were, on average, 2.3% above the HPLC results. Overall, there was a high and significant correlation for total carotenoid contents determined by both methods ($R = 0.96$, $p<0.001$) (Biehler *et al* 2010). Results for individual food items, however, varied, resulting in overestimation of carotenoid content up to 2.7 fold in the case of watermelon and underestimation in the case of banana by 35% as compared to HPLC. Since the spectrophotometric protocol was only used for screening purposes and does not allow for separation and quantification of single carotenoid species, the results in the following paragraph were exclusively obtained by HPLC analyses.

4.1.2 Carotenoid content in frequently consumed food items in Luxembourg

In total, 50 food items were analysed by HPLC-DAD, with carrots (18.0 ± 2.3 mg/100 g), fresh spinach (13.1± 1.4 mg/100 g) and deep-frozen, creamed spinach (11.1 ± 0.5 mg/100 g) yielding highest total carotenoid concentrations (Table 8). Regarding single carotenoid species, yellow bell peppers showed highest violaxanthin values (4.4 ± 0.9 mg/100 g), followed by spinach (2.8 ± 0.2 mg/100 g) and creamed spinach (2.5 ± 0.1 mg/100 g). Leek (1.0 ± 0.2 mg/100 g) and arugula (1.0 ± 0.3 mg/100 g) ranked highest in neoxanthin content, followed by lamb's lettuce (0.9 ± 0.3 mg/100 g). Carrots showed highest alpha- and beta-carotene (total carotenes: 17.9 ± 2.0 mg/100 g) concentrations, followed by carrot juice (4.9 ± 0.1 mg/100 g) and creamed spinach (4.3 ± 0.2 mg/100 g). Tomato ketchup (8.8 ± 0.4 mg/100 g), tomato juice (3.0 ± 0.2 mg/100 g) and tomatoes (2.7 ± 0.4 mg/100 g) contained highest lycopene concentrations. Spinach was the best source of lutein (4.9 ± 0.4 mg/100 g), ensued by arugula (3.6 ± 0.9 mg/100 g) and orange peppers (3.5 ± 0.4 mg/100 g), also being the best source of zeaxanthin (4.2 ± 0.7 mg/100 g). The second best zeaxanthin source was red peppers (1.5 ± 0.1 mg/100 g), followed by

spinach (0.5 ± 0.1 mg/100 g). Detailed carotenoid contents are presented in Table 8.

4.2 Carotenoid intake

Combining food consumption data [g/day] with the respective carotenoid content, the average carotenoid intake of the Luxembourgish population was estimated. Combining seven major carotenoids detected in local foods, total carotenoid intake per person and day was calculated as 12.9 mg. Main contributors to daily intake were carrots (5 mg total carotenoids/ day), green leafy vegetables (1.9 mg/day) and tomatoes (1.2 mg/day, Table 9). Considering single carotenoid species, alpha- and beta-carotene constituted 58.9% (7.6 mg) of daily intake followed by lycopene 14.0% (1.8 mg) and lutein 11.6% (1.5 mg). The epoxycarotenoids violaxanthin and neoxanthin together contributed to 13.2% of daily total carotenoid intake.

Table 8: Carotenoid concentrations in food items commonly consumed/produced in Luxembourg. Values are given as mean ± standard deviation (SD) in mg/100 g of edible portion. Food items are presented in alphabetical order.

#	Food item	Latin term	violaxanthin		neoxanthin		carotenes[§]		lycopene		lutein		zeaxanthin		total	
			mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
1	Apple (red)	*Pyrus malus*	0.10	0.04	0.15	0.03	0.04	0.01	- *	-	- *	-	- *	-	0.29	0.03
2	Apricot	*Prunus armenica*	- *	-	- *	-	2.41	0.43	0.32	0.04	-	-	-	-	2.73	0.47
3	Arugula	*Eruca sativa*	2.41	0.32	0.97	0.27	3.42	0.71	-	-	3.56	0.86	-	-	10.36	1.89
4	Aubergine	*Solanum melongena*	0.04	0.01	0.03	0.00	0.04	0.01	-	-	0.14	0.05	-	-	0.25	0.08
5	Banana	*Musa paradisia*	-	-	-	-	0.24	0.20	-	-	0.08	0.05	0.02	0.01	0.34	0.19
6	Butter		-	-	-	-	0.32	0.01	-	-	-	-	-	-	0.32	0.01
7	Bean (green)	*Phaseolus vulgaris*	0.63	0.01	0.07	0.01	0.80	0.02	-	-	0.67	0.03	0.02	0.00	2.19	0.05
8	Blackberry	*Rubus ulmifolius*	-	-	-	-	0.12	0.01			0.15	0.02	0.02	0.00	0.29	0.03
9	Broccoli	*Brassica oleracea*	-	-	-	-	0.18	0.01			0.02	0.00	-	-	0.20	0.01
10	Cabbage (kale)	*Brassica oleracea*	0.43	0.06	0.21	0.04	0.65	0.09			0.70	0.10	-	-	2.00	0.29
11	Carrot	*Daucus carota*	-	-	-	-	17.93	2.06	-	-	0.08	0.01	-	-	18.01	2.25
12	Carrot (juice)		-	-	-	-	4.88	0.09	-	-	0.20	0.00	-	-	5.08	0.11
13	Cheese (25% fat)		-	-	-	-	0.08	0.02	-	-	-	-	-	-	0.08	0.02
14	Cheese (32% fat)		-	-	-	-	0.08	0.01	-	-	-	-	-	-	0.08	0.01
15	Cherry	*Prunus avium*	0.30	0.03	-	-	0.03	0.01	-	-	-	-	-	-	0.33	0.05
16	Corn, sweet	*Zea mays*	-	-	-	-	- *	-	-	-	0.62	0.09	0.44	0.07	1.06	0.16
17	Courgette	*Curcubita pepo*	0.33	0.04	0.10	0.02	0.08	0.02	-	-	0.86	0.23	0.02	0.00	1.39	0.31
18	Eggs (whole)		-	-	-	-	-	-	-	-	0.63	0.11	0.05	0.01	0.69	0.09

#	Food item	Latin term	violaxanthin		neoxanthin		carotenes[§]		lycopene		lutein		zeaxanthin		total	
			mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
19	Endive (curly)	*Cichorium endivia*	0.43	0.09	0.61	0.16	0.71	0.06	-	-	0.22	0.04	0.02	0.01	1.99	0.36
20	Grapefruit (pink)	*Citrus paradisi*	-	-	-	-	1.72	0.14	2.55	0.32	0.08	0.01	0.19	0.04	4.53	0.48
21	Grapefruit (juice)		-	-	-	-	0.63	0.07	0.94	0.13	0.03	0.01	0.07	0.02	1.67	0.22
22	Grapes (green)	*Vitis vinifera*	0.04	0.00	0.03	0.00	0.05	0.01	-	-	0.03	0.01	-	-	0.15	0.02
23	Lamb's lettuce	*Valerianella locusta*	1.41	0.08	0.86	0.06	2.49	0.34	-	-	2.94	0.17	-	-	7.70	0.60
24	Leek	*Allium ampeloprasum*	0.70	0.13	0.99	0.24	-	-	-	-	0.36	0.06	-	-	2.04	0.45
25	Lentil, green, dry	*Lens culinaris*	0.05	0.02	0.04	0.01	0.02	0.00	-	-	1.20	0.16	0.32	0.05	1.63	0.22
26	Lentil, white, dry	*Lens culinaris*	0.04	0.01	0.03	0.00	0.03	0.00	-	-	1.06	0.06	0.29	0.03	1.45	0.09
27	Lettuce	*Lactuca sativa*	0.36	0.12	0.08	0.04	0.72	0.16	-	-	0.66	0.18	-	-	1.82	0.63
28	Margerine	*80 % fat*	-	-	-	-	0.60	0.03	-	-	0.05	0.01	0.06	0.02	0.71	0.05
29	Margerine (light)	*40 % fat*	-	-	-	-	0.51	0.01	-	-	0.04	0.02	0.03	0.03	0.58	0.04
30	Melon (orange)	*Cucumis melo*	-	-	-	-	3.21	0.59	-	-	0.03	0.00	-	-	3.24	0.62
31	Orange	*Citrus reticulata*	0.04	0.04	-	-	-	-	-	-	-	-	-	-	0.04	0.04
32	Orange (mandarin)	*Citrus sinensis*	-	-	-	-	0.17	0.02	-	-	-	-	-	-	0.17	0.27
33	Oil (olive)	*(extra vierge)*	-	-	-	-	0.14	0.01	-	-	0.16	0.02	-	-	0.29	0.02
34	Peach	*Prunus persica*	1.16	0.06	-	-	0.09	0.00	-	-	-	-	-	-	1.24	0.05
35	Pear	*Pyrus communis*	-	-	-	-	0.01	0.00	-	-	0.05	0.02	-	-	0.06	0.02
36	Peas (garden)	*Pisum sativum*	0.37	0.05	0.52	0.08	0.91	0.04	-*	-	1.77	0.06	0.03	0.01	3.59	0.11
37	Pepper (green)	*Capsicum annuum*	0.46	0.01	0.50	0.06	0.46	0.01	-	-	0.37	0.02	0.36	0.02	2.15	0.06
38	Pepper (orange)	*Capsicum annuum*	0.25	0.01	-	-	0.78	0.10	-	-	3.52	0.36	4.21	0.72	8.76	1.68

#	Food item	Latin term	violaxanthin		neoxanthin		carotenes[§]		lycopene		lutein		zeaxanthin		total	
			mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
39	Pepper (red)	*Capsicum annuum*	0.58	0.18	-	-	3.65	0.78	-	-	0.51	0.01	1.52	0.04	6.25	1.26
40	Pepper (yellow)	*Capsicum annuum*	4.16	0.90	-	-	-	-	-	-	2.22	0.62	0.03	0.01	6.41	1.82
41	Plum	*Prunus domestica*	0.05	0.01	0.11	0.02	0.03	0.00	-	-	0.03	0.01	-	-	0.23	0.04
42	Potato (‘parisienne’)	*Solanum tuberosum*	0.29	0.00	0.24	0.01	-	-	-	-	0.13	0.00	-	-	0.67	0.01
43	Potato (‘victoria’)	*Solanum tuberosum*	0.24	0.01	0.17	0.02	-	-	-	-	0.10	0.01	-	-	0.51	0.03
44	Sour cream		-	-	-	-	0.03	0.00	-	-	-	-	-	-	0.03	0.00
45	Spinach	*Spinacia oleracea*	2.76	0.24	0.84	0.14	4.22	0.66	-	-	4.87	0.39	0.45	0.02	13.13	1.37
46	Spinach (creamed)		2.45	0.05	0.60	0.05	4.26	0.20	-	-	3.44	0.20	0.33	0.02	11.08	0.50
47	Tomato	*Solanum lycopersicum*	-	-	-	-	0.52	0.11	2.72	0.36	-	-	-	-	3.24	0.58
48	Tomato (juice)		-	-	-	-	0.13	0.00	3.04	0.22	-	-	-	-	3.17	0.26
49	Tomato (ketchup)		-	-	-	-	0.17	0.02	8.77	0.44	-	-	-	-	8.94	0.83
50	Watermelon	*Citrullus lanatus*	-	-	-	-	0.88	0.05	0.31	0.02	-	-	-	-	1.19	0.15

[§]carotenes = alpha- and beta-carotene combined

* not detected (LOD for beta-carotene 12 µg/100 g; zeaxanthin 22 µg/100 g; lutein 28 µg/100 g, violaxanthin 44 µg/100g, neoxanthin 30 µg/100 g, lycopene 16 µg/100 g)

Table 9: Estimated carotenoid intake in Luxembourg. Food intake [g/day] from Luxembourgish food groups was combined with average carotenoid concentrations in the consumed food group to yield carotenoid intake [mg/day].

rank	food items	Mean (g/day)[1]	SEM[1]	carotenes[2]	lycopene	lutein	zeaxanthin	violaxanthin	neoxanthin	total
1	carrots (fresh and processed)	27.5	1.0	4.932	-[3]	0.022	-[3]	-[3]	-[1]	4.955
2	green-leafy vegetables (spinach, endive, ..)	25.6	1.2	0.603	-	0.616	0.057	0.391	0.184	1.850
3	tomatoes (fresh and processed)	36.6	1.0	0.191	0.994	-[3]	-	-	-	1.185
4	bell peppers (fresh and processed)	18.3	1.4	0.223	-	0.161	0.186	0.223	0.021	0.815
5	pear, apple, pineapple, melons	90.3	3.8	0.485	0.014	0.021	0.004	0.056	0.079	0.659
6	fruit juices (canned, bottled, tetra-packed)	114.1	5.0	0.240	0.358	0.011	0.026	-	-	0.635
7	salads	21.1	0.6	0.193	-	0.182	-	0.104	0.032	0.511
8	potatoes, total	65.8	1.7	-[3]	-	0.077	0.001	0.175	0.137	0.391
9	aubergine, cucumber, leek, squash, green beans, peas	39.9	1.3	0.074	-	0.162	0.002	0.134	-	0.371
10	vegetable juice	6.9	0.9	0.172	0.104	0.007	-	-	-	0.284
11	citrus fruits	82.4	4.6	0.135	0.143	0.004	-	-	-	0.283
12	ketchup	1.9	0.2	0.033	0.170	-	-	-	-	0.203
13	beans, lentils, peas (chick), sweet corn	15.0	0.7	0.038	-	0.101	0.014	0.024	0.014	0.191
14	plum, grape, nectarine, cherry, peach, apricot	22.0	1.4	0.093	0.012	0.001	-	0.042	-	0.148
15	banana	37.6	1.7	0.091	-	0.031	0.006	-	-	0.128
16	cabbage, average	18.9	0.7	0.037	-	0.025	0.001	0.023	0.022	0.108
17	eggs	13.4	0.6	-	-	0.085	0.007	-	-	0.092
18	butter, margarine, crème fraîche	14.1	0.9	0.054	-	0.005	0.003	-	-	0.061
19	olive oil	11.9	0.4	0.016	-	0.019	-	-	-	0.035
20	cheese total	21.5	1.3	0.017	-	0.002	-	-	-	0.019
	Total ∑ :	**684.6**		**7.6**	**1.8**	**1.5**	**0.3**	**1.2**	**0.5**	**12.9**

[1] consumption data provided by the Luxembourgish ORISCAV-LUX study (Alkerwi *et al.*, 2010); mean = mean intake; SEM = standard error of the mean

[2] carotenes = alpha- and beta-carotene;

[3] carotenoid content of the food group below limit of detection (see chapter 'Limit of detection/quantification, repeatability/reproducibility', Result section)

4.3 Gastro-intestinal digestion: carotenoid micellarisation

The determination of carotenoid intake is only the first step when aiming to investigate total amount of ingested carotenoid and is potentially available to the human body. The so called bioavailability, that is the fraction of a compound that is taken up, reaches the systemic circulation and is available for various physiological functions, is - in the case of carotenoids – largely if not mainly determined by solubility (micellarisation) in the gut and uptake by the intestinal mucosa. The first of the above mentioned processes was investigated by the means of an *in vitro* digestion model.

4.3.1 Validation and optimisation of the *in vitro* digestion model

Spinach was chosen as a basic test meal due to its high nutritional significance, its abundance of carotenoids and due to its broader carotenoid spectrum as opposed to, e.g. carrots. Pilot studies were undertaken in order to test basic parameters in the *in vitro* model. For example, the repeatability was increased when 50 ml centrifuge tubes were put into a plastic bag longitudinal to shaking direction in the water bath. Additionally, a homogenisation step (shaking the 50 ml tubes vigorously for 5 seconds), after the small intestinal phase and before taking aliquots for carotenoid analysis, further minimised variations.

In further preliminary trials, different fat-containing additives, such as cow and soya milk, sausage and a liquid meal substitute were tested for their impact on carotenoid release and solubility. Additionally, in order to validate and optimize different parameters, different enzyme/bile salt concentrations were tested, starting with concentrations as described by Garrett and co-workers (1999).

4.3.1.1 Carotenoid availability in dependency of various test meal compositions

The addition of condensed milk (4% fat) to spinach resulted in highest fractional (total) carotenoid micellarisation in combination with highest reproducibility (Figure 11) and was hence consequently chosen for further experiments. Supplementing milk containing less fat (1.5 %), liver sausage or soya milk resulted in significantly reduced fractional carotenoid micellarisation ($p<0.001$, Bonferroni, Figure 11). Lowest total micellarisation was obtained with the addition of 2 g of the Fresubin® drink (7.1 ±

0.7%). In additional trials, the relative low total carotenoid micellarisation of 17.9% for spinach + condensed milk was substantially increased to over 30% by an additional possessing step (spinach was blended for 5 min in a household blender), highlighting the importance of a homogenisation step not unlikely as occurring during mechanical chewing. Results obtained for total beta-carotene, (9Z)-beta-carotene, lutein and zeaxanthin showed the same pattern as compared to total carotenoids (Figure 11, only total carotenoid micellarisation displayed).

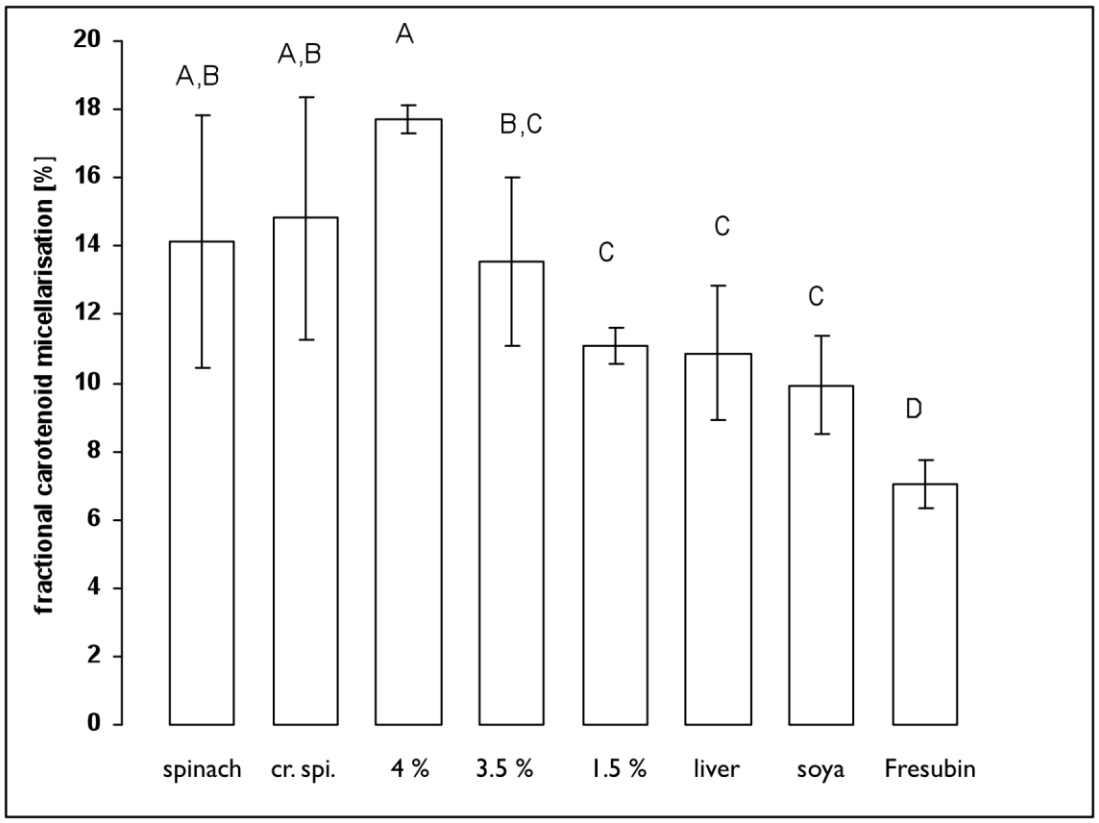

Figure 11: Impact of various test meal compositions on absolute carotenoid micellarisation. Letters indicate levels of statistical significance; bars not sharing a common letter are significantly different, A>B>C>D ($p<0.05$, Bonferroni post-hoc test); n = 3 for each group.

spinach = 4 g pure spinach
cr. spi. = 4 g cream spinach
4 % = 4 g spinach + 2 g condensed milk (4% fat)
3.5 % = 4 g spinach + 2 g whole milk (3.5% fat)
1.5 % = 4 g spinach + 2 g skimmed milk (1.5% fat)
liver = 4 g spinach + 0.5 g liver sausage (32% fat)
soya = 4 g spinach + 2 g soya milk (2% fat)
Fresubin = 4 g of spinach + 2 g of Fresubin (3.4 % fat)

4.3.1.2 Impact of the presence of enzymes and bile

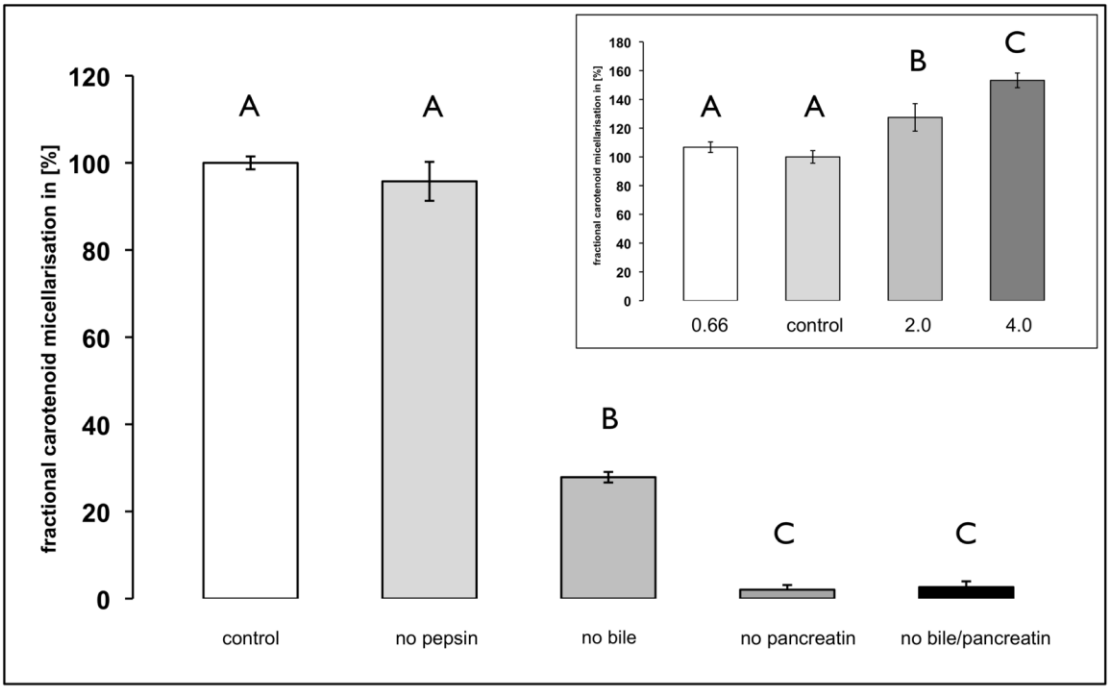

Figure 12: Impact of different concentrations of bile salts and digestive enzymes on carotenoid micellarisation.

A series of experiments was conducted, omitting single digestive enzymes and/or bile salts, while all other basic conditions, such as test meal, pH, incubation time, shaking speed and temperature were kept constant. The omission of pepsin during gastric phase did only slightly, but not significantly decrease carotenoid micellarisation (main panel), while the omission of bile salts, pancreatin, or both resulted in drastic reductions. Control samples (set as 100%) contained 4 g of spinach and 2 g of condensed milk (4 % fat) as a basic meal. The digestion protocols for 'no pepsin', 'no bile', 'no pancreatin' and 'no pancreatin/bile' were similar to the control protocol, without adding the indicated substance into the tubes. The upper right panel displays the fractional carotenoid micellarisation relative to the control (set as 100%). For these experiments, the amount of test meal was varied, yielding different 'matrix: enzyme' ratios (6 g of spinach and 3 g of milk, i.e. 0.66 x enzyme concentration to 1 g of spinach and 0.5 g of milk, i.e. 4.0 x enzyme concentration compared to the control). Different letters (A>B>C) indicate levels of statistical significance – bars not sharing a common letter are significantly different, $p<0.001$, Bonferroni post-hoc test; n = 4 for each group. Values are presented for total carotenoids - similar trends were also observed for single carotenoid species.

A series of experiments was conducted, omitting single digestive enzymes or bile salts, while all other basic conditions, such as pH, incubation time, shaking speed and temperature were kept constant. The omission of pepsin during gastric phase did only slightly, but not significantly decrease micellarisation (Figure 12), while the omission of bile salts, pancreatin, or both resulted in drastical reductions of carotenoid micellarisation below 5% of the control. Results obtained for total beta-carotene, cis-beta-carotene, lutein and zeaxanthin showed the same pattern as compared to total

carotenoids. Significant increases in the fractional micellarisation of total carotenoids were also found with increasing enzyme concentrations (Figure 12, insert), up to 50% increase at 4.0 x enzyme concentration used by Garrett et al. (1999). In further trials, saturation of fractional carotenoid micellarisation according to increased enzyme and bile salt concentrations was determined and these concentrations were consequently chosen as standard conditions.

4.3.2 Impact of potential enhancers and inhibitors on carotenoid micellarisation

4.3.2.1 Validation

The repeatability for the micellarisation experiments was determined for the final conditions, yielding 6.8% (RSD) for 5 independent samples (total carotenoids) treated in parallel under control conditions. Systematic effects could not be differentiated. The reproducibility was assessed while taking into account samples under control conditions on 6 different days of analyses (16.1% RSD) (total carotenoids). In order to decrease day-to-day variances, values were consequently expressed as percent of the control.

4.3.2.2 Carotenoid content of spinach (test meal) and micelles

The carotenoid content in spinach used for micellarisation experiments is presented in Table 10, with concentrations being highest for beta-carotene followed by lutein and violaxanthin. The major Z-isomer of beta-carotene was tentatively identified as the (9Z)-isomer by spectrophotometry (λ_{max}: 339/423(shoulder)/447/472 nm), according to Britton and co-authors (2004) and Thakkar et al. (2007). Carotenoid precursors, such as phytoene and phytofluene, were not detected in the test meal.

Fractional carotenoid micellarisation was substantially higher for xanthophylls as compared to beta-carotene (61.9% vs. 2.6%, respectively). The (9Z)-beta-carotene isomer (5.1%) was slightly better micellarised than the corresponding all-trans form (2.6%). Fractional micellarisation of violaxanthin and neoxanthin digestion products was 48.5% and 30.1% (Table 10), respectively.

Table 10: Carotenoid content in spinach, gastro-intestinal solutions (prior to digestion), micelles and Caco-2 cells under control conditions.

Carotenoid species	Carotenoids in spinach [mg/100 g]	Carotenoids in GI-solution prior to digestion [ng/ml][3,4]	Carotenoids in mixed micelles [ng/ml]	Carotenoids in mixed micelle fraction [%]
total carotenoids	22.8 ± 1.9	18,747 ± 857.8	6,398.4 ± 394.0	34.1
(all-E)-ß-carotene	6.2 ± 0.7	5,204 ± 190.0	133.3 ± 11.8	2.6
(9Z)-ß-carotene[1]	0.9 ± 0.1	763 ± 11.0	39.0 ± 0.5	5.1
lutein	6.0 ± 0.6	5,027 ± 244.9	3110.0 ± 232.8	61.9
violaxanthin[2]	5.3 ± 0.2	4,266 ± 146.5	2067.0 ± 167.5	48.5
neoxanthin[2]	4.4 ± 0.3	3,487 ± 302.6	1049.1 ± 81.8	30.1

[1] tentatively identified based on retention times, spectrum, and literature (Thakkar *et al.*, 2007)

[2] during simulated gastro-intestinal digestion (gastric phase: pH 2), violaxanthin was converted to auroxanthin and luteoxanthin epimers; and neoxanthin to neochrome, respectively. In micelles and cells, only these conversion products were detected. Auroxanthin and luteoxanthin concentrations were summed up and compared to the parental concentration of violaxanthin.

[3] calculated from original spinach content of the test meal; total digestion volume was 50 mL

[4] GI = gastro-intestinal

4.3.2.3 Impact of lipophilic dietary compounds

The addition of low amounts of dietary lipophilic compounds did not result in significantly altered fractional micellarisation (Biehler *et al* 2011). Only cholesterol at highest concentrations (114 mg blended into the test meal) significantly reduced total carotenoids to 61.3%, compared to the control ($p<0.001$, Dunnett's). Lutein and zeaxanthin fractional micellarisation was also diminished to 57.0% and 58.0%, respectively, while (9Z)-beta-carotene and total beta-carotene were not impacted. Table 11 displays carotenoid micellarisation at highest inhibitor/enhancer concentrations. Although similar in trend compared to cholesterol, the addition of stigmasterol led only to a slight, but non-significant decreased micellarisation (total carotenoids) of approx. 15% compared to the control. Similar trends were obtained for single carotenoid species. α-tocopherol in contrast, employing concentrations of physiological relevance, slightly but not significantly increased carotenoid micellarisation.

Table 11: Micellarisation of individual carotenoids following *in vitro* digestion experiments conducted in the presence of potential inhibitors at different concentrations (cholesterol, stigmasterol and α-tocopherol).

		micellarisation[3]									
Added food compound	amount [mg][4]	total carotenoids %		(9Z)-beta carotene %		total beta-carotene %		lutein %		zeaxanthin %	
control[2]	0	100.0	1.8	100.0	8.3	100.0	8.3	100.0	2.6	100.0	6.5
cholesterol	20[1]	99.0	4.8	130.4	10.0	129.3	8.8	94.2	4.2	89.5	2.8
cholesterol	114[1]	61.3*	2.9	102.7	5.5	89.6	7.7	57.0*	3.1	58.0*	8.6
stigmasterol	108	85.5	10.6	100.7	11.7	89.8	11.9	84.5	10.6	85.1	7.6
α-tocopherol	1[1]	111.9	5.2	134.8	25.4	133.7	20.0	108.6	5.3	125.7	7.9

[1] lower concentrations were also tested, but only results of highest concentrations are displayed
[2] control = no inhibitor
[3] results are given as the mean micellarisation in percent of the control (standard test-meal) ± SD (n=4, control n=14). A detailed procedure of the addition process is presented in the method section.
[4] amount per 50 ml test meal
* significantly different from the control ($p<0.001$, Dunnett's, 2-sided).

4.3.2.4 Different concentrations of sodium

The impact of sodium chloride concentration on micellarisation efficiency was investigated. Adding high 280 mM and very high 530 mM concentrated sodium chloride solutions significantly increased the micellarisation of (9Z)-beta-carotene and total beta-carotene compared to the control (150 mM NaCl). In contrast, both xanthophylls were less efficiently micellarised compared to the control, with lutein being slightly, but not significantly stronger affected than zeaxanthin (Figure 13). Total carotenoid micellarisation was also diminished, however, only significantly at 280 mM NaCl final concentration.

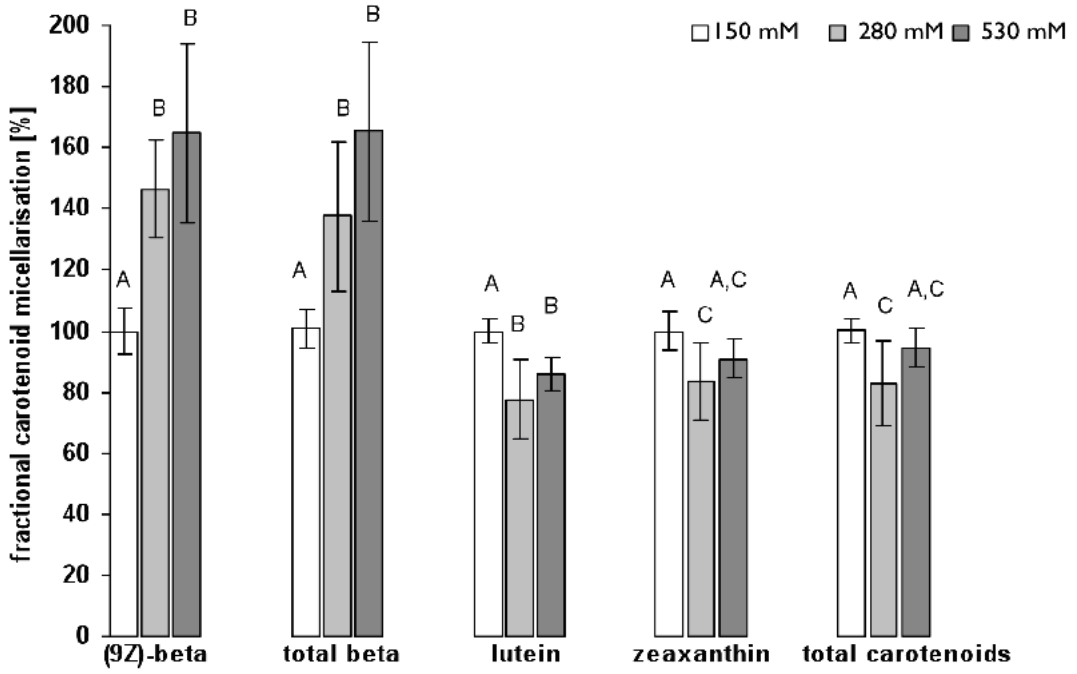

Figure 13: Fractional carotenoid micellarisation is differently affected by increasing sodium chloride concentrations (control = 150 mM, high sodium chloride = 280 mM and very high sodium chloride = 530 mM). Total beta = total beta-carotene, (9Z)-beta = (9Z)-beta-carotene. Letters indicate levels of statistical significance; bars without a common letter significantly differ (A compared to B, $p<0.005$ Bonferroni, B compared to C, $p<0.05$ Bonferroni (2-sided)). Bars indicate mean +/- SD; n = 8 for each of the three groups.

4.3.2.5 Impact of divalent ions on carotenoid micellarisation

After having detected an impact of sodium chloride on carotenoid solubility, divalent ions were additionally investigated. Carotenoid solubility in mixed micelles was compromised in the presence of divalent salts present during simulated gastro-intestinal digestion with the strength of effects following the order $Fe^{2+}>Zn^{2+}>Ca^{2+}>Mg^{2+}$ (Figures 14 and 16, panels A-D, details of test meal composition in Table 7, Material and Methods). Magnesium had the weakest inhibitory effect of the different ions investigated, even slightly increasing total carotenoid micellarisation at 7.5 mM, while the effect of iron, on average, was strongest. Regarding individual carotenoid species, beta-carotene reduction compared to control was more pronounced than the attenuation of lutein micellarisation.

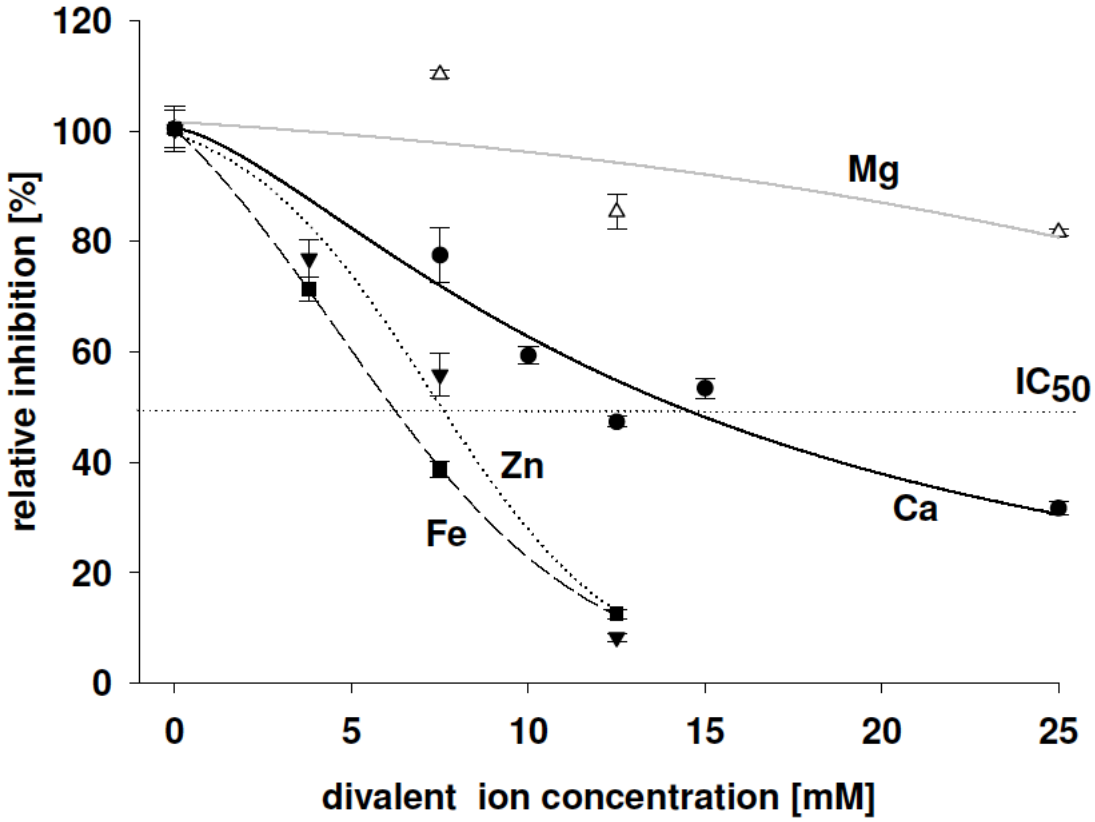

Figure 14: Micellarisation inhibition curves. The image displays concentration dependent attenuation of total carotenoid micellarisation in the presence of divalent ions. Ca = calcium chloride (full circles, black line), Fe = iron(II)chloride (full squares + black dashed line), Mg = magnesium chloride (empty triangles, grey line), Zn = zinc sulphate (full triangles + grey dotted line); concentration range 0 – 25 mM; Values = mean ± SD (n=3). The dotted horizontal line indicates the 50% inhibition threshold (IC_{50}).

The mean inhibitory concentration to reduce total carotenoid micellarisation to 50% [IC_{50}] was calculated as 4.6 ± 0.6 mM for Fe^{2+} ($R = 0.99$, $p<0.001$), 7.2 ± 0.4 mM for Zn^{2+} ($R = 0.98$, $p<0.001$), 14.2 ± 0.5 mM for Ca^{2+} ($R = 0.98$, $p<0.001$) and 41.2 ± 10.7 mM for Mg^{2+} ($R = 0.73$, $p<0.005$), respectively (Figure 14).

In an additional trial, the impact of bile salt concentration on the micellarisation inhibition of calcium and magnesium was tested. At lower bile concentration of 2.2 mg/mL (50% of the concentration in the above experiments), the inhibition effect of the divalent ions was substantially stronger, especially for magnesium. [IC_{50}] values in the presence of the 50% reduced bile salt concentration were 12.1 mM for Ca^{2+} ($R = 0.96$, $p<0.001$) and 14.7 for Mg^{2+} ($R = 0.95$, $p<0.001$).

4.4 Carotenoid uptake in Caco-2 intestinal cells

In addition to carotenoid micellarisation, carotenoid uptake by the intestinal mucosa is considered as a major factor for carotenoid bioavailability. With the help of the human Caco-2 cell line, uptake processes and factors impacting these processes were studied.

4.4.1 Repeatability/reproducibility

The repeatability of total carotenoid uptake for the cell studies was calculated at 15.4% (RSD) for 8 independent samples treated in parallel under control conditions, yielding similar results compared to the reproducibility that was assessed for control samples on 4 different days of analyses (15.8% RSD). Results were always expressed as percent of the control in order to increase comparability between micellarisation and uptake studies and cells of 3 wells were pooled to increase sensitivity. Conditions for cellular uptake experiments are described in chapters 3.10-3.12.

Table 12: Carotenoid content in spinach and Caco-2 cells under control conditions after 4 hours of incubation with digesta:DMEM mixture (1:1, v/v) [1,2].

Carotenoid species	Carotenoids in spinach [mg/100 g]	Carotenoids in cells [ng/mg cell protein][1,2]
total carotenoids	22.8 ± 1.9	77.2 ± 9.5
(all-E)-ß-carotene	6.2 ± 0.7	1.2 ± 0.2
(9Z)-ß-carotene	0.9 ± 0.1	n.d.[4]
lutein	6.0 ± 0.6	23.6 ± 5.1
violaxanthin[3]	5.3 ± 0.2	19.1 ± 2.2
neoxanthin[3]	4.4 ± 0.3	33.3 ± 4.5

[1] Prior to incubation, no carotenoids were detected/present in the cells

[2] For cell studies, digesta was diluted with DMEM (1:4, v/v)

[3] during simulated gastro-intestinal digestion (gastric phase: pH 2), violaxanthin was converted to auroxanthin and luteoxanthin epimers; and neoxanthin to neochrome, respectively. In micelles and cells, only these conversion products were detected. Auroxanthin and luteoxanthin concentrations were summed up and compared to the parental concentration of violaxanthin.

[4] n.d. = below limit of detection

A typical chromatogram of cell extracts incubated for 4 hours with spinach-derived digesta is shown in Figure 16. Area under curves for cis- and trans- isomers were combined and final concentrations were determined via external calibration curves (details in chapter 3.5.3). Under control conditions (no divalent ions added), total cellular carotenoid concentration was 77.2 ± 9.5 ng/mg cell protein (Table 12). Regarding single carotenoid species, all-trans-beta-carotene showed lowest concentrations (1.2 ± 0.2 ng/mg cell protein), with the (9Z)-isomer being under the limit of detection. Lutein concentration was substantially higher (23.6 ± 5.1 ng /mg), closer to the violaxanthin and neoxanthin digestion products (19.1 ± 2.2 ng/mg and 33.3 ± 4.5 ng/mg, respectively). Chlorophyll-degradation products (tentatively identified by comparison with literature (Ferruzzi *et al.*, 2001)), were also taken up into the intestinal cells, but concentration was not determined.

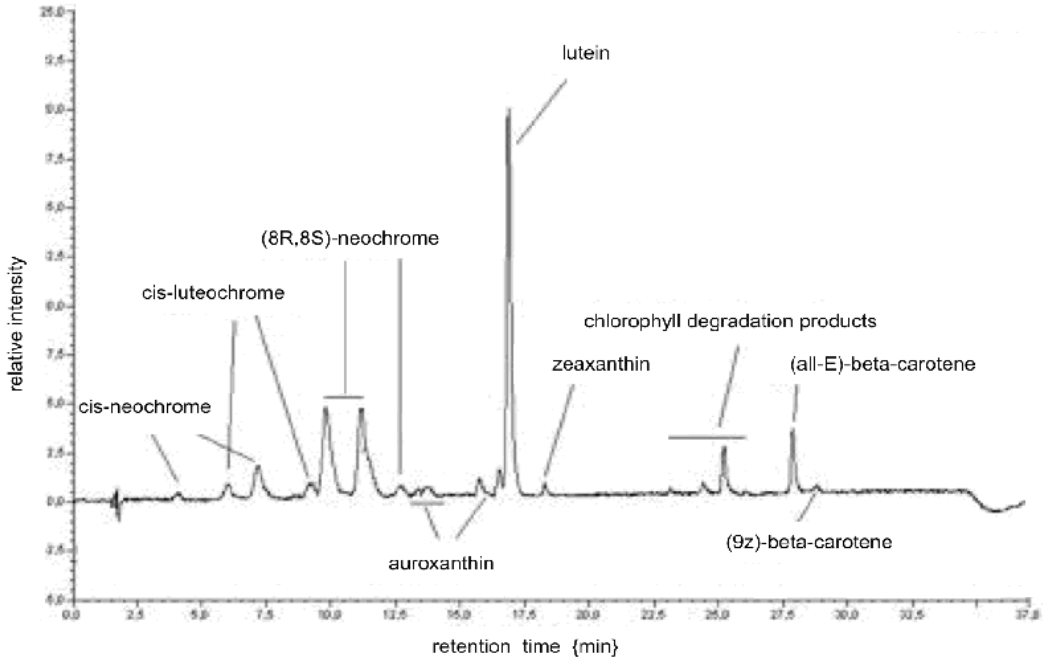

Figure 15: Typical HPLC chromatogram (details in Material and Methods) of a cell extract after 4 hours of incubation with micellarised carotenoids from a spinach-based test meal. HPLC separation protocol was shortened from 44 min to 37 min because samples did not contain lycopene (retention time 39 min). Carotenoids were detected based on their retention times, absorption spectra and comparison to (digested) carotenoid standards. Cis-carotenoid spectra usually show a hypsochromic shift of 5-6 nm compared to the all-trans-form and a less defined fine spectrum (Britton *et al* 2004). Carotenoids were not detected in cells incubated with cell medium only.

4.4.2 Impact of divalent ions on carotenoid cellular uptake

The presence of divalent ions did not only influence carotenoid micellarisation, they additionally modified overall carotenoid uptake (in % of the original spinach meal; chapter 3.1.13.1) into Caco-2/TC7-cells (Figure 17) compared to cells incubated with 'control digesta'. For all minerals at maximum concentration tested (12.5 – 25 mM), lutein and total carotenoid uptake into Caco-2 cells from test meals was, compared to the control, significantly reduced ($p<0.001$ for Ca^{2+}, Fe^{2+}, Zn^{2+} and $p<0.01$ for Mg^{2+}, Bonferroni), equally or even more pronounced compared to the respective reduction of their fractional micellarisation (Figure 16). Results from Fe^{2+} and Zn^{2+} at lower concentrations showed similar trends. In contrast to lutein, beta-carotene uptake was not significantly reduced compared to the control at highest mineral concentrations for all minerals except for Mg^{2+}, showing a significant reduction at 25 mM ($p<0.001$, Bonferroni).

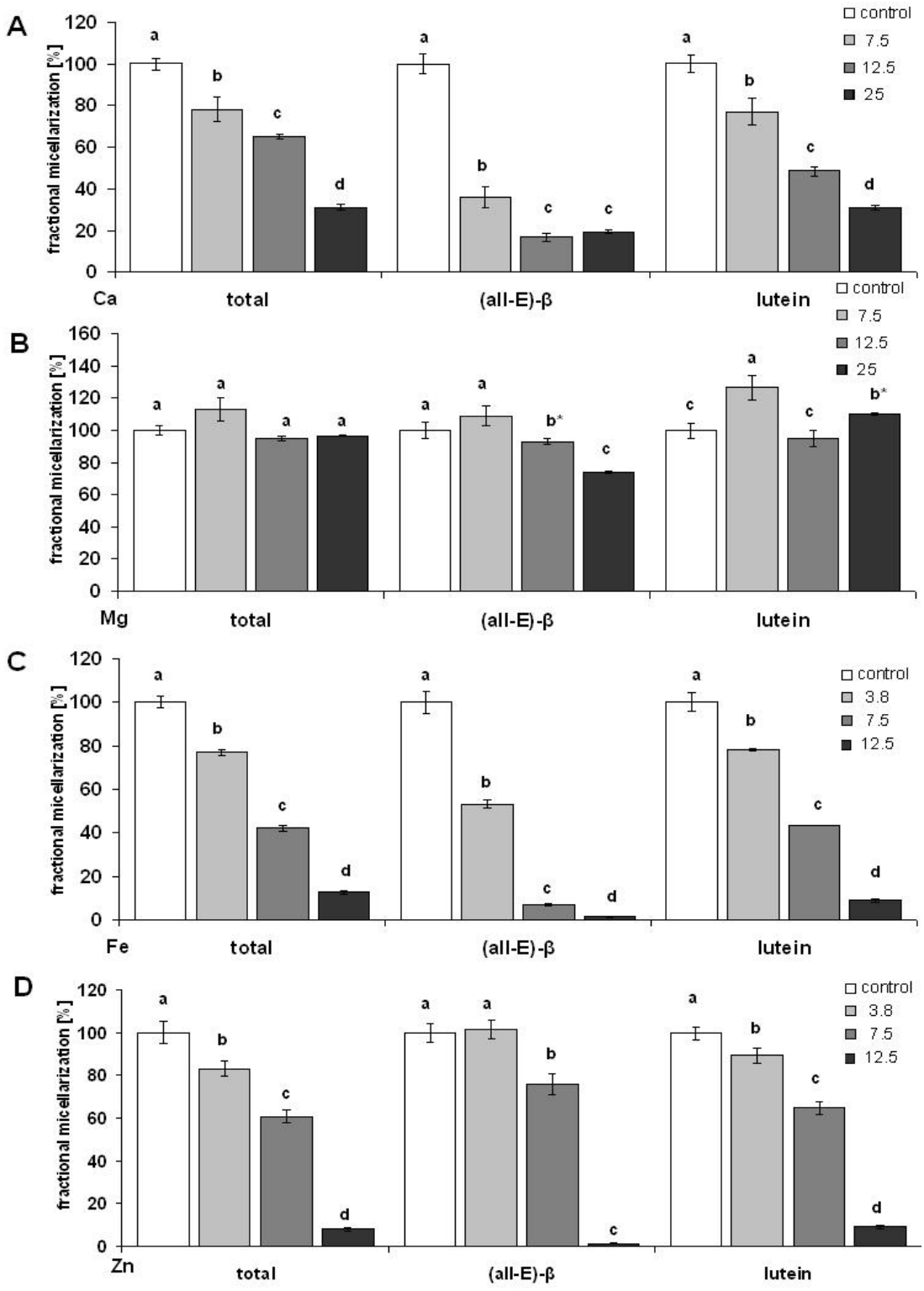

Figure 16: Carotenoid micellarisation was affected by the presence of divalent ions. Panels A-D show fractional carotenoid micellarisation in [%] of the control (no divalent ions added), in the presence of calcium (A), magnesium (B), iron (C) and zinc (D). Empty bar: control (no divalent ions added), light grey bar: 3.8 mM (Fe, Zn) and 7.5 mM (Ca, Mg), dark grey bar: 7.5 mM (Fe, Zn) and 12.5 mM (Ca, Mg) and full bar: 12.5 mM (Fe, Zn) and 25 mM (Ca, Zn). Bars referring to one category tested (beta-carotene (all-E- β), lutein and total carotenoids) not sharing the same letter are significantly different (a>b>c>d; $p<0.001$, Bonferroni post-hoc test; a>b*, $p<0.05$). Bars = mean ± SD, n=3.

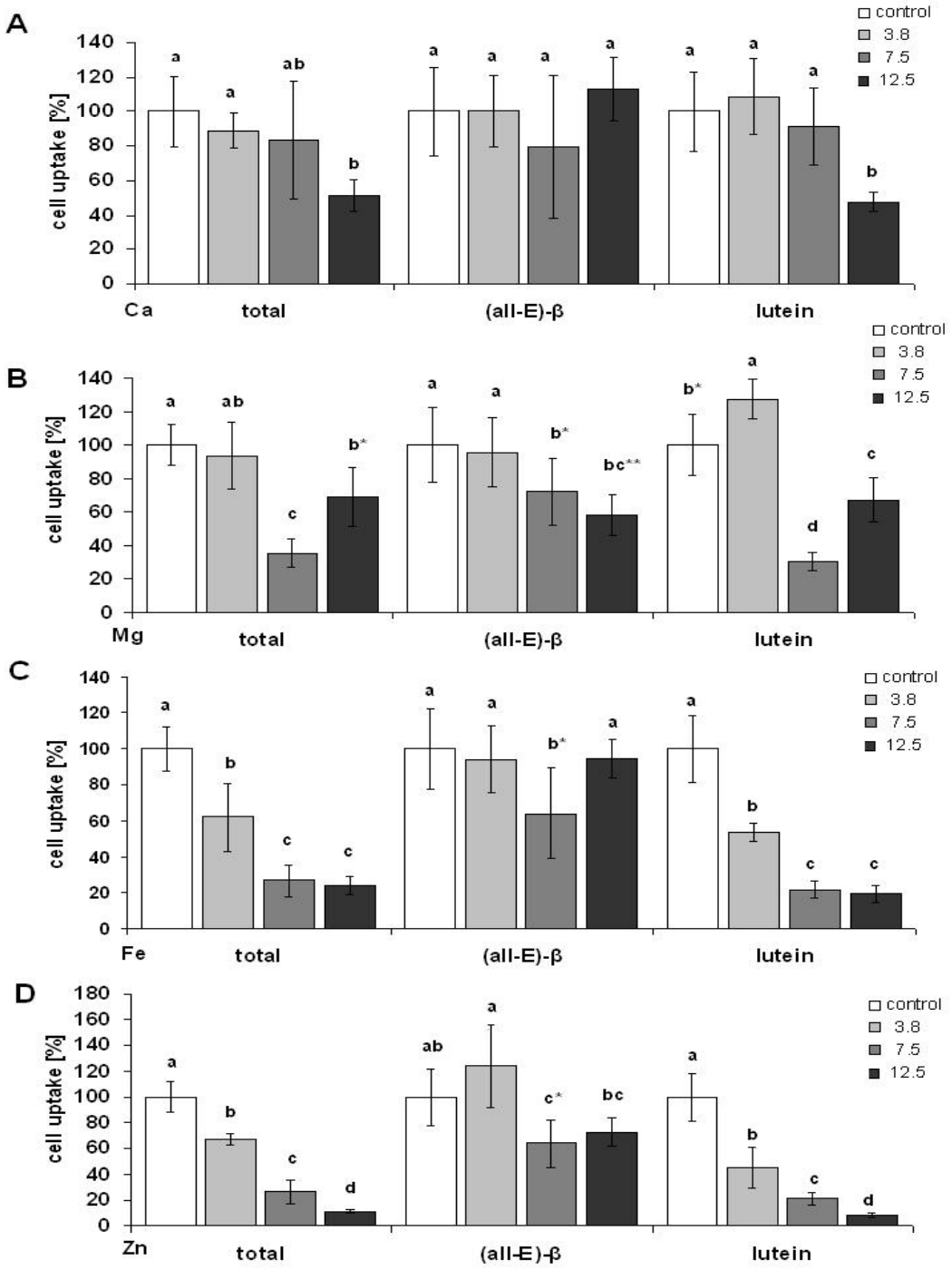

Figure 17: Carotenoid cellular uptake ('total uptake' compared to the original spinach content) was affected by the presence of divalent ions. Panels A-D show fractional carotenoid cellular uptake in [%] of the control (no divalent ions added), in the presence of calcium (A), magnesium (B), iron (C) and zinc (D). Empty bar: control (no divalent ions added), light grey bar: 3.8 mM (Fe, Zn) and 7.5 mM (Ca, Mg), dark grey bar: 7.5 mM (Fe, Zn) and 12.5 mM (Ca, Mg) and full bar: 12.5 mM (Fe, Zn) and 25 mM (Ca, Zn). Bars referring to one category tested (beta-carotene (all-E- β), lutein and total carotenoids) not sharing the same letter are significantly different (a>b>c>d; $p<0.001$, Bonferroni post-hoc test; a>b* and b>c*, $p<0.05$). Bars = mean ± SD (n=6-10).

Increasing divalent ion concentration present during digestion resulted in decreased carotenoid micellar concentrations. When incubating Caco-2 cells with digesta containing increasing amounts of divalent ions, lutein cellular uptake was reduced with decreasing lutein digesta content added on the cells, while beta-carotene cellular uptake was not or only slightly diminished, independently from the digesta (micellar) content (Figures 17 and 18). Compared to lutein (Figure 18, insert), beta-carotene (Figure 18) cellular uptake was saturated at 120-times lower concentrations (approx. 5 ng/mL digesta:DMEM vs. 600 ng/mL digesta:DMEM).

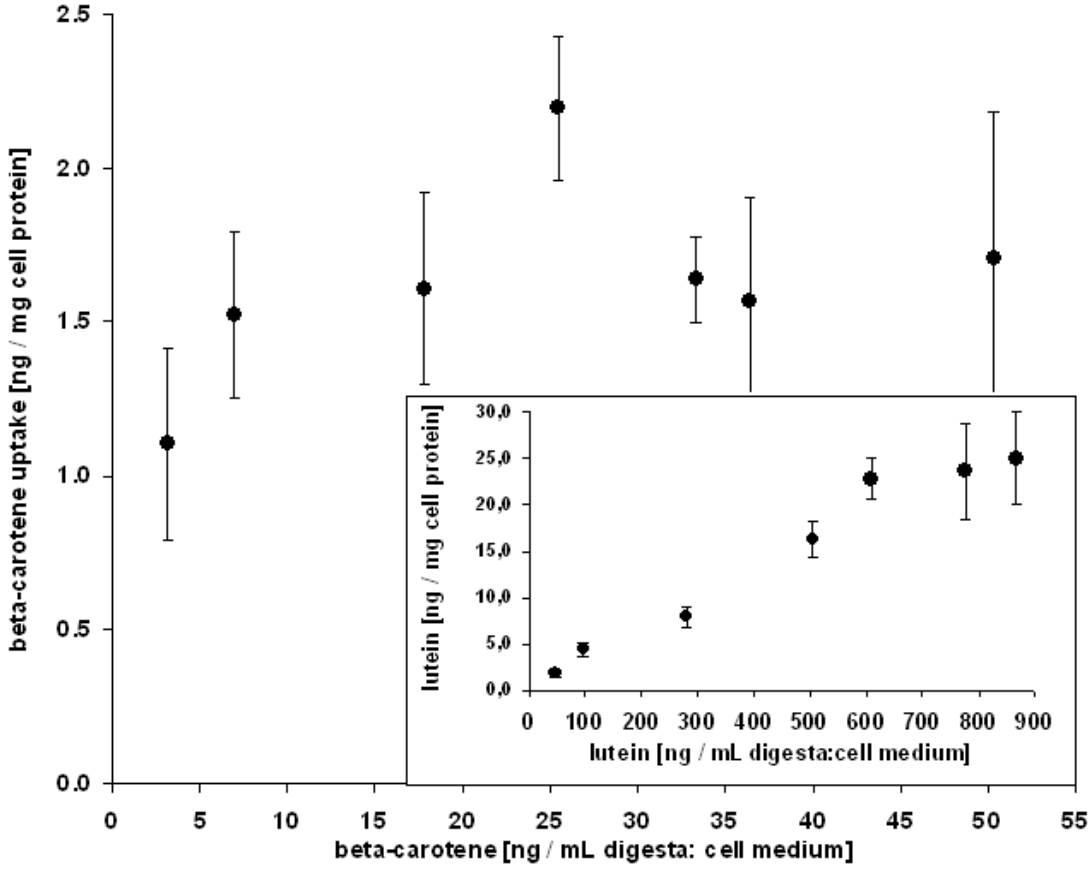

Figure 18: Early saturation of beta-carotene cellular uptake. Beta-carotene concentrations [ng/mg cell protein] in Caco-2 cells after 4 hours of incubation with digesta:DMEM mixture [1:4, v:v]. Different beta-carotene concentrations during simulated digestion resulting from the presence of iron or zinc ions (0 – 12.5 mM). The insert panel shows lutein concentrations under similar conditions. Results from iron and zinc experiments are presented as one set of data in order to increase the number of data points.

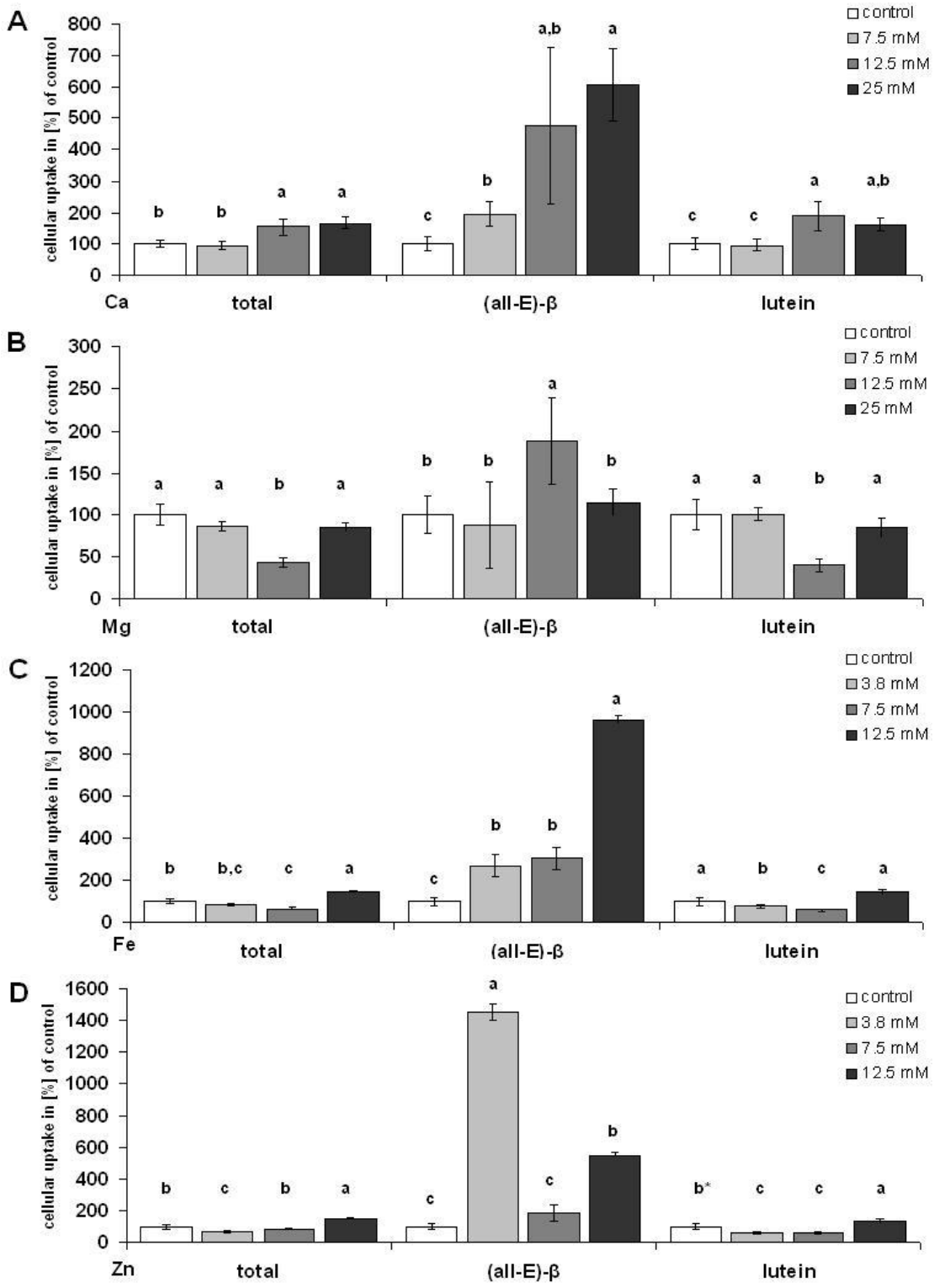

Table 19: Fractional cellular carotenoid uptake from digesta:DMEM mixture [1:4, v:v] by Caco-2/TC-7 cells (for definition see chapter 3.13.2). Ca = calcium, Fe = iron(II), Mg = magnesium, Zn = zinc; total = total carotenoids, (all-E)-β car = (all-E)-β-carotene. Empty bar: control, light grey bar: 3.8 mM (Fe, Zn) and 7.5 mM (Ca, Mg), dark grey bar: 7.5 mM (Fe, Zn) and 12.5 mM (Ca, Mg) and full bar: 12.5 mM (Fe, Zn) and 25 mM (Ca, Zn). Bars referring to one category tested (beta-carotene (all-E- β), lutein and total carotenoids) not sharing the same letter are significantly different, with a>b>c>d ($p<0.001$) and a>b* ($p<0.05$, Bonferroni post-hoc test). Bars = mean ± SD (n=6-10).

Calculating fractional cellular uptake from the micellar carotenoid content (termed 'relative uptake' from digesta, chapter 3.13.2) instead of the original spinach meal content (termed 'overall uptake'), beta-carotene uptake was 5.5 to 9.5 times increased compared to the control at highest mineral concentrations (for Ca^{2+}, Fe^{2+} and Zn^{2+}, $p<0.001$, Bonferroni), except for magnesium (no significant changes; Figure 19), i.e. lower micellar beta-carotene content of digesta was, to a certain extent, counterbalanced by improved cellular uptake from the micelles. Lutein uptake was only slightly, though significantly increased ($p<0.001$), by the factor 1.3 – 1.6 for Ca^{2+}, Fe^{2+} and Zn^{2+}, also showing a weak counterbalancing effect for decreased micellar carotenoid concentration.

4.5 Carotenoid conversion/degradation products

Additionally to micellarisation and cellular uptake of the major carotenoids found in spinach, such as lutein and beta-carotene, the digestive fate of other abundant carotenoid species, especially the fate of the epoxycarotenoids violaxanthin and neoxanthin, was also monitored during simulated gastro-intestinal digestion.

4.5.1 Epoxycarotenoid digestion products

Concentrations of both epoxycarotenoids violaxanthin (λ_{max}: 416/439/468 nm) and neoxanthin (λ_{max}: 413/436/464 nm) diminished during simulated gastro-intestinal passage (Figures 20+21) due to the acidic pH, as described by Asai and coworkers (2004b), following opening of the epoxy-structure and recyclization. Neoxanthin digestion products were tentatively identified as (8'R)- and (8'S)-neochrome epimers by comparing retention times, absorption maxima (λ_{max}: 399/422/448 nm) and the peak-III-to-peak-II-ratio (III/II: 0.94-0.95) by a neoxanthin standard digested under similar conditions (chapter 3.9, Material and Methods) and by comparison with literature (Asai *et al.*, 2004). Violaxanthin digestion resulted in two main products, one with λ_{max}: 400/421/448 nm and a III/II-peak ratio of 0.98 and the second one with λ_{max}: 380/401/426 and a III/II-peak ratio of 0.99. Both products were tentatively identified as luteoxanthin and auroxanthin isomers (with a ratio of approximately 2:1 for luteoxanthin: auroxanthin) by comparing results with literature (Asai *et al* 2004b) and by matching with auroxanthin standards digested under similar conditions.

The hypsochromic shift of λ_{max} (-14 nm) for neochrome, the conversion product of neoxanthin, was less pronounced than for the violaxanthin digestion product auroxanthin (-38 nm; loss of two double bonds from the conjugated system). λ_{max} of luteoxanthin, the potential intermediate in violaxanthin conversion, was shifted from 439 nm to 421 nm during digestion, likely due to the reduction of the conjugated system by one double bond (similar for the conversion of neoxanthin – neochrome).

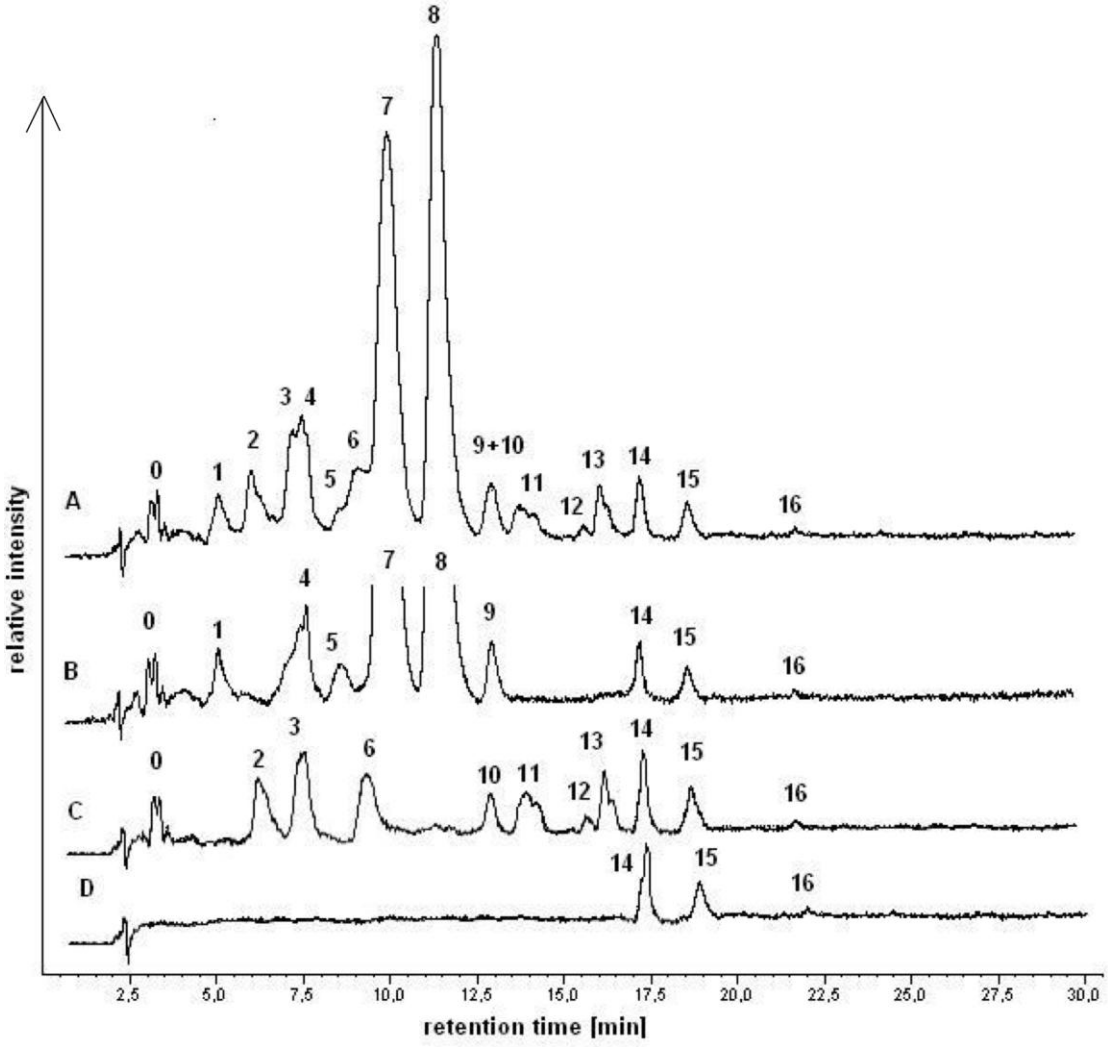

Figure 20: HPLC chromatograms of neoxanthin/violaxanthin (A), neoxanthin (B), and violaxanthin (C) standards of equal concentrations after simulated gastro-intestinal digestion (details in Materials and Methods; tentatively identified). (D) blank, 0 = unidentified degradation products, 1+4+5+9: = cis-neochrome; 2+3+6: cis-luteochrome; 7-8: 8'R and 8'S neochrome; 10: luteochrome; 11-13: auroxanthin epimers; 14: lutein; 15: zeaxanthin; 16: unidentified (peaks 14-16 from lecithin, an chicken egg derived emulsifier present during digestion). The chromatogram was recorded at 472 nm.

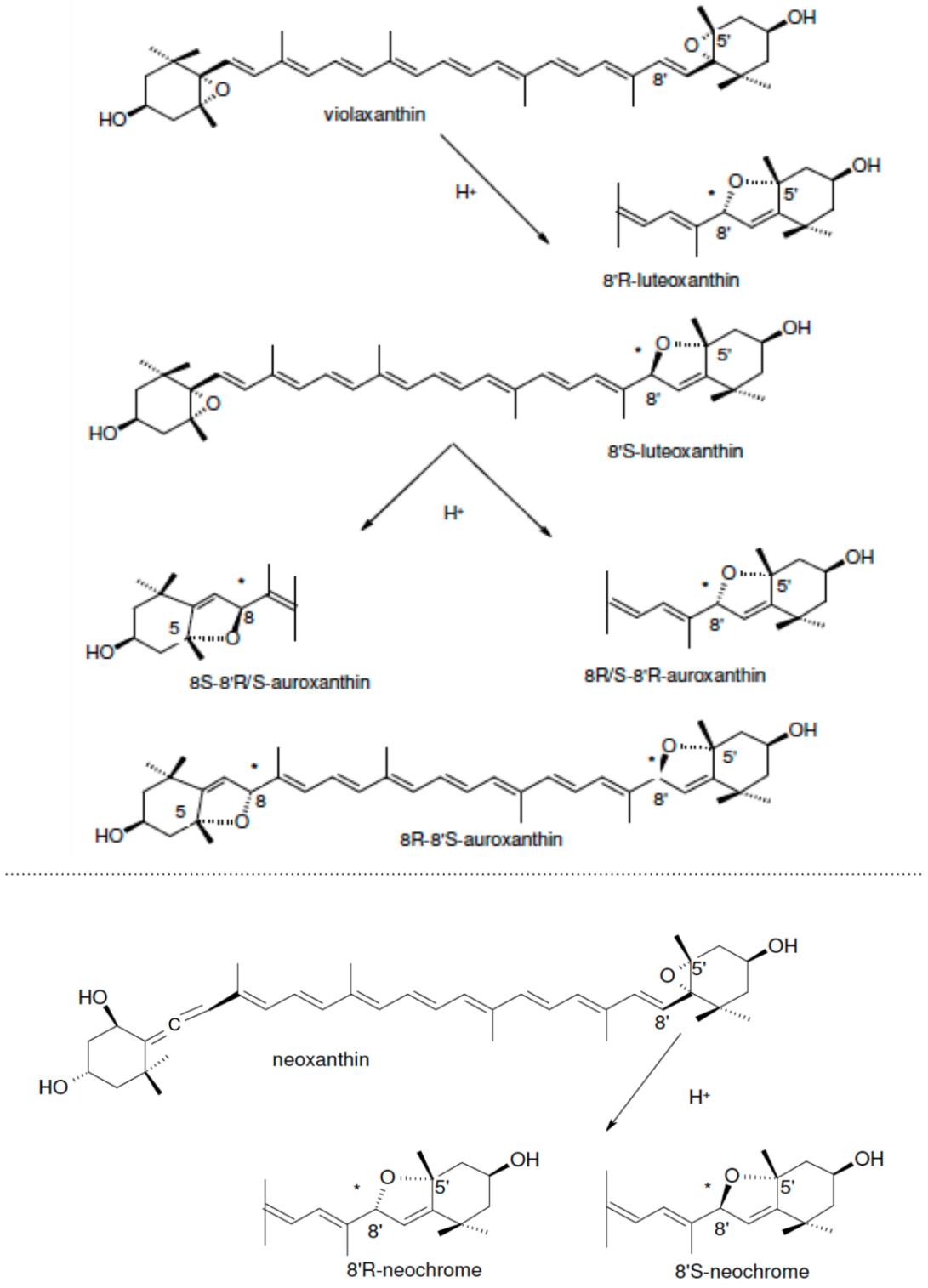

Figure 21: Acid catalysed epoxycarotenoid conversion. Violaxanthin is converted to luteoxanthin and auroxanthin (upper panel), while neoxanthin converts to neochrome (lower panel). A general, acid catalysed mechanism for epoxycarotenoids was originally proposed by Eugster (1995). Asterisks indicate newly formed stereogenic centres.

4.5.2 Micellarisation and cellular uptake of epoxycarotenoid conversion products

All three main digestion products, neochrome, auroxanthin, and luteoxanthin and their respective epimers, were detected in the micellar fraction after simulated gastro-intestinal digestion and in Caco-2/TC-7 cells after 4hrs of exposure (Table 12). Considering the original concentration present in the test meal ('overall uptake'), the less polar (8'R/S)-neochrome showed highest uptake compared to other carotenoids investigated ($p<0.001$, Bonferroni). The cellular uptake for the violaxanthin conversion products auroxanthin and luteoxanthin from the test meals was similar as compared to lutein ($p<0.001$, Bonferroni). By comparison, the least polar species studied, beta-carotene was found to be the carotenoid with the lowest 'overall cellular uptake' compared to all other carotenoids ($p<0.001$, Bonferroni, Figure 22).

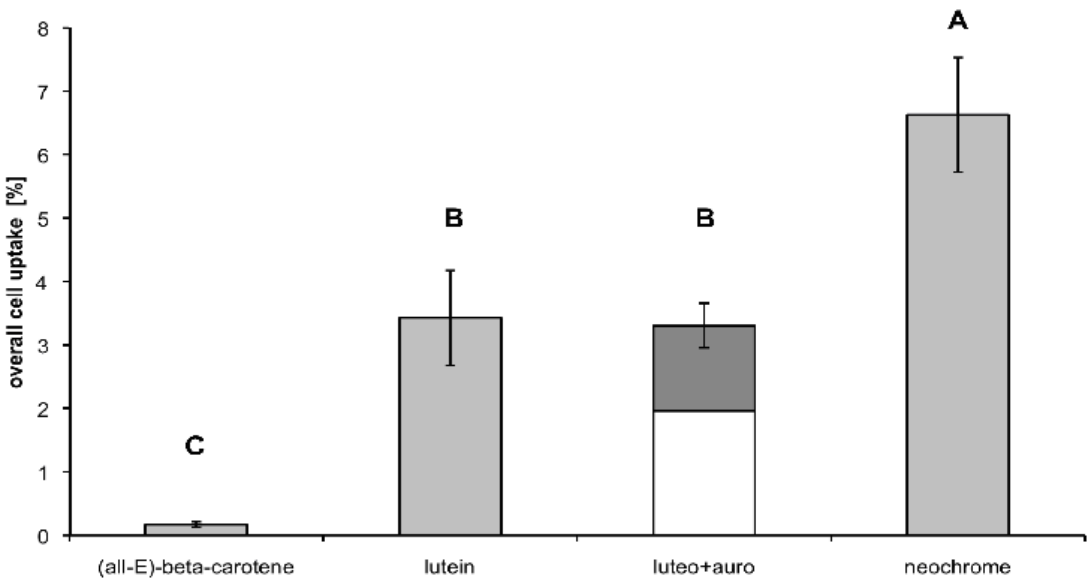

Figure 22: Fractional neochrome uptake into Caco-2 cells from test meal exceeds that of other carotenoids present. Values are given as: carotenoids in cells /carotenoids present in original test meal * 100 ± SD. Violaxanthin was converted during the digestion to 2 main products, i.e. auroxanthin (auro, white bar) and luteoxanthin (luteo, solid bar). For the calculation of cellular uptake, both species were combined and compared to the original violaxanthin concentration in the test meal. The two neochrome epimers (8'R/S) found in the cells, i.e. the neoxanthin digestion products, were compared to the amount of neoxanthin present in the test meal. Letters indicate statistical significance level – bars not sharing a common letter do significantly differ (A>B>C, $p<0.001$, Bonferroni post hoc test). Values are given as mean ± SD; n=6 for each group.

5 Discussion

In the first part of this section, carotenoid content in locally produced and/or consumed food items and their intake in Luxembourg are discussed. In the second part, results from carotenoid micellarisation and cell uptake studies will be elaborated.

5.1 Food composition

Food databases including information on phytonutrients, such as beta-carotene, lutein/ zeaxanthin and lycopene, have existed for many years, mostly in European countries and the US (BAG 2004, Mangels *et al* 1993, O'Neill *et al* 2001, Souci *et al* 2000, USDA 2010), but also in others, such as Israel (Chaiter *et al* 2007). Since neither general information on the concentration of nutrients nor on non-nutrients, such as on vitamins, polyphenols and carotenoids has been available for Luxembourgish food items, it was aimed to create primary data on major carotenoids present in local and locally consumed foods. Screening for a broad variety of food items potentially rich in carotenoids, 50 items were finally chosen according to frequency of consumption (Alkerwi *et al* 2010) and carotenoid content (Souci *et al* 2000), and were analyzed in detail.

5.1.1 Total carotenoids

In general, carotenoid content in Luxembourgish food items (Table 8, Results) were, on average, similar to those reported in other studies in the US and 6 European countries, i.e. France, Germany, Ireland, the Netherlands, Spain and the UK (Bauernfeind 1972, Mangels *et al* 1993, Müller 1997, O'Neill *et al* 2001). Food carotenoid content ranged from 0.03 mg/100 g (sour cream) to 18.0 mg/100 g (carrots) (Table 8, Results). Some food items, though, such as several fruits and vegetables, showed considerable differences compared to published data from other countries. For example, total carotenoid content in watermelon in the study by O'Neill (2001) was three times higher compared to the locally purchased product. There exists a multitude of factors possibly influencing carotenoid content, such as differences in climate, the availability of water, the amount of nutrients in the soil and the variety of cultivars (Chenard *et al* 2005).

5.1.2 Individual carotenoids

Luxembourgish food items were rich in alpha- and beta-carotene compared to other European countries. However, considerable differences compared to published data from these countries existed. For example, in Luxembourgish carrots, the sum of alpha- and beta-carotene was 17.9 mg/100 g, identically to the content described in the Israeli database (17.9 mg/100 g), while the US database suggests substantially higher (28.9 mg/100 g) (USDA 2010) and the database from 5 European countries considerably lower concentrations (10.2 mg/100 g). The carrot cultivars in these studies were unknown, and additionally, differences could be, at least partly, due to the time of harvest and the ripening state. For example, carotenoid contents in the same carrot cultivar in May were found approx. 25% lower compared to carrots analyzed in September (Fraser *et al* 2004).

For lutein, which is thought to protect the retina from UV-damage (Landrum *et al* 1997b), discrepancies in vegetable content were detected when evaluated against data from other countries: for example, lutein content in lettuce was determined at 0.7 mg/100 g (Table 8, Results), while Müller (1997) found 4.5-times higher concentrations. O'Neill and colleagues (2001) though detected lutein concentrations between 0.2 an 1.3 mg/100 g depending on the cultivar, indicating that there is a substantial diversity even in different cultivars of the same species.

Although not acting as a vitamin A precursor, lycopene is considered as a potent antioxidant (Bohn 2008). Its content in local products was also found to be different from other countries. Lycopene concentrations in locally purchased watermelons were 0.9 mg/100 g, only 25% compared to data from O'Neill and colleagues (2001) and only 15% compared to Souci, Fachmann and Kraut (2000), while the Israeli database suggested even lower concentrations (0.3 mg/100 g) (Chaiter *et al* 2007). It appears that, besides the effect of ripening, a highly aqueous matrix, which is found in watermelon, poses analytical challenges, such as for weighing and extraction, likely influencing results (Chaiter *et al* 2007). In another lycopene containing matrix, such as tomatoes, substantial differences were detected by Müller (1997), showing differences in concentrations of 300% between varieties matured in green house vs. grown on fields.

Concentrations of epoxycarotenoids in foods are generally not described in food databases (BAG 2004, Chaiter *et al* 2007, Mangels *et al* 1993, Souci *et al* 2000). Comparing Luxembourgish data to the results from a study on selected German food items (Müller 1997), violaxanthin and neoxanthin were, on average, found to be similar.

Taken together, it seems likely that the above variations in plant foods might be due to a multitude of factors, such as different cultivars, presence of nutrients in the soil, water availability, time of harvest and storage and also due to analytical methods. The HPLC column containing a C30 stationary phase employed in this study allowed for detection of carotenoid isomers. These were added to the concentrations of the all-E-isomers. Other data sources, especially older ones (Mangels *et al* 1993), employed mostly C18 columns with lower separation capacity. Since the presented values are in the range of other studies, it appears that the detected differences were not systematical or substantial.

5.1.3 Animal derived food items

While plant foods generally show a high variability, carotenoid content in animal products, such as in eggs, appear very consistent, perhaps due the strict regulations by the European Union concerning additives in foods and feeds (reviewed by Breithaupt 2008). Lutein concentrations in Luxembourgish eggs were almost identical compared to data published by O'Neill and colleagues (O'Neill *et al* 2001), showing only differences of approx. 3%. Findings in dairy products, such as butter, margarine and cheese were 10 - 30% different compared to reference databases (O'Neill *et al* 2001, Souci *et al* 2000), indicating the strong impact of costumer perception preferences concerning food colour and appearance.

5.1.4 Summary food composition

Since the general food databases in the US (USDA 2010), Germany (BLS 2007) and Switzerland (BAG 2004) as well as the carotenoid specialized US (Mangels *et al* 1993) and European (O'Neill *et al* 2001) databases only focus on a few selected carotenoid species, such as lycopene, beta-carotene and lutein/zeaxanthin, the here provided data for Luxembourg have the advantage of offering additional information on the less studied epoxycarotenoids violaxanthin and neoxanthin, and can be

considered as being more detailed. Although generally of lower abundance, for some food items, such as yellow bell pepper, epoxycarotenoids constitute up to 68% of total carotenoids (Table 8, Results).

An additional benefit of this study is, similar to the Israeli study (Chaiter *et al* 2007), that all analyses were performed in the same laboratory, including the same HPLC apparatus including column and detector, while other studies, such as the one by O'Neill and coworkers (2001) and the study in the US (Mangels *et al* 1993) compiled information from a large number of laboratories and investigators, including different extraction and detection protocols, such as open column separation (Mangels *et al* 1993).

5.2 Carotenoid intake

5.2.1 Total carotenoids

Combining carotenoid content of locally produced and/or consumed food items with consumption data obtained from the first nationwide Luxembourgish risk factor study (ORISCAV-LUX) (Alkerwi *et al* 2010), the average local carotenoid consumption was estimated. Although differences in total carotenoid intake exist (Table 9, Results), main contributing items to total Luxembourgish carotenoid intake (carrots and green, leafy vegetables including spinach as main contributor) were similar to all other countries (carrots and spinach), except for Italy (carrots, tomatoes and spinach). Compared to carotenoid intake in other European countries, total carotenoid intake in Luxembourg was between 8% (the Netherlands) and 30% (Italy) lower (Figure 23), while national carotenoid intake was 25% higher as compared to Spain. Larger distinctions exist when comparing to Israel, where carotenoid intake was 70% higher (Figure 23).

5.2.2 Individual carotenoid intake

Compared to other European countries, local alpha- and beta-carotene intake was estimated to be high (7.6 mg/day), lutein/zeaxanthin intake rather low (1.5 mg/day) and lycopene intake very low (1.8 mg/day) (Figure 23, Discussion). In the view of antioxidant and provitamin A properties, alpha- and beta-carotene intake was of major interest. It appears, however, that existing knowledge is not sufficient to establish an

'adequate intake' or a 'recommended dietary allowance' for beta-carotene and other carotenoids (Institute of Medicine 2001). To cover the recommended dietary allowance of vitamin A (800 µg for females and males in average), from beta-carotene in the diet alone, approximately 9.6 mg of beta-carotene would be necessary. Considering the already high intake of vitamin A (approximately 2200 µg) in industrialised countries, such as the US (Chasan-Taber *et al* 1999), it appears that, at least in regard to the vitamin A provision, the intake of provitamin A carotenoids is more than sufficient.

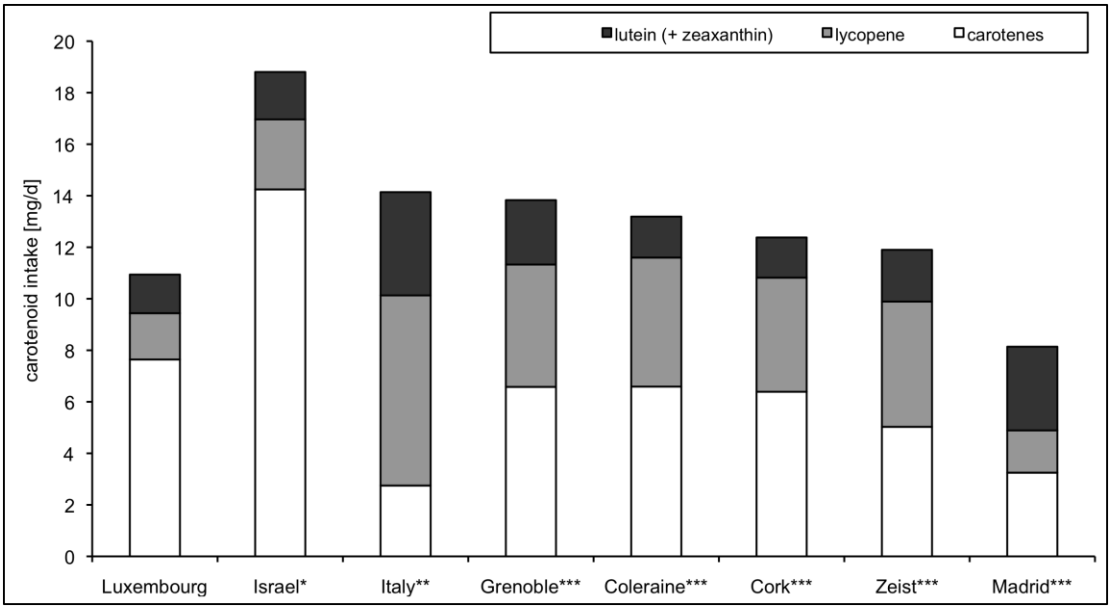

Figure 23: Carotenoid intake in Luxembourg (mean consumption in mg*person^{-1}*day^{-1}) as opposed to data from Israel, Italy and 5 European Cities.* Nutritional study in Israel conducted by Chaiter et al., 2008; ** Italian total diet study (Lucarini *et al* 2006);***carotenoid intake study in 5 European Countries (O'Neill et al., 2001): Madrid (Spain), Grenoble (France), Coleraine (N. Ireland/UK), Cork (Ireland) and Zeist (The Netherlands). Black bar: lutein+ zeaxanthin; grey bar: lycopene; white bar: alpha- and beta-carotene. Note: data for Luxembourg as for other countries without violaxanthin and neoxanthin for improved comparison.

A low intake of lycopene was found, especially in comparison to the Italian study, where lycopene intake exceeded that of Luxembourg by the factor 4 (Figure 23). This difference might be, at least to some extent, due to the incomplete local carotenoid database with respect to complete dishes, fast- and convenient foods, which are often rich in processed tomato products, such as tomato sauce and ketchup, and hence in lycopene (Khachik *et al* 1986, O'Neill *et al* 2001). These food items were not included in the local database – detailed analyses of every potential carotenoid

containing food would simply have burst the frame of the presented thesis. Thus, these dishes are naturally missing in the calculation of daily carotenoid intake in Luxembourg. Nonetheless, specific food consumption behaviour in Italy, that is the lycopene-rich Mediterranean diet (tomato sauce, pasta, soup, pizza, etc.) appears to be the main reason for the detected differences in lycopene intake compared to Luxembourg and the other European countries.

When comparing to Israel, an example for a southern, non-European country, carotenoid intake in Luxembourg, especially that of alpha- and beta-carotenes showed considerable differences. According to the authors of the Israeli study (Chaiter *et al* 2007), this is due to a different consumption behaviour, especially because of a high consumption of fresh, green vegetables.

Carotenoid intake in neighbouring Germany was described to be very low, approx. 44% of the average of the above-mentioned European countries, reporting especially low alpha- and beta-carotene (2.1 mg/day) and lycopene intake (1.3 mg/day) (Pelz *et al* 1998), for reasons unknown. Additionally, a similar total carotenoid intake may not necessarily correlate with a similar carotenoid intake pattern. For example, Australians from the Blue Mountain region west of Sydney (aged >55 years), were described to take in a similar amount of carotenoids compared to the Luxembourgers, however, with a high intake of alpha-and beta-carotene (10.0 mg/day), a medium intake of lycopene (3.6 mg/day) and low intake lutein/zeaxanthin (0.9 mg/day) (Manzi *et al* 2002) (Figure 23).

Comparing studies from different countries, employing various study designs, questionnaires and databases is an extremely difficult task. For example, carotenoid intake data from the study covering 5 European countries (O'Neill *et al* 2001) and the one from Israel (Chaiter *et al* 2007), are presented in medians + range, while the Italian (Lucarni *et al* 2006), Australian (Manzi *et al* 2002) and German (Pelz *et al* 1998) data are given as means + SD, suggesting different distribution patterns (Gaussian vs. non-Gaussian). Furthermore, there are considerable differences in the age of participants, ranging from a total population (> 1 year) in Italy (Lucarini *et al* 2006) to a cohort of 70 years on average in the Israeli study (Chaiter *et al* 2007).

Employing the Luxembourgish nutritional consumption data of the risk factor study (Alkerwi *et al* 2010), which was originally not foreseen for estimating carotenoid intake, had some limitations for our purpose:

First, in the study's questionnaires, food items, which had similar nutritional and energy values but not strictly the same or a similar carotenoid content, were merged into consumption groups (for example 'green, leafy vegetables'). Therefore, the relative frequency of consumption of the individual food items in each of these groups had to be assessed additionally by the means of national food disappearance data, and completed by data from Switzerland (Gremaud *et al* 2005). Food disappearance data do not completely reflect consumption. However, since disappearance data were only employed to estimate relative consumption ratio (percentages) of individual food species inside the food groups and not to calculate total food intake, these errors can be considered as minor.

Second, nutritional data from the ORISCAV-Lux study indicated a substantial consumption of pasta products, of vegetable soup and mixed vegetables with varying fractions of meat, making it difficult to estimate the proportion of each ingredient, and resulting in underestimation of actual uptake, simply because these food groups were excluded from the further calculations of carotenoid intake.

Third, approximately 3000 out of 4500 contacted persons contacted for participation in the ORISCAV-Lux study did not participate. It might be possible that the behaviour of non-responders was different from that of the responders. This is, however, a general drawback of population-based studies. Additionally, there are typical biases connected to the dietary recall methods: small snacks, consumed between larger meal, are easily forgotten and can result in slight underestimation of food intake.

On the other hand, the ORICLAV-Lux study was done on a sufficient large scale, enabling to cover a sufficient proportion of the Luxembourgish population, especially if one considers the low number of residents (approx. 500.000 people). For example, the carotenoid intake study in 5 European countries (O'Neill *et al* 2001) was conducted in small scale, including only 73 people/ city.

Taken together, carotenoid intake (Figure 24) in Luxembourg was estimated for the first time. Differences between study design and population as well as in carotenoid content of investigated foods were considerable and the carotenoid database created for Luxembourg relatively small, complicating comparisons between different studies and countries. Considering these potential biases, it appears that total carotenoid intake in Luxembourg is not considerably different from other European countries.

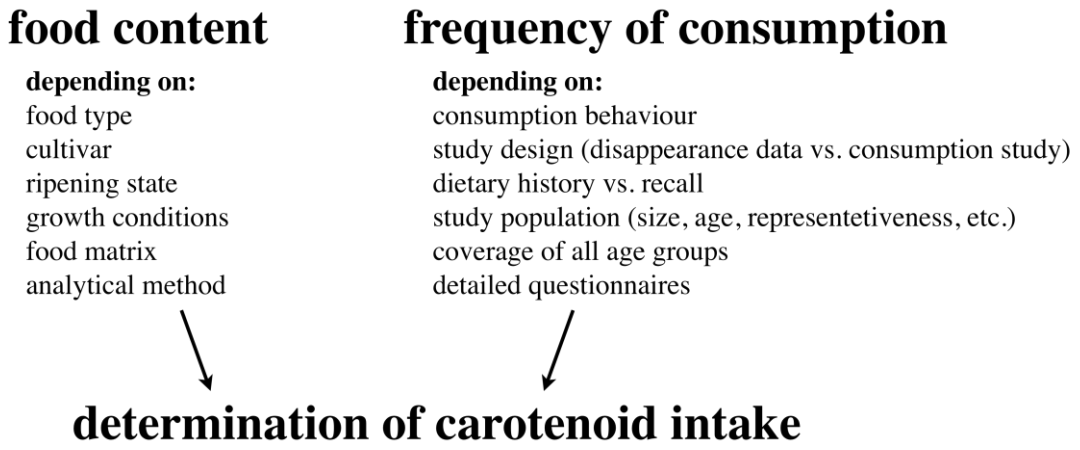

Figure 24: Summary of factors impacting the determination of carotenoid food content and intake

Carotenoid intake is, however, only one parameter that determines the amount of carotenoid taken up by the body. A second, important factor is the bioavailability – the fraction of carotenoids (out of the total amount ingested) that is taken up, reaches the systemic circulation and is available for various functions. Factors potentially impacting the availability of carotenoids are discussed in the following chapter.

5.3 Gastro-intestinal digestion: carotenoid micellarisation

The second part of this thesis aimed to investigate factors influencing carotenoid bioavailability by the means of an *in vitro* gastro-intestinal model coupled to cellular uptake into the enterocytic Caco-2 cells. Due to the apolar character of carotenoids, micellarisation followed by cellular uptake are deemed to be major determining steps in the process of carotenoid absorption, consisting of breakdown of plant cell walls for carotenoid release, transfer into lipid droplets, emulsification into mixed micelles and uptake by the intestinal mucosa.

5.3.1 Host-related factors influencing carotenoid micellarisation

Bile salts and digested lipids are crucial for the emulsification of carotenoids into mixed micelles, hence directly influencing carotenoid availability (Borel 2003, El-Gorab *et al* 1975). In the present study, carotenoid micellarisation was strongly decreased when omitting bile salts, pancreatin, or both, being comparable, if not stronger, to previous studies (Garrett *et al* 1999a, Hedren *et al* 2002, Wright *et al* 2008). This situation may occur *in vivo*, such as in cystic fibrosis patients lacking the ability to secrete sufficient amounts of pancreatin, possibly resulting in inadequate uptake of lutein, zeaxanthin and beta-carotene (Rust *et al* 1998, Schupp *et al* 2004). Pancreatic hydrolases are required to cleave triglycerides into mono- and diglycerides, which are part of the formed mixed micelles. The low carotenoid micellarisation observed in the absence of both pancreatin and bile salts (>90%, Figure 12, Results) is in line with earlier studies indicating a reduction of carotenoid micellarisation by 80-100% when omitting bile salts (Garrett *et al* 1999a, Hedren *et al* 2002, Wright *et al* 2008) or the entire intestinal digestion phase (Garrett *et al* 1999a, Hedren *et al* 2002). The omission of pepsin during the gastric phase, however, did not significantly influence carotenoid micellarisation, similar as found by Garrett et al. (1999a). It appears that, at least in an *in vitro* model with a relatively simple, low-protein meal as investigated in this study, the enzymatic activity of pepsin was sufficient for matrix disruption.

5.3.1.1 Effect of food matrix on carotenoid micellarisation

Additionally to the host-related factors, several food-related factors were studied. Disruption of the matrix (Castenmiller *et al* 1999, Gärtner *et al* 1997) and presence of lipids (De Jesus Ornelas-Paz *et al* 2008, Hedren *et al* 2002, Huo *et al* 2007) were shown to improve the release of different carotenoid species *in vitro* and *in vivo*. In the present investigation, processing of spinach, such as by mixing in a household blender, considerably increased total carotenoid micellarisation as opposed to simple, crude chopping by a kitchen knife by 40%. Additional processing steps, such as heating, that were not investigated in the present thesis, were shown to further increase carotenoid uptake (Unlu *et al* 2007).

The addition of milk (4% fat) resulted in highest micellarisation and significantly increased carotenoid solubility compared to low fat milk. Other meal components tested, such as liver sausage and soya milk, although delivering comparable or higher amounts of fat (2 g of milk containing 2 % or 0.5 g of sausage containing 32 %), were less effective in increasing carotenoid bioaccessibility. It is possible that the complex nature of the matrix, i.e. present fiber (for a soya milk) attenuated carotenoid accessibility, similar to findings for other matrices (De Jesus Ornelas-Paz *et al* 2008). Fresubin as an example of a ready-to-drink meal used e.g. as a meal replacement or as part of a balanced diet resulted in strongest reduction of carotenoid micellarisation (>50%) compared to other meals tested. This effect may be due to highly increased viscosity of the digesta, which has also been suggested to negatively impact micellarisation (Yonekura *et al* 2009).

Although the static *in vitro* digestion model does not fully mimic the gastro-intestinal conditions *in vivo*, especially the dynamics of bile/enzyme secretion in relation to the food matrix composition, the results suggest that carotenoids may be more accessible from simple, processed matrices compared to more complex sources.

5.3.2 Dietary factors influencing carotenoid micellarisation

During optimisation and validation, so-called dietay factors, such as the impact of food matrix (food-processing, presence of lipids) and presence of potential enhancers/ inhibitors (cholesterol, stigmasterol and α-tocopherol) of carotenoid micellarisation were investigated.

5.3.2.1 Effect of lipophilic compounds on carotenoid micellarisation

Lipophilic compounds with sufficiently high concentrations in the diet, including α-tocopherol, cholesterol and stigmasterol, may compete for micellarisation, hence potentially attenuating carotenoid bioaccessibility. A significant reduction of total carotenoids, lutein and zeaxanthin was only found for high but still realistic cholesterol concentrations of 1.9 g/100 g test meal, as it is present in e.g. animal entrails (Souci *et al* 2000). Cholesterol has already been described to attenuate beta-carotene incorporation in artificial systems (Socaciu *et al* 2000), similar to certain phytosterols in Caco-2 cells (Fahy *et al* 2004).

Interestingly, at similar high concentrations of 1.8 g/100 g test meal, the cholesterol resembling free stigmasterol did only slightly but non-significantly reduce total carotenoid micellarisation (Table 10, Results). It can be speculated that a reduction of carotenoid serum levels, found in an *in vivo* trial following the consumption of phytosterols (Mensink *et al* 2002), might be governed by additional factors, such as by the regulation of the uptake into the enterocytes, sequestration into chylomicrons, or by increased excretion into the gastro-intestinal tract.

In the case of α-tocopherol, at very high concentrations up to 16.6 mg/100 g test meal, which is present e.g. in colza oil (Souci *et al* 2000), no significant impact on carotenoid micellarisation was detected. Since higher concentrations are unrealistic, it appears that this compound does not affect carotenoid micellarisation processes, at least not in this *in vitro* approach.

5.3.3 Impact of minerals on carotenoid micellarisation

5.3.3.1 Impact of sodium chloride on carotenoid micellarisation

Minerals can impact carotenoid availability, by complexation and/or precipitation of bile salts, fatty acids, or by compromising micelle stability by e.g. ion-micelle interactions. When conducting experiments by adding high sodium concentrations of 530 mM sodium chloride as opposed to physiological standard conditions (150 mM), a significantly reduced bioaccessibility of xanthophylls was found (15% reduction, Figure 13, Results), while beta-carotene isomers were significantly better micellarised compared to the control (160%). As bile or fatty acid precipitation at high sodium concentrations was not observed, other mechanisms such as viscosity or ionic

interactions between aqueous phase and micelles must play a role. It is possible that the increased ion strength of the aqueous phase especially favours the incorporation of the hydrophobic carotenes into the micelles, while the interaction with the micelle surface and the xanthophylls is disturbed. The high sodium concentration is well out of a typical dietary range and was tested to investigate whether the differentiation of carotenoid micellarisation between xanthophylls and carotenes extends to high salt concentrations. However, at concentrations of 280 mM, similar findings compared to 530 mM were obtained. Although this concentration cannot be easily reached from the diet, the consumption of a meal mostly containing sausages or salty ham is likely to reach sodium concentrations that may impact micellarisation patterns in a similar way.

5.3.3.2 Impact of divalent minerals on carotenoid micellarisation

This is, to the author's knowledge, the first investigation highlighting negative effects of dietary abundant minerals, such as calcium and magnesium and trace elements, such as iron and zinc, on carotenoid bioaccessibility. The potential impact of divalent minerals has never been thoroughly investigated so far, even though it has been suggested that high mineral concentrations might compromise micelle stability (Bohn 2008, Failla *et al* 2005), and that minerals could reduce concentrations of free soluble bile salts and fatty acids required for micelle formation (Feroci *et al* 1995, Fini *et al* 1997, Graham *et al* 1982, Graham *et al* 1983).

In the present study, it was shown that macro-minerals, i.e. calcium, and trace elements, i.e. iron and zinc, in concentrations that could be reached from the intake of supplements, reduced micellarisation significantly following simulated gastro-intestinal ingestion, by up to 55% (Ca^{2+}) and 90% (Fe^{2+}, Zn^{2+}), respectively.

Ca^{2+}, Fe^{2+} and Zn^{2+} were found to strongly inhibit carotenoid bioaccessibility, as determined by the amount of carotenoids solubilised in the mixed micelles, with strongest effects for Zn^{2+}>Fe^{2+}>Ca^{2+}, while the effect for Mg^{2+} was much less pronounced. Divalent minerals could react with free fatty acids and can form insoluble soaps (Graham *et al* 1983). Similarly, they could react with bile acids and bilirubin, further compromising the emulsification process of the carotenoids (Apstein 1998, Feroci *et al* 1995, Fini *et al* 1997, Hofmann *et al* 1992). In pilot studies, divalent salts caused severe precipitations when mixed with bile solution, again with

strongest effects detected for Fe^{2+} and Zn^{2+}, followed by Ca^{2+}, and only weak precipitation with Mg^{2+}.

In addition to the mineral, the tendency to form complexes would also depend on the type of bile salt, for example, in the presence of Ca^{2+}, low solubility has been shown for glycine-conjugated bile salts (Graham *et al* 1983), the predominant species in the human bile (Schindlbeck *et al* 1987) while higher solubility was shown for taurine-conjugated ones (Graham *et al* 1982, Hofmann *et al* 1992). In the presence of iron(II), a decreased solubility was found for bile salts in the following order: glycocholate > cholate > chenodeoxycholate > deoxycholate (Fini *et al* 1997).

Interestingly, calcium and magnesium both showed similar effects on total carotenoid micellarisation at 50% lower bile concentrations (4.4 mM) compared to the usually used one of 8.8 mM, with only slightly stronger effects for Ca^{2+} (IC_{50} 12.0 ± 0.9 mM) than for Mg^{2+} (IC_{50} = 14.8 ± 1.1 mM). Doubling bile concentration (8.8 mM) during digestion affected the inhibitory effect of Ca^{2+} only marginally (IC_{50} 14.2 ± 0.5 mM), while the impact of Mg (IC_{50} 41.2 ± 10.7 mM) on carotenoid micellarisation was very low. In experiments with bile and pancreatic duct ligated rats, Mg^{2+} was only about 10% less efficient in insoluble soap formation as compared to Ca^{2+} (Graham *et al* 1982). Since these rats were fed a defined amount of bile salts and digestive enzymes along with their diet, it appears that the artificially created situation within the animals was comparable to the conditions at 50% bile concentration in the model described in this study. Regarding bile salt concentrations in the human gut, usually ranging from 4 - 20 mM (Garidel *et al* 2007), concentrations used in the present *in vitro* model (4.4 – 8.8 mM) seem plausible.

5.3.3.3 Impact of divalent minerals on micellarisation of single carotenoid species

Under control conditions, micellarisation efficiency for (all-E)-beta-carotene was low compared to lutein, being in accordance with the literature (Garrett *et al* 2000, Garrett *et al* 1999b). Solubility in the mixed micelles has been suggested to decrease with increasing apolarity (Garrett *et al* 1999b). For example, beta-carotene and lycopene were reported to have a very low solubility in aqueous environments and crystallize easily when removed from the original matrix (Britton 1995), while, for lutein, a bioaccessibility of up to 80% was described (Chitchumroonchokchai *et al* 2004b).

The potential (9Z)-beta-carotene isomer showed a slightly better solubility as opposed to the (all-E)-beta-carotene (Table 10, Results), similar to results in a human study investigating small intestinal contents (Tyssandier *et al* 2003), suggesting that the higher bioavailability of the (all-E)-isomer found *in vivo* (Deming *et al* 2002) might be due to preferential uptake or due to cis-trans isomerisation during or following cellular absorption.

Increasing concentrations of Ca^{2+}, Fe^{2+}, Zn^{2+} and to some extent Mg^{2+} presumably reduced either the quantity of formed micelles or their size. This resulted in a markedly decrease of especially beta-carotene in the micelles (Figure 16 Results), as compared to the control, by approx. 98% (for iron and zinc at 12.5 mM). When compared to lutein instead of the control, beta-carotene also showed a decreased bioaccessibility. Earlier investigations (Garrett *et al* 1999a, Garrett *et al* 2000) indicated an equally decreased solubility for lutein and beta-carotene when reducing the concentrations of bile salts during digestion. However, the complexation of fatty acids by divalent ions might additionally reduce the size of the micelles, compromising especially the emulsification of the apolar carotenoids, i.e. beta-carotene, normally present in the core of the mixed micelles (Borel *et al* 1996).

5.4 Cellular uptake

5.4.1 Impact of divalent minerals on carotenoid cellular uptake

Total carotenoid uptake in Caco-2 cells was about proportional to micelle concentrations, which depended on the type and concentration of the mineral present during gastro-intestinal digestion (Figures 16 - 18, Results). Under control conditions, where total carotenoid concentration was highest, also cellular uptake was greatest. The more efficient an added mineral reduced carotenoid micellarisation, the lower the total carotenoid cellular uptake, with Fe being the most effective inhibitor (Figure 17).

Interestingly, beta-carotene and lutein uptake were differentially affected by high salt concentrations for all minerals tested, except for Mg^{2+}. The pronounced reduced concentration of beta-carotene in the mixed micelles was counterbalanced by an increased fractional cellular (‘relative‘) uptake of beta-carotene from the micelles in the digesta, up to 10-times compared to the control, and up to 1.6 times for lutein (for Ca^{2+} at maximal concentrations, Figure 19, Results). It is possible that this increased

fractional carotenoid uptake from the depleted micellar fraction is due to a simple reduction of beta-carotene concentration and a consequent reduced uptake inhibition due to competitive mechanisms. However, recent publications have shown that facilitated diffusion, e.g. via the scavenger receptor class B, type I (see Figure 25, Discussion) seem to be involved in the uptake of especially beta-carotene, and to a lesser extent lutein (Garrett *et al* 2000, Reboul *et al* 2005), across the apical cell membrane of intestinal cells (During *et al* 2007, van Bennekum *et al* 2005), a hypothesis that is additionally supported by satiable beta-carotene uptake kinetics described in other investigations (During *et al* 2002, Garrett *et al* 2000). When critically reviewing a pioneer study in this regard (El-Gorab *et al* 1975), satiable kinetics could also be detected, however, results were then interpreted differently.

Although the presented results support the involvement of facilitated diffusion as carotenoid - especially beta-carotene - uptake mechanism into Caco-2 cells (Figure 25), this mechanism appears of minor importance in regard to the total carotenoid fluxes. Under the conditions investigated, total cellular uptake was dominated by lutein, which was also the predominant carotenoid in the digesta (>50% of total carotenoids). Lutein uptake was found to be linear over a broad concentration range – in contrast to beta-carotene, which showed only a slight increase (Figure 18, Results). However, only relatively low concentrations of beta-carotene were tested, which was due to the low beta-carotene micellarisation compared to lutein (Table 10, Results).

In addition to facilitated uptake, interactions between beta-carotene and other carotenoids, i.e. lutein, could have impacted the uptake from the mixed micelles at higher concentrations (Tyssandier *et al* 2002, van den Berg *et al* 1998). In the present study, lutein micelle concentration exceeded that of beta-carotene by over 20-times (Figure 18, Results). It could thus be speculated that the relative high beta-carotene cellular uptake into intestinal cells is necessary to secure the supply of this physiologically important pro-vitamin. However, in other specialized tissue, such as in the macula lutea of the eye, the particular need for zeaxanthin/lutein seems to be similarly favoured by facilitated uptake, potentially by another subtype of SR-B1 (During *et al* 2008).

Investigations of other potential mechanisms, such as paracellular diffusion processes (Figure 25), could not be conducted with the relative simple cellular uptake model. However, this mechanism was suggested to play only a minor role in Caco-2 cells (Artursson *et al* 2001).

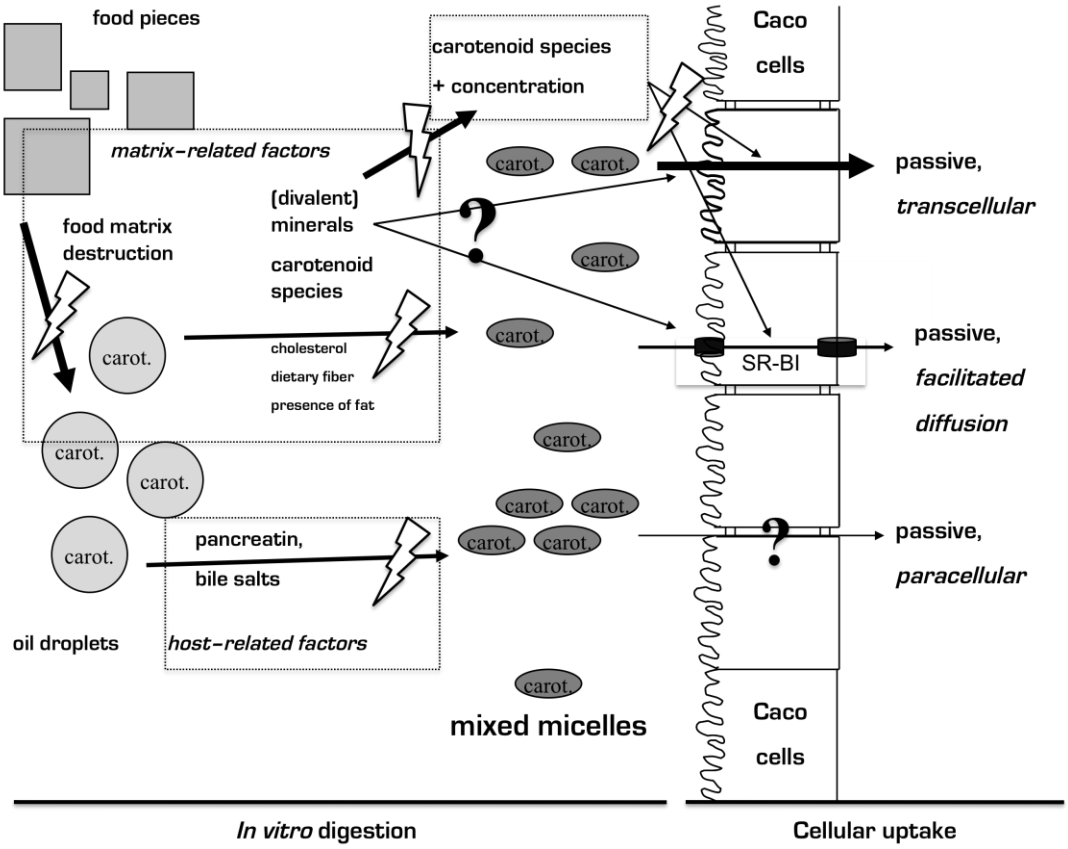

Figure 25: Factors impacting carotenoid micellarisation and cellular uptake. Carot. = carotenoids., caco cells = differentiated Caco-2/TC7 grown in monolayers. SR-BI = cholesterol scavenger (scavenger receptor calls B type I); carot = carotenoid. A 'flash' indicates a severe impact on the respective process, while the 'question mark' suggests a potential influence. While the process of micellarisation during '*in vitro* digestion' is mainly influenced by host- and matrix-related factors, 'cellular uptake' processes seem to be mainly impacted by the species and concentration of carotenoid present in the micelles (which are in turn influenced by the presence of divalent ions).

Results from beta-carotene uptake studies can be theoretically influenced by conversion to retinol, especially in cell lines showing cleaving activity, as it is the case in Caco2/TC-7 cells (During *et al* 2004). By carefully observing chromatograms of cell extracts after 4 hrs of incubation at a retinol specific wavelength of 340 nm (Hemley *et al* 1979), no retinol peak could be detected. It might have been the case that the enzymatic conversion rate was too low or the time of incubation was too short to yield a cellular retinol concentration high enough for detection, suggesting that the

intracellular beta-carotene concentration was not significantly negative impacted by conversion losses.

Despite the findings that micellarisation and cellular uptake were significantly impaired by the presence of divalent minerals in the presented *in vitro* system, it should be considered that the situation in the human body is considerably more complex. For example, the *in vitro* system used reflects a non-dynamic model. In the human gut, bile acids are continuously secreted. For example, the excretion seems to be well regulated in a way that precipitation of bile acids in the intestine could be counterbalanced by increased secretion of bile salts into the gut (Meier *et al* 2002), lowering the negative impact that the minerals may have. For example, the strong inhibiting effect of Mg^{2+} at 50% bile salt concentration was relatively moderate compared to 100% bile salt concentration. It is thus possible that the effect of divalent ions on micelle formation is attenuated in the more adaptive *in vivo* situation in the human intestine. However, if the sensitive equilibrium of the digestive tract is disturbed, i.e. in subjects with digestibility problems including e.g. pancreatitis or subjects with impaired digestion, i.e. cystic fibrosis patients, who are known to be prone to carotenoid malabsorption (Rust *et al* 1998, Schupp *et al* 2004), a high mineral concentration might further impair carotenoid uptake.

Recently, Bengtsson and colleagues (2009a) described an inhibitory effect of iron at low concentrations (>30 μM) on beta-carotene uptake into Caco-2 cells. They used synthetic micelles including 10-times higher beta-carotene concentration compared to the present study, resulting in a molecular ratio of mineral/carotenoids of approx. 15 - 25. The ‘artificial micelles’ employed in this study contained approximately 2 mM of mixed bile salts and cannot be compared directly to the ‘naturally’ produced micelles in the present study. It can be hypothesized that also in the study by Bengtsson et al. (2009a) iron might have precipitated bile salts and fatty acids, reducing the available beta-carotene, while not changing total extractable beta-carotene in the medium. This is in contrast to the present study, where precipitated carotenoids were removed after *in vitro* digestion by centrifugation and filtering.

Compared to magnesium and calcium dietary intake (ca. 12.3 and 25 mM/day, respectively), iron and zinc are minor abundant minerals in the human diet, with a recommended intake of about 0.24 (zinc) and 0.27 mM/day (iron), respectively (DGE *et al* 2000). Considering the intake of mineral supplements, ranging up to 1 mM/capsule for iron, taken twice daily (as recommended by CDC 1998) or supplements containing up to 25 mM of calcium and the combinatory effect of the four minerals investigated, as e.g. found in some mineral supplements for athletes (containing e.g. 14.4 mM Mg, 12.5 mM Ca, 0.08 mM Zn^{2+} and 0.9 mM), an impact on carotenoid micellarisation and cellular uptake could be possible.

5.4.2 Carotenoid digestion products

Two major xanthophylls in (frozen) spinach following the predominant species lutein (26% of total carotenoids), violaxanthin (23%) and neoxanthin (19%), belong to the epoxycarotenoid family. Both neoxanthin and violaxanthin were shown to undergo rapid epoxide–furanoid rearrangements under acidic conditions, converting to neochrome and to luteoxanthin and auroxanthin (Figures 20 and 21, Results), respectively (Asai *et al* 2004b). The digestion products of both species were highly abundant in the formed micelles following *in vitro* digestion, and were also taken up by the Caco-2 cells, with a fractional uptake similar to lutein (for luteoxanthin and auroxanthin) or higher (neochrome).

It might be the case that the conversion from epoxycarotenoids to furanoid products (Figure 21) slightly decreases solubility as compared to the parental species, simply due to a reduction of polarity. This might also explain the lower solubility of luteoxanthin/auroxanthin and neochrome when compared to lutein (Table 10, Results), which contains two hydroxyl groups. On the other hand, a decreased polarity could enhance cellular uptake compared to the epoxy parental molecules, since polarity was described to be correlated with uptake in Caco-2 cells (Sugawara *et al* 2001). Perhaps, a medium polarity is key factor to yield high cellular uptake (Figure 22).

Using a pH of 2 for gastric digestion (1 hour, 37°C), parental molecules were neither detected in the micelles nor in the cells. This is in contrast to the study of Asai and colleagues (2004b), who administered pre-micellarised neoxanthin to stomach-intubated mice and found both neoxanthin and neochrome to be absorbed and distributed in blood plasma, at a ratio of approx. 1:2. This incomplete conversion of neoxanthin in the mouse stomach could have been due to a pH greater than 2 or low stomach residing time. With the feeding conditions chosen (the 'meal' in this study consisted of carotenoid standards dissolved in artificial micelles that were administered to the mouse stomach by intubation), carotenoid-containing micelles might have been rapidly transferred to the mouse intestine before complete conversion, even if conditions in the mouse stomach were (highly) acidic, and the pre-existing micelles could have protected carotenoid from degradation.

In a human study, absorption of violaxanthin as measured in blood plasma was not detectable after admission of a high, single dose (10 mg) (Barua *et al* 2001). Similar results were described by Asai and colleagues (Asai *et al* 2008) in another human trial, where neoxanthin and its conversion product neochrome was absorbed in low amounts, even after one week of intensive spinach ingestion. These results support the hypothesis that the majority of epoxycarotenoids undergo epoxide-furanoid transition prior to absorption. For example, Yonekura and colleagues (2010) detected mostly furanoid products in mouse tissues after 2 weeks of intensive food supplementation.

5.5 Conclusion and perspectives

Carotenoids are colorants ubiquitous in nature with a high antioxidant potential and partially pro-vitamin A activity (Bendich *et al* 1989). High and regular consumption of carotenoid rich foods, such as fruits and vegetables, has been associated with the prevention of cardio-vascular disease (Voutilainen *et al* 2006), several types of cancer (Giovannucci *et al* 2002, Wright *et al* 2003, Ziegler *et al* 1996) and might play a role in bone health (Sahni *et al* 2009, Wattanapenpaiboon *et al* 2003).

5.5.1 Carotenoid content and intake

For the first time, carotenoid intake in Luxembourg was estimated. Additionally, substantial contributors to total carotenoid intake in Luxembourg, missing in other food databases and surveys, such as neoxanthin and violaxanthin, were included. Overall, carotenoid intake in the Grand-Duchy of Luxembourg was in the range of published data from other countries. It appeared to be slightly lower compared to several neighbouring countries, but the uncertainty of the reported data can be estimated to be high for all estimates of food consumption. Therefore, it cannot be concluded that the intake in Luxembourg is somewhat different from other European countries.

Although only focusing on one member of phytonutrients, these carotenoid data can, however, be regarded as a first step toward the creation of a national food database in Luxembourg. Additional effort has to be invested in the future as no ‘complete (complex) dishes’, such as vegetable soups, pizza, and pasta as well as fast and convenient foods have been analyzed so far. In a future study, potentially in the frame of the envisioned, detailed nutritional study in the member states of the European Union until the year 2017 employing 24-hour diet recall protocols, questionnaires should be designed in more detail to allow for separating carotenoid containing foods without the further need of secondary data sources.

5.5.2 Carotenoid micellarisation and cellular uptake

This study highlighted the importance of the food matrix, concentrations of sodium chloride, presence and concentration of digestive enzymes and bile salts on carotenoid bioaccessibility, while the impact of other inhibitors such as cholesterol, stigmasterol and especially α-tocopherol showed to be rather limited in this *in vitro* approach.

An inhibitory effect of the divalent ions Ca^{2+}, Fe^{2+}, Zn^{2+} and partly Mg^{2+} on carotenoid micellarisation and uptake into Caco-2/TC-7 cells was highlighted. This inhibitory effect manifested in a diminished carotenoid quantity solubilized in the aqueous phase during *in vitro* digestion. In the case of beta-carotene, the reduced solubility was partly counterbalanced during cellular uptake by enhanced cellular uptake. This effect was less pronounced for lutein. Future studies investigating the observed effects in an *in vivo* situation seem warranted. Additionally, the underlaying mechanism should be investigated in more detail. Furthermore, complex mixtures of divalent minerals should be tested, since usually no isolated minerals are present in foods and additive or even synergistic inhibitory effects may result. It would be also interesting to investigate the bioavailability of complex supplements, which contain minerals, such as concentrated mixtures of Zn^{2+}, Ca^{2+} and Mg^{2+}, together with vitamines and carotenoids and compare the results to supplements only containing carotenoids.

Epoxycarotenoids were completely converted to furanoid products during simulated gastro-intestinal passage under the chosen conditions. These digestion products were micellarised and taken up into Caco/2/TC-7 cells, indicating that the low bioavailability of epoxycarotenods described *in vivo* might be due to acid catalized epoxide conversion. However, uptake of the conversion products was comparatively high. Thus, future investigations should also focus on the detection of respective conversion products.

Overall, the *in vitro* digestion model has proved to be an important screening tool for the estimation of carotenoid bioavailability, especially in combination with the simulation of intestinal uptake (Caco-2 cells). Further validation of the obtained results in human studies seem warranted.

6 Literature

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7 Publications/Acknowledgements

Journal publications

E. Biehler, F. Mayer, E. Krause, L. Hoffmann and T. Bohn (2010). Comparison of Three Spectrophotometric Methods for Carotenoid Determination in Frequently Consumed Fruits and Vegetables. Journal of Food Science 75: C55-C61

E. Biehler and T. Bohn (2010). Methods for Assessing Aspects of Carotenoid Bioavailability. Current Nutrition and Food Science 6: 44-69

E. Biehler, A. Kaulmann, E. Krause, L. Hoffmann and T. Bohn (2011). Dietary and Host-Related Factors Influencing Carotenoid Bioaccessibility from Spinach (*Spinacia oleracea).* Food Chemistry 125: 1328-1334

E. Biehler, L. Hoffmann, E. Krause and T. Bohn. Divalent Minerals Decrease Micellarization and Uptake of Carotenoids and Digestion Products into Caco-2 Cells. *Submitted for publication to the Journal of Nutrition*

E. Biehler, A. Alkerwi, L. Hoffmann, E. Krause, M.L. Lair and T. Bohn. Contribution of Violaxanthin, Neoxanthin, Phytoene and Phytofluene to Total Carotenoid Intake - Assessment in Luxembourg. *In preparation*

Abstracts/Poster presentations

E. Biehler and T. Bohn. "Carotenoid Content and Consumption of Luxembourgish Fruits and Vegetables", presented on the 13th of March, 2009, during the 46th Scientific Congress of the German Nutrition Society (DGE), Giessen, Germany.

E. Biehler, T. Bohn, E. Krause and L. Hoffmann. "Carotenoid content in frequently consumed fruits and vegetables and their contribution to carotenoid intake in Luxembourg", presented on the 22nd of April, 2009, during the 1st Luxembourg Food and Nutrition Conference (NULUX) Luxembourg City, Luxembourg.

E. Biehler, A. Kaulmann, L. Hoffmann, E. Krause and T. Bohn. "Dietary and Host-Related Factors Influencing Carotenoid Bioaccessibility from Spinach (*Spinacia oleracea*)", presented on the 11th of March, 2010, during the 47th Scientific Congress of the German Nutrition Society (DGE), Jena, Germany.

Oral presentations

E. Biehler, L. Hoffmann, T. Bohn. "Carotenoid Consumption in Luxembourg" presented on the 3rd of June, 2009, during the 2nd Meeting of the Luxembourgish European Food Safety Authority focal point (OSQCA), Neumünster Abbey, Luxembourg City.

E. Biehler, L. Hoffmann, E. Krause and T. Bohn. "Divalent Minerals Decrease Micellarization and Uptake of Carotenoids and Digestion Products into Caco-2 Cells", presented on the 18th of March, 2011, during the 48th Scientific Congress of the German Nutrition Society (DGE), Potsdam, Germany.

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8 Curriculum Vitae

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10/2001 - 10/2004	First Diploma (Vordiplom) in general Biology, Saarland University, Germany
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- German triathlon team champion (2005 and 2007, hansgrohe-Team Schwarzwald)
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