Influence of dietary proteins on relevant aspects of carotenoid bioavailability

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Influence of dietary proteins on relevant aspects of carotenoid bioavailability

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This thesis is dedicated to my parents…

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Summary

Background: Carotenoids are bioactive secondary plant metabolites, presumably with health beneficial effects for humans. Their intake from the diet and their concentrations in body tissues (e.g. blood plasma) have been related to the reduced incidence of several chronic diseases, including type 2 diabetes, cardiovascular diseases, and certain types of cancer. In addition, several carotenoids are provitamin-A constituents, and some appear to be involved in the prevention of the age-related macular degeneration, the major cause of vision loss in the elderly. Owing to their rather low water solubility requiring emulsification prior to their potential absorption, carotenoids are of low and varying bioavailability (10-20% for e.g. β-carotene). This depends on host, but also on dietary factors including the presence of dietary fiber, lipids, and possibly other nutrients such as minerals or compounds competing for absorption (e.g. fat-soluble vitamins). However, not much is known on the interaction between proteins and carotenoids during gastric and small intestinal digestion, and the potential influence of these macromolecules on the solubilization and micellization of carotenoids, a prerequisite for their bioavailability. Several proteins have been shown to have emulsifying properties during digestion, stabilizing oil-in-water emulsions, which can be expected to result in smaller sizes of lipid droplets, possibly enhancing carotenoid micellization. While proteins have been employed in the production of encapsulated carotenoids, the interactions between proteins and carotenoids in test meals during gastro-intestinal (GI) digestion have, to our knowledge, never been studied systematically.

Summary

Objective of the thesis: The purpose of the overall PhD thesis was to study the influence of various frequently consumed proteins on aspects of carotenoid bioavailability, i.e. bioaccessibility and absorption processes. The final goal was to conduct a human trial to investigate the effect of added proteins on carotenoid bioavailability from plant food items rich in carotenoids. Preceding the human trial, *in vitro* studies were to be conducted, by means of simulated GI digestion, coupled to Caco-2 cellular uptake models representing the small intestinal lining. These studies aimed at selecting, for the human trial, the proteins interacting the most strongly with carotenoid solubilization, mixed micelle formation, and cellular uptake aspects.

Materials and methods: A digestion consensus protocol (based on EU-Cost action FA-1005) was employed to study the interaction of frequently consumed proteins on bioavailability aspects of carotenoids (bioaccessibility, Caco-2 cellular uptake). Whey protein isolate (WPI), sodium caseinate (SC), soy protein isolate (SPI), and gelatin (GEL), at concentrations equivalent to 0/10/25/50% of the recommended dietary allowance (RDA, approx. 60g/d), were co-digested gastro-intestinally with individual carotenoid (solubilized) standards (lutein, β-carotene, lycopene), as well as carotenoid-containing solid and liquid matrices (spinach, carrot juice, tomato juice). Microscopic visualization, and protein and lipid digestion were also carried out, and physico-chemical properties of the digesta (e.g. surface tension, protein digestion, lipid digestion, micelle size, and zeta-potential) were evaluated. Protein-rich matrices (instead of adding individual proteins) including cod and turkey were also studied (low-fat items). The interaction with gastric lipases (e.g. *R. niveus*, *R.*

oryzae and rabbit lipase (RGE)) was also investigated as their negligence may have influences on lipid-protein interactions in the stomach. The human trial consisted of a randomized, postprandial crossover trial with 24 subjects (12 male, 12 female), receiving the test meal that consisted of a mixture of 350 g of tomato and carrot juice (1:1, v/v, approx. 32 mg carotenoids, mostly lycopene and β-carotene) that contained or not 30 g of either SPI or WPI (equivalent to ca. 50% of the RDA). Carotenoid bioavailability was assessed by HPLC in the triacyl-rich-lipoprotein fraction (TRL), representing newly absorbed carotenoids.

Results: It was recognized that WPI could significantly enhance the bioaccessibility of β-carotene, by a fractional increase of up to 20% ($p < 0.001$). The effects strongly depended on the digestive conditions, especially on pancreatin amounts (based on trypsin activity) and simulated peristalsis. A drop of 1/3 in β-carotene bioaccessibility was also observed under rather incomplete digestive conditions (high oil, low peristalsis, low pancreatin and low bile) ($p < 0.001$). When investigating the effect of WPI, SPI, GEL and SC on different individual carotenoids, there were marked differences between the rather apolar carotenes and the more polar xanthophylls. While the latter were rather reduced in their bioaccessibility to about half in the presence of proteins ($p < 0.001$), carotene bioaccessibility increased by almost 2-fold ($p < 0.001$). Higher protein concentration correlated generally with higher bioaccessibility ($R = 0.57$, $p < 0.01$), higher surface tension ($R = 0.44$, $p < 0.01$), lower micelle size ($R = -0.83$, $p < 0.01$), and decreased repulsive forces (zeta-potential, $R = -0.72$, $p < 0.01$). When investigating the effect of the often neglected gastric lipase, RGE

increased total carotenoid bioaccessibility by almost 2-fold ($p < 0.001$), which is higher than what was obtained with lipases from *R. oryzae* or *R. niveus*. However, all three lipases improved lipid digestion/fatty acid release, by an average of 10-fold ($p < 0.001$). The positive effect of RGE on carotenoid bioaccessibility was partly maintained in the presence of co-digested proteins. When switching to real food matrices, findings depended on the individual conditions. In general, the presence of proteins positively influenced carotene bioaccessibility from food matrices by up to 3-fold ($p < 0.001$), while it resulted generally in decreased bioaccessibility of xanthophylls. An increased cellular uptake was observed for xanthophylls/carotenes (up to 12/33%, $p < 0.001$). Also here, bioaccessibility and uptake were better for rather apolar carotenoids compared to xanthophylls, but cellular uptake counterbalanced to some extend the poor bioaccessibility of xanthophylls. Equivalent amounts of proteins in form of cod and turkey had generally weaker effects than the added proteins. Co-digested proteins generally improved lipid digestion in all matrices ($p < 0.001$), though slight decreases were observed for GEL. With regard to the human trial, WPI significantly improved bioavailability (AUC of the plasma-TRL fraction) of carotenoids in general from the tomato/carrot mixed juice ($p<0.05$), by about 1/3, while SPI had rather a borderline negative effect. The effect in men was slightly more pronounced than in women. Cmax for total carotenoids was positively influenced by WPI, while significantly lowered by SPI, compared to the control ($p < 0.001$). Not all carotenoids were increased in their absorption, the effects were especially visible for the already strongly absorbed ones such as phytoene and phytofluene.

Conclusions: The tested proteins, especially some of the added ones (notably WPI) had the potential to increase aspects of carotenoid bioavailability. This was most pronounced for complete digestion conditions (sufficient enzymes, bile, and peristalsis), and especially for the normally more poorly solubilized carotenes (lycopene and β-carotene). Proteins from food matrices appeared to have weaker influences compared to individual proteins, possibly due to the stronger food-matrix and slower digestion of these proteins. In humans, the positive effects of proteins prevailed, but was only shown for WPI, and an increased positive absorption of the more highly absorbed carotenoids such as phytoene and phytofluene was recognized. Women had a generally lower fractional absorption of carotenoids from the test-meal, which may have precluded finding significant effects in this gender. These findings may help to improve bioavailability of provitamin A and other carotenoids, especially for consumers that heavily rely on these compounds for meeting vitamin A requirements, such as vegetarians, vegans, or people living in developing countries, where pre-formed vitamin A intake from meat and animal products is low. The obtained results may also be of interest for food and supplement producers, offering more insights into the formulation of carotenoids in functional foods, e.g. in terms of the amount and/or type of protein.

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ABA	Abscisic acid
ACN	Acetonitrile
AMD	Age-related macular degeneration
AUC	Area under the curve
BCO1	β-Carotene oxygenase 1
BCO2	β-Carotene oxygenase 2
BSA	Bovine serum albumin
CHLG	Chlorophyll synthetase
CHYB	β-Carotene hydroxylase
CHYE	ε-Carotene hydroxylase
CRTISO	Carotenoids isomerase
CVD	Cardiovascular disease
DCM	Dichloromethane
DMAPP	Dimethylallylpyrophosphate
DMEM	Dulbecco's modified Eagle's medium
FFA	Free fatty acid
GEL	Gelatin
GGDR	Geranylgeranyl reductase
GGPP	Geranylgeranyl pyrophosphate
GGPPS	Geranylgeranyl pyrophosphate synthase
GI	Gastro-intestinal
HCl	Hydrochloric acid
HDR	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase)
Ikβ	Inhibitor of nuclear factor kappa-B
IKK	Iκ-β kinase
IL	Interleukin

IPI	Isopentenyl pyrophosphate isomerase
IPP	Isopentenyl pyrophosphate
IS	Internal standard
Keap1	Kelch-like ECH-associated protein 1
LCYB	Lycopene β cyclase
LCYE	Lycopene ε-cyclase
LHC	Light-harvesting complex
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MeOH	Methanol
MEP	Non-mevalonate pathway
MEV	Mevalonate pathway
MTBE	Methyl tert-butyl ether
NCED	9-Cis-epoxycarotenoid dioxygenase
NF-κB	Nuclear factor kappa -light-chain-enhancer of activated B cells
Nrf-2	Nuclear factor erythroid 2-related factor 2
NSY	Neoxanthin synthase
O/W	Oil-in-Water
PDS	Phytoene desaturase
pI	Iso-electric point
PPI	Pea protein isolate
PS	Phytoene synthase
PS II	Photosynthetic reaction center II
RA	Retinoic acid
RAR	Retinoic acid receptor
RDA	Recommended dietary allowance

ROS	Reactive oxygen species
RXR	Retinoid X receptor
SC	Sodium caseinate
SPI	Soy protein isolate
TAG	Triacylglycerides
TNFα	Tumor necrosis factor α
TRL	Triacylglycerol-rich lipoprotein
WPI	Whey protein isolate
ZDS	ζ-Carotene desaturase
ZEP	Zeaxanthin epoxidase
Z-ISO	ζ-Carotene isomerase

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Introduction

Carotenoids are lipophilic phytochemicals associated with several health benefits, such as their potential role in preventing chronic diseases, including type 2 diabetes, cardiovascular diseases, certain types of cancer, as well as age-related macular degeneration [1-4]. In addition, a low intake of provitamin A carotenoids within a mixed diet represents a major health concern in populations with low preformed vitamin A intake (such as those living in developing countries where the access to meat and animal products is often limited, or vegans/vegetarians), leading to increased susceptibility to infections, growth disorders, skin diseases, xerophthalmia and night-blindness (nyctalopia) [5].

Micellization of carotenoids, which includes the release from the food matrix, transfer into lipid droplets and incorporation into mixed micelles, can be considered a determining factor for further carotenoid bioavailability. Due to their lipophilic nature, the bioavailability of carotenoids, including β-carotene, is relatively low, typically between 10 and 20% [6, 7]. This depends on host and dietary factors influencing carotenoid bioavailability aspects (i.e. their bioaccessibility and absorptive processes), including dietary lipids [8, 9], dietary fiber [10], and higher concentrations of divalent minerals [11-13].

One factor that may impinge on carotenoid absorption, but has not yet received much attention, is the presence of proteins. Several proteins have been shown to have emulsifying properties during digestion, and have been successfully employed for encapsulation of

liposoluble bioactive compounds, including β-carotene, significantly improving their bioaccessibility. In fact, following their adsorption to lipid droplet surfaces, proteins may stabilize the resulted emulsions in the gastro-intestinal (GI) tract, and limit lipid oxidation and degradation of sensitive molecules [14], attributed to the fact that proteins can be highly surface active molecules [15, 16]. The formed particles tend to be highly negatively charged, preventing the aggregation of lipid droplets [17]. This would facilitate the formation of smaller lipid droplets, and their processing into mixed micelles. However, some proteins in contrast may decrease the enzymatic degradation of lipids, likely by hindering digestive enzymes to adsorb to the droplet surfaces or binding to enzymes directly, implying potential negative influences of proteins on the micellization process [18].

While proteins have been employed for encapsulation and delivery of poorly soluble compounds [19], including carotenoids [20], the interactions between proteins and carotenoids in food matrices under conditions resembling the *in vivo* environment have, to our knowledge, never been studied systematically.

The present thesis aims to study the influence of proteins on carotenoid digestion and aspects of bioavailability. Chapter 1 presents a brief overview on carotenoids, while Chapter 2 (adapted from a review submitted to an international peer-reviewed journal) reviews the state of knowledge on the interactions of protein and liposoluble bioactive compounds. The specific objectives of the thesis are set in Chapter 3. The experimental part of the thesis includes Chapters 4, 5, 6, 7 and 8, which are articles published in international peer-reviewed

journals. These Chapters include *in vitro* studies, by means of simulated GI digestion protocol [21], coupled to Caco-2 cellular uptake model, in order to select proteins most strongly interacting with carotenoid solubilization, mixed micelle formation, and absorption aspects, preceding a human trial aiming at investigating the effect of added proteins on carotenoid bioavailability from a test food rich in carotenoids. All of this work is discussed in Chapter 9, and Chapter 10 presents some prospects on global nutrition aspects and future research directions.

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Chapter 1

Carotenoids: A brief overview

1.1. Carotenoid structures and biosynthesis

1.1.1. Characteristics and classification

Carotenoids belong typically to the category of tetraterpenoids. They are present in living organisms in the form of lipophilic pigments. They have attracted the attention of chemists and biologists since at least the early 1800s, after the isolation of the first carotenoid, β-carotene, by Wackenroder in 1831, while its empirical formula, C40H56, was established by Willstatter and Mieg in 1907 [1]. To date, approximately 800 carotenoid structures have been isolated from natural sources [2], among which over 500 structures have been fully characterized [3].

Several aspects contribute to the diversity of carotenoids. Firstly, the number of carbons making up the backbone of the molecule can vary from 30 to 50, with a majority of molecules having a C40 structure. Then, the structure of these pigments can be completely linear or comprise one or two cyclic structures, which are located at the ends of the molecule. Thirdly, these cyclic structures can also vary (Figure 1.1). A further element allowing this structural diversity is the presence or absence of oxygen atoms in the molecule, giving rise to non-oxygenated carotenoids known as carotenes, and oxygenated carotenoids termed xanthophylls. The oxygen atoms are usually inserted at the ends of the molecule, and can be present in the form of hydroxyl-, ketone-, epoxy- or methoxy-groups (Figure 1.1).

Color is the characteristic element of these molecules. Indeed, most carotenoids show a yellow to red color. Their system of conjugated double bonds (i.e. 3-13) creates a chromophore inducing

the absorption of visible light in the range between 400 nm and 500 nm. Only a few carotenoids, mostly those constituting precursors for further unsaturated carotenoids, such as phytoene, do not share that property. Thus, the degree of chromophore conjugation determines the absorption characteristics of the carotenoid [4]. The higher number of conjugated double bonds, the higher the maximum absorbance wavelength (λ_{max}) of the carotenoid and the darker its red color.

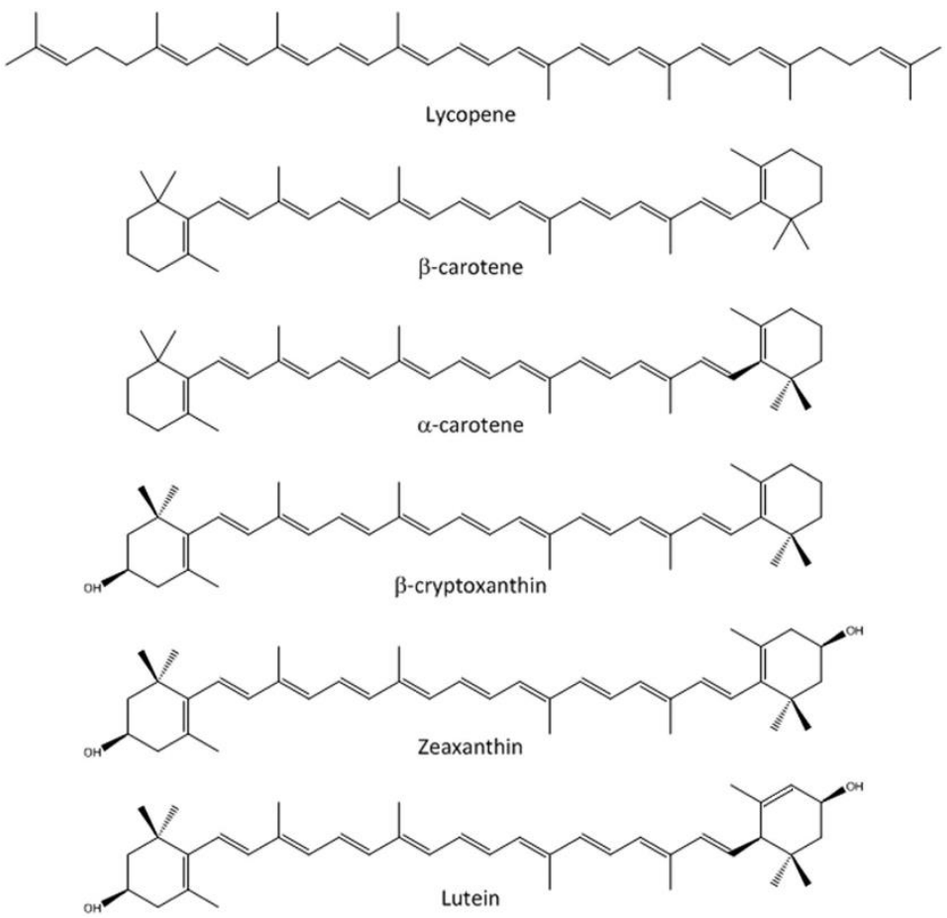

Figure 1.1. Chemical structure of the major carotenoids present in the diet and human plasma. Some structural differences exist between carotenoids, with can be of a linear (trans-lycopene) or also cyclic structure (β-carotene, α-carotene, lutein and neoxanthin). Structures can also be non-oxygenated (carotenes; trans-lycopene, β-carotene and α-carotene) or oxygenated (xanthophylls; lutein and zeaxanthin).

1.1.2. Carotenoid-producing organisms

Photosynthetic organisms such as anoxygenic photosynthetic bacteria and cyanobacteria (prokaryotes), as well as algae and higher plants (eukaryotes) synthesize carotenoids. Likewise, many non-photosynthetic organisms such as actinobacteria and fungi also synthesize these compounds [5].

In plants, carotenoids are clearly identifiable by the color they impart to fruits, flowers and roots (Figure 1.2). In fact, carotenoids are located either in the chloroplasts of the green vegetable leaves, associated to proteins together with chlorophylls, as part of the light-harvesting complex (LHC), or present in the chromoplasts such as in carrots and tomatoes, in the form of crystalline agglomerations [6]. Animals are unable to synthesize carotenoids, with the recently added exception of aphids [7]. Their presence in certain organisms such as crustaceans, insects, fish, and birds results from dietary absorption [8], and likewise contributes to the coloration of these animals.

Figure 1.2. Presence of carotenoids in living organisms.

1.1.3. Biosynthesis

Carotenoid biosynthesis begins within the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, which results in the formation of isopentenyl pyrophosphate (IPP) for the synthesis of the intermediate geranylgeranyl pyrophosphate (GGPP) (Figure 1.3). In fact, IPP molecules synthesized in plastids are isomerized via IPP isomerase (IPI) to the allylic isomer, dimethylallyl pyrophosphate (DMAPP). Three IPP molecules condense with DMAPP to generate GGPP, in a process involving GGPP synthase (GGPPS) (Figure 1.3). GGPPS plays a central intermediary role in the synthesis of plastid isoprenoids including chlorophylls, carotenoids and prenylquinones (Figure 1.3).

Regarding the carotenoid pathway, phytoene, a colorless carotenoid, is synthesized from two molecules of GGPP, via phytoene synthase (PSY). Biosynthesis continues with a set of desaturations and isomerizations of phytoene to produce trans-lycopene. The first step includes the action of phytoene desaturase (PDS), producing phytofluene, followed by the action of both PDS and ζ-carotene isomerase (Z-ISO) to produce ζ-carotene. The second step comprises the action of ζ-carotene desaturase (ZDS), followed by that of carotenoid isomerase (CRTISO), leading to the production of all-trans lycopene [9].

Subsequently, lycopene is converted into either β-carotene, via lycopene β-cyclase (LCYB), or into α-carotene, by the successive action of lycopene ε-cyclase (LCYE) and LCYB.

The hydroxylation of β-carotene by the enzyme β-carotene hydroxylase (CHYB) results in the synthesis of zeaxanthin, while the hydroxylation of α-carotene by ε-carotene hydroxylase (CHYE) and CHYB results in the formation of lutein.

The double action of zeaxanthin epoxidase (ZEP) on zeaxanthin and then on antheraxantin results in the formation of violaxanthin. Finally, the cleavage of violaxanthin by neoxanthin synthase (NSY) results in the formation of neoxanthin [9].

1.2. Biological functions of carotenoids

1.2.1. In photosynthetic organisms

In photosynthetic organisms, carotenoids are synthesized in chloroplasts and accumulate in the thylakoid membranes of these organelles [9], near the photosynthetic reaction center II (PS II) of LHC, with chlorophyll a and b, where carotenoids transfer the absorbed energy to the latter during photosynthesis, as carotenoids are able to absorb blue light in an even wider range than chlorophylls [4].

A second role of carotenoids is their protective effect against photo-oxidative damage in plant cells due to their antioxidant property given by the conjugated bonds of the polyene chain [10]. In fact, carotenoids have been reported to absorb the excess of energy resulted from reactive oxygen species (ROS), and are able to neutralize singlet oxygen ($^1O_2$) produced by the chlorophyll triplet in the PS II [11]. Furthermore, carotenoids paly also a role in protecting plants against photo-oxidative damage through dissipation of heat energy [12]. This process occurs when excess energy increases the

transmembrane pH gradient of thylakoids, because of an accumulation of protons, which induce protein conformational changes of individual LHCs, promoting heat dissipation [13, 14].

In flowers and fruits, carotenoids produce bright colors. They can be cleaved to produce volatile compounds such as scent and flavor active compounds, with the primary aim to attract insects, birds and other animals for pollination and seed dispersal [15].

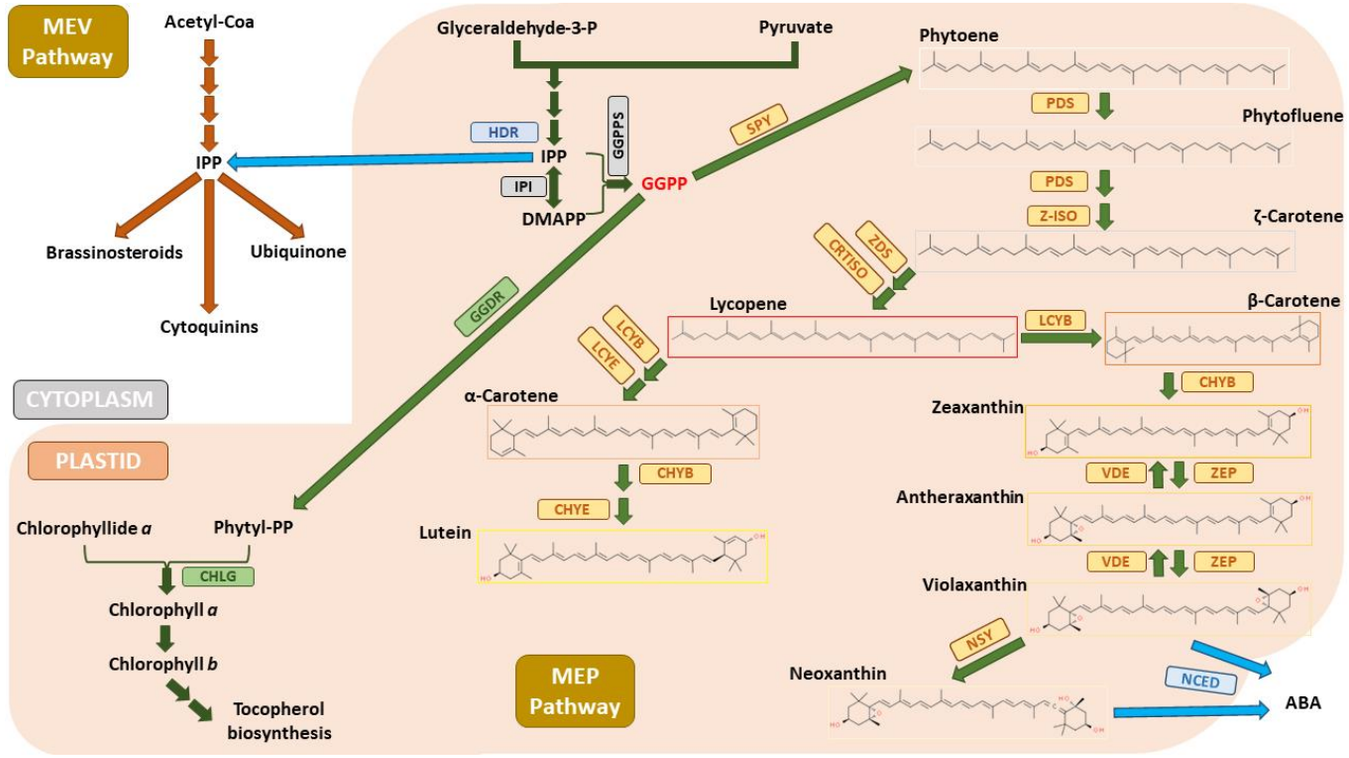

Figure 1.3. Scheme of carotenoid biosynthesis in plants (adapted from [16]). The non-mevalonate pathway (MEP) occurs in the plastids, while the mevalonate pathway (MEV) occurs in the cytoplasm of the cell. Isopentenyl pyrophosphate (IPP) and geranylgeranyl pyrophosphate (GGPP) are the key metabolites in the biosynthesis of chlorophylls and carotenoids. Abbreviations: 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR); isopentenyl pyrophosphate isomerase (IPI); dimethylallylpyrophosphate (DMAPP); geranylgeranyl pyrophosphate synthase (GGPPS); phytoene synthase (PS); phytoene desaturase (PDS); ζ-carotene isomerase (Z-ISO); ζ-carotene desaturase (ZDS), carotenoids isomerase (CRTISO); lycopene β-cyclase (LCYB), lycopene ε-cyclase (LCYE); β-carotene hydroxylase (CHYB); ε-carotene hydroxylase (CHYE); zeaxanthin epoxidase (ZEP); neoxanthin synthase (NSY); abscisic acid (ABA); geranylgeranyl reductase (GGDR), chlorophyll synthetase (CHLG); 9-cis-epoxycarotenoid dioxygenase (NCED).

1.2.2. In non-photosynthetic organisms

In animals, some carotenoids (such as β-carotene, α-carotene and β-cryptoxanthin) are precursors of vitamin A, which plays important roles, notably in vision and cell differentiation [17, 18]. In fact, the symmetrical cleavage of β-carotene at the central 15,15' double bond is facilitated by the enzyme β-carotene 15,15'-oxygenase 1 (BCO1) in the enterocytes, and results in the generation of two molecules of retinal (Figure 1.4) [19]. An asymmetric cleavage could also occur at the 9',10' double bond by the action of β-carotene 9',10'-oxygenase 2 (BCO2), which yields β-apo-10'-carotenal and β-ionone [20].

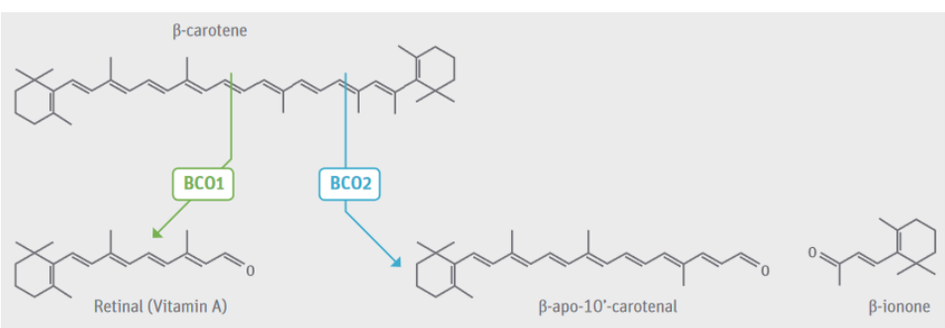

Figure 1.4. Enzymatic cleavage of β-carotene (adapted from [19, 20]). Oxidative cleavage of β-carotene at the central 15,15' double bond is catalyzed by the enzyme β-carotene 15,15'-oxygenase 1 (BCO1) and leads to the generation of two molecules of retinal (β-apo-15-carotenal). Cleavage at the 9',10' double bond is catalyzed by β-carotene 9',10'-oxygenase 2 (BCO2) and yields β-apo-10'-carotenal and β-ionone.

Carotenoids are also believed to be involved in the prevention of certain diseases [21]. The ingestion of carotenoids and their presence at different tissue levels and in blood plasma/serum have been inversely correlated in epidemiological studies, with several chronic diseases such as age-related macular degeneration and cataracts, caused by photo-damage of the retina, especially among the elderly [22, 23]. Likewise, several prospective studies have suggested that the consumption of carotenoids in the diet is associated with a reduction in the risk of cardiovascular diseases [24], development of type 2 diabetes [25], certain cancers [26], and a multitude of other mortality causes [27]. Furthermore, carotenoids have recently been demonstrated to influence microbiota composition and improve blood lipids in subjects with obesity [28].

Carotenoids including possibly the action of BCO1 and BCO2 would have an important role in the regulation of the inflammatory response [29], by interacting with the cysteine residues of the I kappa-B kinase (IKK) and/or nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB) subunits, leading to the inactivation of the NF-κB pathway [30]. Carotenoids are also involved in the protection of cells against oxidative stress caused by ROS [31]. Indeed, during a redox imbalance, carotenoids or their derivatives interact with Kelch-like ECH-associated protein 1 (Keap1) and change its physical properties, which fosters the dissociation of the Keap1- Nuclear factor erythroid 2-related factor 2 (Nrf-2) complex, leading to Nrf-2 activation and thus expression of downstream genes [32] (Figure 1.5). In addition, carotenoids are efficient physical and chemical quenchers of 1O2, via deactivation of the latter by conversion of an excess of

energy to heat, or via auto-oxygenation of carotenoids, respectively [33]. Carotenoids have also been reported to effectively scavenge ROS and other free radicals, via multiple types of reactions such as electron transfer reaction between carotenoids and free radicals [34].

Some studies have also hypothesized a possible involvement of carotenoids in the MAPK (mitogen-activated protein kinase) signaling pathway [35] (Figure 1.5).

Finally, the products of the asymmetric cleavage of β-carotene, i.e. β-apocarotenoids, regulate transcriptional responses by interfering with specific nuclear receptors, including the retinoic acid receptor (RAR), the retinoid X receptor (RXR), as well as the peroxisome proliferator-activated receptors (PPARs) [36].

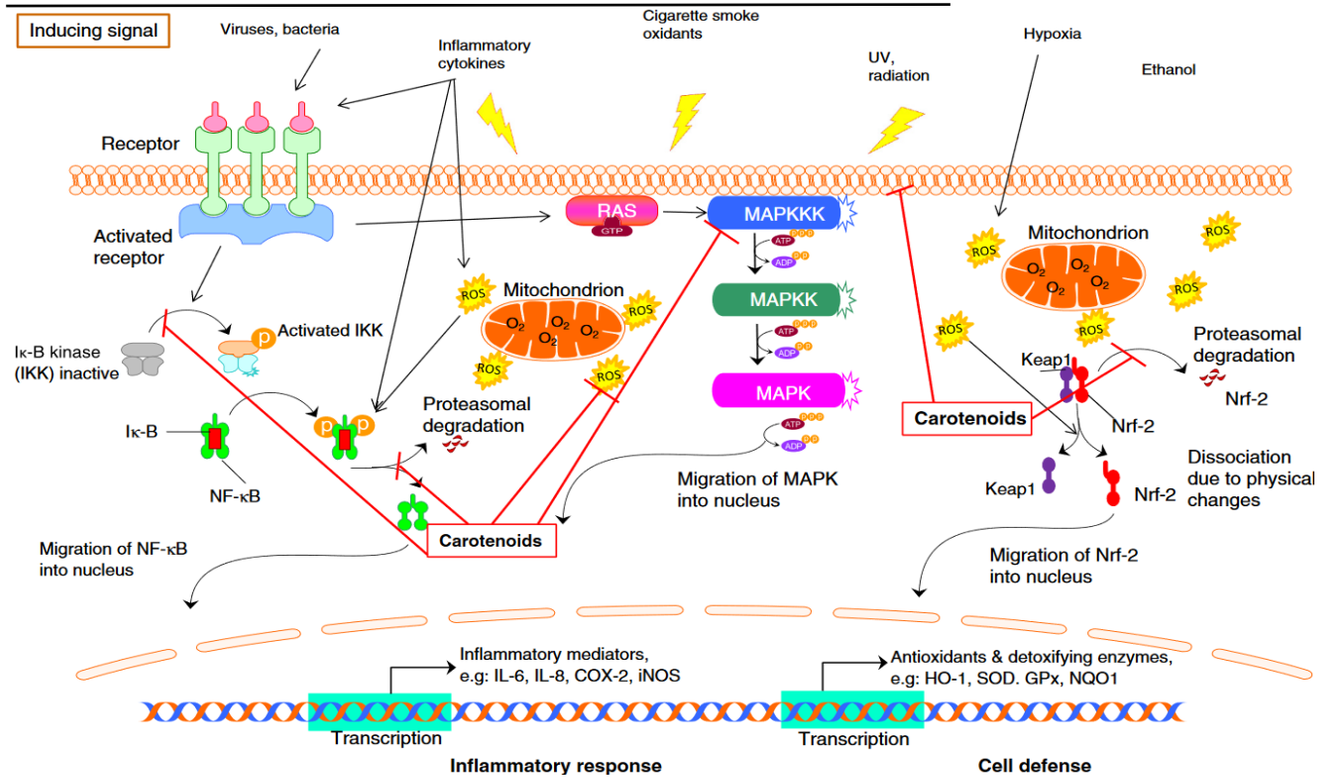

Figure 1.5. Inflammatory signaling pathways and carotenoids (Figure from reference [30]). the key metabolites in the biosynthesis of chlorophylls and carotenoids. Carotenoids or their derivatives would have an important role in the regulation of the inflammatory response, by interacting with the cysteine residues of the I kappa-B kinase (IKK) and/or nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB) subunits [26],by interacting with Kelch-like ECH-associated protein 1 (Keap1) [32], by being involved in the signaling pathway of (mitogen-activated protein kinase) MAPK [37]. Other abbreviations: Nuclear factor erythroid 2-related factor 2 (Nrf-2); I kappa-B (Iκ-B); MAPK kinase (MAPKK); MAPKK kinase (MAPKKK); reactive oxygen species (ROS); interleukin (IL); cyclooxygenase-2 (cox-2); inducible nitric oxide synthase (iNOS); heme oxygenase-1 (HO-1); superoxide dismutase (SOD), glutathione peroxidase (GPx); NAD(P)H quinone dehydrogenase 1 (NQO1).

1.3. Diet and carotenoids

1.3.1. Food sources and dietary intakes

Carotenoids are a large heterogeneous group of phytochemicals present in distinctive patterns in numerous food sources. The consumption of colorful fruits and vegetables is the main natural source of carotenoids for humans, with a predominance of lutein, β-carotene, violaxanthin, and neoxanthin in green leafy vegetables [38]. Carotenoid content of plant products vary according to the species, the season, the place and the cultivation techniques, as well as the stage of maturity of the products [39-41]. There are also other sources of carotenoids, such as from animals, including eggs, certain fish, seafood, as well as certain processed foods, including juice/vegetable beverages, butter, or margarine [42]. In recent years, a number of supplements containing significant amounts of these compounds have also become available [43].

Based on dietary patterns, the daily intake of carotenoids may vary between cultures and geographic areas, as this depends on the intake of carotenoid-containing food sources. In Europe, the daily intake of carotenoids is estimated between 10 and 15 mg/day [44], which could be achieved by the daily consumption of 100-200 g of vegetables and fruits with a particularly high carotenoid content [45, 46]. Although national recommendations for the daily intake of fruits and vegetables agree to some extent, there are currently no recommendations for the daily intake of carotenoids.

In human serum, the most common carotenoids are the same as those most commonly encountered in the diet: β-carotene, α-carotene,

β-cryptoxanthin, lycopene, lutein and zeaxanthin (Table 1.1) [47]. However, the concentrations of various carotenoids in human serum and tissues remain highly variable (Table 1.1), and depend on a number of factors.

Table 1.1. Carotenoid concentrations found in human serum and in human liver [48].

Carotenoids	Serum (μmol/L)	Liver (μmol/g)
α-Carotene	0.02 - 0.47 (1.0 - 25.3 μg/dL)	0.075 – 10.8 (0.04 – 5.8 μg/g)
β-Carotene	0.04 – 2.26 2.2 – 122.7 μg/dL)	0.39 – 19.4 (0.21 –6.3 μg/g)
β-Cryptoxanthin	0.03 - 0.70 (1.4 - 38.2 μg/dL)	0.037– 20.0 (0.05 – 11.0 μg/g)
Lutein	0.10 - 1.23 (5.8 - 69.8 μg/dL)	0.10 – 3.0 (0.06 – 6.9 μg/g)
All-trans-lycopene	0.05 - 1.05 (2.7 - 54.6 μg/dL)	0.20 – 17.2 (0.11 – 11.1 μg/g)

1.4. Determination of consumed carotenoid levels

To determine the intake of carotenoids in a population, two important points must be considered. The first being the quantity and frequency of food consumption, while the second concerns the carotenoid content of food products consumed. The estimation of the quantity of carotenoids consumed by an individual can be carried out directly or indirectly.

Direct estimation is a double serving technique. For each food consumed, the equivalent is collected and the total for the day is analyzed for its carotenoid content. As for the indirect measurements, these are based on a food survey (e.g. questionnaire on the frequency of consumption and the quantity consumed from a list of foods). From the quantities of food declared, the quantities of carotenoids consumed are calculated using food composition tables (example shown in Table 1.2).

Table 1.2. Carotenoid composition and content of different vegetal food items (mg/100 g of food) (adapted from [44]).

Food source	-Carotenoids-				
	α-carotene	**β-carotene**	**Lutein**	**Trans-lycopene**	**β-cryptoxanthin**
Apple	-	0.022	0.052	-	0.010
Apricot	0.037	0.953	0.066	-	0.130
Beans	-	-	-	-	
Broccoli	-	0.944	1.596	-	0.024
Brussels sprout	-	0.324	0.669	-	-
Butter	-	0.410	-	-	-
Carrot	2.186	7 .975	0 .271	-	-
Celery	-	0.570	0.860	-	-
Melon	-	0.03	-	-	-
Grapefruit	0.000	1.310	0.00	3.362	0.000
Cherries	0.002	0.013	0.048	0.010	0.005
Mango	-	1.30	-	-	0.054
Orange	0.016	0.152	0.106	-	1.309
Parsley	-	5.062	8.006	-	-
Pizza	-	0.34	0.23	4.30	-
Potato	-	0.00	0.06	-	-
Pumpkin	-	0.49	0.63	-	-
Spinach	-	0.000	2.255	-	0.023
Maize	0.033	0.024	0.819	-	-
Tomato	-	0.608	0.077	2.718	-
Watermelon	0.000	0.154	0.027	3.477	0.062
Onion	-	0.001	0.002	-	-

1.5. Digestion process of carotenoids

1.5.1. Micellar solubilization

The release of carotenoids from the food matrix starts generally in the mouth, after chewing (Figure 1.6). Subsequently, carotenoids as well as other lipidic microconstituents follow the fate of lipids in the gastro-intestinal (GI) tract [49]. Gastric lipase promotes the formation of lipid droplets following the cleavage of about 10 to 30% of the triacylglycerols (TAGs) in the stomach. In addition to containing other constituents such as di- and triglycerides, free fatty acids and cholesterol [50], lipid droplets also allow the solubilization of the previously released carotenoids from the food matrix (Figure 1.6) [51].

Partially digested food is then directed via the stomach pylorus to the duodenum, where intestinal digestion begins with the secretions of bile and pancreatic enzymes. Bile acids emulsify lipid droplets, while pancreatic lipase, activated following an increase in pH, hydrolyzes TAGs. This action induces the formation of mixed micelles about 3-8 nm in diameter [52, 53]. These micelles can contain cholesterol, phospholipids, free fatty acids, monoglycerides, lysophospholipids, liposoluble vitamins (A, D, E, and K), carotenoids, and bile salts that are needed for their structure [54] (Figure 1.6). Some carotenoids may also interact with proteins, as demonstrated earlier by Mensi and co-workers, such as the ability of β-lactoglobulin to bind β-carotene [55].

Bioaccessibility of carotenoids, which includes release from the food matrix, transfer into lipid droplets and incorporation into mixed micelles (micellization), can be considered as a determining factor for bioavailability. The micellar incorporation of carotenoids is rather

incomplete, typically below 40% for carotenes, while estimated at around 60% for xanthophylls. This variability is probably due to the presence of hydroxyl groups in xanthophyll structures that increase their solubility towards the surface of mixed micelles, whereas this solubility is rather limited for the linear hydrocarbon-based and very apolar carotenes, preferably located in the hydrophobic internal part of the particles [50]. Interestingly, the linear but flexible molecular structures of phytoene and phytofluene (precursors of all other carotenoids, Figure 1.3), compared to carotenes, increase their incorporation into mixed micelles, resulting in a very high bioaccessibility [56]. In humans, the incorporation of carotenoids into mixed micelles seems to be a limiting factor in case of disorders associated with micellar solubilization: lipid malabsorption, bilio-pancreatic damage, and hepatic damage are associated with carotenoid malabsorption [57].

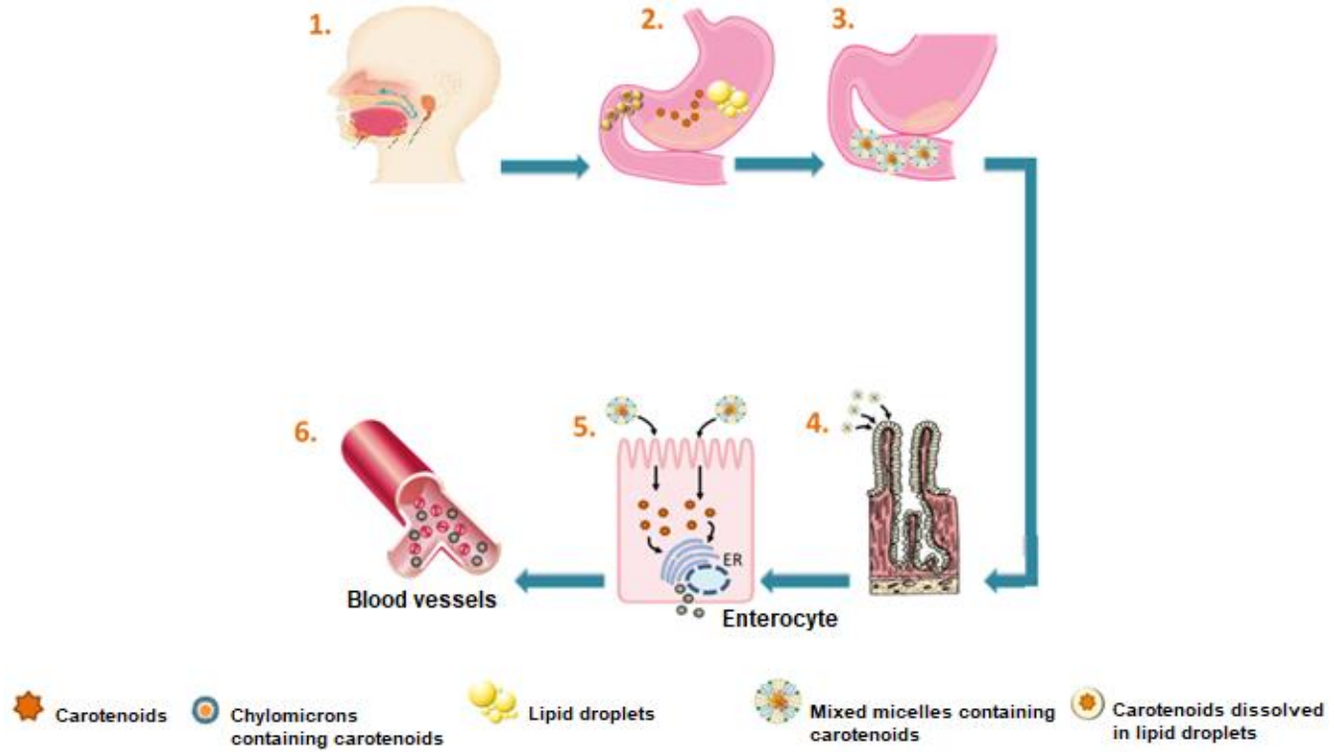

Figure 1.6. The key steps in the absorption of carotenoids. Step 1: Onset of carotenoid release from the food matrix. Step 2: Dissolution into lipid droplets. Step 3: Incorporation into mixed micelles. Step 4: Intestinal absorption by enterocytes. Step 5: Intracellular transfer of carotenoids followed by their incorporation into chylomicrons. Step 6: Transport into the bloodstream and distribution throughout the body via lipoproteins.

1.6. Transport across the intestinal epithelial brush border membrane

Carotenoids can be transported across the brush border membrane of the enterocyte by passive and/or facilitated diffusion. The passive diffusion mechanism is determined by a concentration gradient between the micellized carotenoids and the enterocyte membrane (Figure 1.6), while the facilitated diffusion occurs via a transporter-dependent process.

In vitro study findings employing mostly Caco-2 cell models, have favored carotenoid uptake facilitated by membrane receptors such as scavenger receptor class B type I (SR-BI), cluster of differentiation 36 molecule (CD36), and Niemann-Pick type C1 Like 1 protein (NPC1L1), at least for certain carotenoids [49], and when present at not too high, i.e. physiological, concentrations. For instance, SR-BI has been described as a main transporter in the intestinal uptake of lycopene, lutein [58], α-carotene, β-carotene, β-cryptoxanthin [59], phytoene and phytofluene [60]. CD36, another scavenger receptor expressed in the duodenum and jejunum, has been reported to be involved in the uptake of lycopene, provitamin A carotenoids, and lutein [59, 61]. Finally, NPC1L1 has been suggested to facilitate β-carotene, α-carotene, lutein, and β-cryptoxanthin intestinal uptake [62, 63].

1.6.1. Cytosolic transfer and intracellular metabolism

Some protein carriers have been hypothesized to be involved in the cytosolic transfer of carotenoids. For instance, the intestinal fatty

acid binding protein (IFABP), a fatty acid transfer protein, may affect the transfer of carotenoids from the membrane to the intracellular organelles, in particular the Golgi apparatus, the site of assembly of chylomicrons [64]; however, this has never been confirmed. Similarly, the human retinal lutein-binding protein (HR-LBP), present in human retina, has been suggested to be an intestinal intracellular transporter of xanthophylls [65].

It is noteworthy mentioning that up to 60% of the absorbed carotenoids are metabolized [66]. Depending on the species, different processes can be involved in carotenoid cleavage. For instance, β-carotene can be cleaved into retinal through the single action of BCO1 [19], while β-cryptoxanthin can be cleaved into retinal by the action of mitochondrial BCO2, followed by the action of the cytosolic BCO1 [67]. The asymmetric cleavage of provitamin A carotenoids gives rise to an alternative pathway to produce vitamin A [68]. However, it has been reported that BCO2 can be involved in the asymmetrical cleavage of both provitamin A and non-provitamin A carotenoids into apo-carotenoids [69].

1.6.2. Transport, body distribution and excretion

Following their cleavage, the resulting carotenoid metabolites are incorporated into chylomicrons, that are then secreted into the lymph to join the bloodstream [70]. Subsequently, carotenoids are taken up by the liver, remodeled and secreted into various lipoproteins (Figure 1.6).

In healthy humans, the distribution of total carotenoids between the different lipoproteins is estimated at 55% in LDL (low density lipoproteins), 31% in HDL (high density lipoproteins) and 14% in VLDL (lipoproteins of very low density) [71]. It is also noted that the distribution varies according to the structure of the carotenoids, 58 to 73% of α-carotene, β-carotene and lycopene are found in LDL, 53% of lutein and zeaxanthin are in HDL, while β-cryptoxanthin is equally distributed between LDL and HDL [71].

The main stores of carotenoids in humans are in the adipose tissue (containing around 80% of total body carotenoids) and in the liver (around 10%). However, other organs show high concentrations, such as in testes, the ovaries, and the adrenal glands [72], possibly due to the fact that these tissues are rich in LDL receptors, which allow the accumulation of high levels of carotenoids. Carotenoids are also found in many other organs (skin, pancreas, kidney, spleen, heart, thyroid, and eye) [73]. Similarly, carotenoids have been determined in a variety of tissues, such as the eye, which has the particularity of a predominance of lutein and zeaxanthin in the retina, while their metabolite, meso-zeaxanthin, is exclusively found in the macula [74]. The tissue distribution of carotenoids could be related to their biological role, and their variable concentration at different tissue levels suggests regulatory mechanisms that are currently not elucidated [75].

The subsequent stages of carotenoid fate is less well documented. Excretion of carotenoids and/or their degradation products occurs primarily through the bile and pancreas in faeces [76], with urinary excretion being negligible [77]. However, it should be

noted that 50-80% of the carotenoids in the diet are not absorbed and are available for the gut microbiota in the large intestine. At this level, carotenoids and/or their metabolites may be (re)absorbed and/or have an effect on the transcription of certain factors and thus on human metabolism [78]. The results from animal trials showed that astaxanthin and β-carotene (in the metabolite form of retinoic acid) may contribute to the gut immune homeostasis by regulating IgA production, thereby preventing gut microbiota dysbiosis [79]. Moreover, carotenoids have recently been demonstrated, in a human intervention trial with subjects with obesity, to influence microbiota composition, related to positive health effects such as improved blood lipids [28]. At this time, further investigations are warranted to study the relationship between the gut microbiota and carotenoids.

1.7. Methods to assess carotenoid bioavailability

1.7.1. *In vivo* models

The use of the human body remains the most appropriate approach to investigate the bioavailability of carotenoids. However, as pointed out previously by Brodkorb et *al*, it is necessary to follow the digestive processes within the human digestive tract in more detail in order to understand the physiological response to specific foods [92]. This can be investigated with invasive procedures, such as aspiration from the stomach [80] or the small intestine [81], or with less invasive processes such as imaging technologies [82].

Animal models are also widely used. Several species of rats and mice have been used to evaluate carotenoid bioavailability [83, 84],

such as Mongolian gerbils (Meriones unguiculatus) [85]. Unfortunately, their use generally involves animal death or surgical approaches to access the contents of the GI tract. In addition, the relevance of animal models for understanding the digestion of food in humans is also regularly questioned, as the physiology of animal species differs significantly from that of humans [84].

In summary, *in vivo* (human or animal) trials can be difficult to undertake, be very expensive and are ethically challenging. Although nutritional studies using human subjects are still being considered the "gold standard", *in vitro* models have been developed as alternatives to simulate the digestion of food, and assess some aspects of carotenoid bioavailability.

1.7.2. *In vitro* models

The development of the static *in vitro* models simulating human digestion processes, by subjecting food samples to sequential oral, gastric and intestinal digestion, has facilitated the assessment of carotenoid bioaccessibility, which is an important prerequisite for their bioavailability [86].

In addition to allowing a relatively large number of samples to be measured in parallel for screening purposes, these models have the advantage of being low costs, more rapid, less personal and work effrot, and without ethical limitations [87]. In addition, *in vitro* models appear very suitable for mechanistic studies and hypothesis building [88]. For instance, they have been widely used to investigate the effect

of various dietary factors on carotenoid bioaccessibility such as lipids [89], fiber [90], and divalent minerals [91].

The growing interest in nutrient digestion has led to the development of the harmonized INFOGEST *in vitro* model, to standardize several key parameters, such as stomach and intestinal transit times, pH, digestive enzymes and their activity, based on available physiological data and practicality aspects [87]. This harmonized static *in vitro* digestion method for foods aids in the generation of more comparable data. An amended and improved digestion protocol (INFOGEST 2.0) has clarified some uncertainties associated with the original method [92].

Another *in vitro* model is the standardized semi-dynamic digestion method, in which the oral and intestinal phases remain identical to the above mentioned static digestion model, but which is more specifically focused on the gastric phase [93]. This semi-dynamic model modulates nutrient release from the gastric chyme, by including crucial kinetic aspects associated with the gastric phase of digestion, such as gradual pH decrease, fluid and enzyme secretion, and gastric emptying, allowing a better evaluation of changes occurring in the structure and disintegration of foods. Several recommendations were proposed for each of these main dynamics of gastric physiology. For instance, a constant emptying rate was suggested, regardless of the physical state of the food sample, and based on the energy content of the three major macronutrients (lipids, carbohydrates and proteins). The emptying is regulated, so that equal numbers of calories are delivered to the duodenum with an average rate of 2 kcal min-1. However, since the objective of this model is the development of a

standard approach, one can note that a critical point of variability is the several emptying stages that could lead to sample losses, depending on the complexity of the nutrients and the property of the structures studied. Therefore, the application of this semi-dynamic model to various food matrices deserves further research.

1.7.3. Caco-2 cell model

The static *in vitro* digestion experiments are often coupled with cell culture studies, such as with Caco-2 cells (derived from a human colonic adenocarcinoma [94]), to address further mechanistic questions. The Caco-2 cell model allows less expensive and time-consuming studies, as compared to those using animal or human models. In addition, this model allows assessing intestinal uptake mechanisms and transport kinetics. It also permits identifying proteins playing a key role in the transport and metabolism of carotenoids in intestinal cells, as well as in the regulation of these processes, all of which relate to understanding the bioavailability of carotenoids [49].

After 2-3 weeks post confluence, Caco-2 cells differentiate spontaneously into mature epithelial cells. After this stage, Caco-2 cells show a cellular polarization and exhibit functional and morphological characteristics typical of absorptive enterocyte with tight junctions and a brush border membrane, simulating the small intestinal environment [95].

To study cellular uptake, only one compartment on the apical side of Caco-2 cells is used, while for transport studies, both

basolateral and apical compartments are used. Differentiated Caco-2 cells express a number of typical membrane transporters, including SR-BI, NPC1L1, and ATP-binding cassette transporter, subfamily A (ABCA1) [62], participating in intestinal efflux like that of cholesterol [96], though the identification of membrane transporters involved in the transport of carotenoids in these cells needs further research [49].

1.8. Dietary factors affecting carotenoid bioavailability

Although carotenoids are present in human blood plasma and tissues, their concentration is relatively low compared to the initial quantities present in the ingested food matrices. For instance, the bioavailability of β-carotene is typically between 10 and 20% [57, 97]. Several factors have been described to influence the bioavailability of carotenoids, and are stated in the mnemonic term "SLAMENGHI", developed by Castenmiller and West [98]: species of carotenoid; linkage at molecular level; amount of carotenoids consumed in a meal; matrix in which the carotenoids are incorporated; effectors of absorption and bioconversion; nutrient status; genetics; host-related factors; interactions of the various factors described above.

The above-mentioned determinants of carotenoid bioavailability can be summarized to intrinsic and extrinsic factors, namely host-related factors such as single nucleotide polymorphisms (SNPs) [99, 100], as well as food matrix-related factors, which affect carotenoid release, absorption, transport, metabolism and storage [101].

Host-related factors have been shown to modulate carotenoid bioavailability, including conditions that alter the GI surface area or digestive enzyme secretion [99]. For instance, bariatric surgery has been associated with modified gastric peristalsis movements that result in a decrease of plasma carotenoid concentrations [102], while Crohn's disease results in carotenoid malabsorption [103]. Abnormalities such as cholecystectomy (reducing the available amount of bile), and pancreatitis (leading to secretory insufficiency of digestive enzymes) have also been proposed to compromise carotenoid bioavailability [57, 104].

Regarding matrix-related factors, it has been acknowledged that dietary fat can significantly increase carotenoid absorption efficiency, by facilitating their transfer to the aqueous micellar fraction during digestion [105, 106]. In contrast, it has been concluded that dietary fiber can decrease carotenoid absorption, by altering mixed micelle formation and micellization processes of carotenoids and/or affecting enzymatic access and activity, which would inhibit carotenoid transfer from lipid droplets to mixed micelles [90]. Similarly, divalent minerals at high though still physiological concentrations were suggested to impair carotenoid bioavailability, likely by precipitating fatty acids and bile salts in the gut and/or affecting the stability of micelles [91, 107, 108].

One factor that may impinge on carotenoid absorption, but has not yet received much attention, is the potential influence of co-consumed dietary proteins on digestion processes of carotenoids. During digestion, co-consumed dietary proteins exhibit emulsifying properties by adsorbing to the lipid droplets, and form a viscoelastic

film that constitutes a physical barrier and prevents coalescence [109]. This may help to emulsify apolar dietary constituents by modulating their transfer into mixed micelles [110]. More recently, the potential effect of proteins to aid in the encapsulation process and delivery of lipophilic compounds [111, 112], including carotenoids [113, 114], has started to gain the attention of the pharmaceutical and food industry, but very little is known about the influence that proteins could have on the bioavailability of carotenoids, under conditions resembling the *in vivo* environment.

1.9. Conclusion

Taking into account the various health benefits of carotenoids and their limited absorption, understanding how factors, so far little explored, influence their availability is essential. One of the dietary factors mentioned above is the presence of proteins, the daily presence of which in the diet may interact with carotenoids and thus modulate their absorption. As a first step, it would be of interest to summarize recent findings on the possible interactions between proteins and liposoluble vitamins as well as secondary plant compounds, and potential influences on their digestion bioavailability aspects.

1.10. References

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Chapter 2

Interaction of proteins with lipophilic vitamins and secondary plant metabolites during digestion

Adapted from: Influence of proteins on the absorption of lipophilic vitamins and secondary plant metabolites – a review

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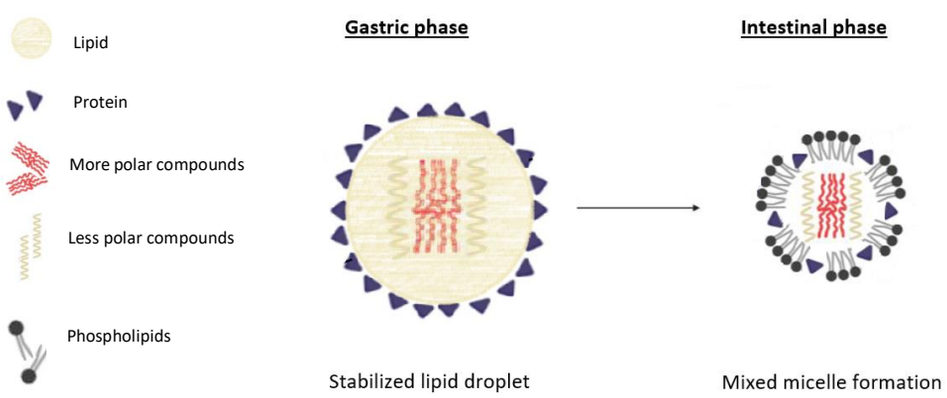
Foreword

In the present Chapter, we aim to focus on the use of typically consumed proteins or those employed for encapsulation purposes, either from animals or plant sources, as effective emulsifiers of apolar bioactive plant compounds during digestion. The functional properties of proteins related to their emulsification capacity, the chemical interactions with digestive enzymes and/or plant bioactive compounds taking place in the stomach and small intestine as well as potentials and limitations of the use of different protein matrices as emulsifiers are emphasized in this Chapter. We also summarize here the results from human, animal and *in vitro* trials investigating the relationship between protein intake and digestion, and bioavailability aspects, i.e. bioaccessibility and absorptive processes of liposoluble food constituents, such as liposoluble vitamins (A, D, E, K) as well as carotenoids and curcumin, as examples of secondary plant compounds.

Abstract

While proteins have been widely used to encapsulate, protect and regulate the release of bioactive food compounds, very little is known about the influence of co-consumed proteins on the absorption of lipophilic constituents during digestion. These compounds comprise vitamins (A, D, E, K) and secondary plant compounds such as carotenoids. Their bioavailability is often low and very variable, depending on the food matrix and host factors. Some proteins can act as emulsifiers during digestion, as their liberated peptides have amphiphilic properties that can facilitate the absorption of micro-constituents by improving their transition from lipid droplets into mixed micelles. Contrarily, the less well digested proteins could negatively impinge on enzymatic accessibility to the lipid droplets, slowing down their further processing into mixed micelles and entrapping apolar food compounds. Interactions with mixed micelles are also plausible, as shown for drugs. In this review, we aimed to focus on the ability of proteins to act as effective emulsifiers of plant lipophilic constituents during digestion. The functional properties of proteins, their chemical interactions with enzymes and food constituents during digestion, potentials and limitations for their use as emulsifiers are emphasized. Finally, protein properties that are essentially linked to their emulsifying activity are outlined, and we summarized findings from human, animal and *in vitro* trials on the evidence that proteins can modulate the absorption of liposoluble vitamins and secondary plant compounds.

Keywords: surface tension, rheology, fatty acids, emulsions, xanthophylls, carotenes, lipase, protease, zeta-potential.



Graphical abstract 2. Role of proteins in the stabilization of lipid droplets in the gastric phase, and in their subsequent processing into mixed micelles during the intestinal phase.

2.1 Introduction

Proteins are of primary importance for the human body, as the individual amino acids (9 of them being essential, and 6 further being conditionally- or semi-essential [1]) are building blocks for all human cells and tissues. Since free amino acids cannot be stored by the human body for later specific usage, a regular consumption of dietary proteins is paramount, and the recommended dietary allowance (RDA) of 60 g/d for healthy adults reflects this necessity [1]. Proteins do vary in their amino acid composition, and the ones comprising a high content in essential amino acids with relative ratios close to the cellular requirements have the highest biological value. These include egg, milk, animal meat and fish flesh proteins, as well as proteins from some plant sources such as those from soy and derived products [2].

Due to their different size and tridimensional structure [3], proteins hugely vary in their properties such as water solubility, amphiphilicity, digestibility in the human digestive tract, net charge (depending on surrounding pH and the protein's iso-electric point (pI)), and interaction of parts of their structure (e.g. hydrophobic pockets) with other molecules [4, 5].

During gastro-intestinal (GI) digestion, some proteins could act as emulsifiers, i.e. stabilizing an emulsion by increasing the interaction between lipophilic constituents and water-soluble ones, thus reducing surface tension [6]. Proteins with suitable emulsifying properties are generally quite amphiphilic, i.e. with both polar and apolar groups [7]. These proteins are thus interesting natural candidates to enhance the bioavailability of liposoluble micro-constituents, especially in the

context of lipid-poor, calorie-restricted diets which may otherwise go along with malabsorption. Indeed, it is well known that the liposoluble vitamins, i.e. vitamins A, D, E, and K, and other bioactive compounds with low polarity such as carotenoids, are poorly absorbed in the absence of dietary lipids [8, 9], which are needed to drive the formation of mixed micelles, consisting of fatty acids, phospholipids, mono- and di-glycerides, bile salts, and cholesterol [10]. It has recently been shown that proteins were able to modify the bioavailability of liposoluble micro-constituents, such as vitamin D3, on postprandial absorption in mice following the simultaneous intake of WPI [11].

The positive effects of proteins on the absorption of liposoluble micro-constituents are thought to be related to the following features:

a) Emulsifying properties, i.e. produced peptides during digestion could foster the solubility and stability of the lipid droplets while interacting with their surface, and promote their processing into mixed micelles [12];

b) Improving the enzymatic access to the surface of lipid droplets by altering surface tension and viscosity, [13];

c) Stabilizing the mixed micelles in the small intestinal phase (i.e. the chyme) by interacting with their surfaces, which prevent their aggregation [7];

d) Improving solubility and perhaps preventing the oxidation of lipophilic constituents following their interaction with proteins, via hydrophobic pockets within peptides [14, 15].

Negative effects are likewise possible, and may be explained by the following mechanisms:

a) Limiting access of lipases to lipid droplets, while forming a protein layer on their surface, which could prevent their processing into mixed micelles , even though the effect appears weaker compared to e.g. lecithin and Tween20 [16];

b) Reducing gastric lipase activity with the addition of certain proteins, such as β-lactoglobulin [17], explained by interfacial depletion of the substrate (lipids) into the interior of the droplets;

c) Causing precipitations of poorly soluble proteins and those of lower digestibility, which could entrap liposoluble constituents [13];

d) Interacting with micro-constituents at the surface of mixed micelles (e.g. xanthophylls), or even penetrating into the mixed micelles (Figure 2.1) causing their destabilization [18].

Considering their health benefits, the bioavailability of liposoluble vitamins and non-nutrients such as carotenoids is of great significance, and the investigation of the potential impact of their interaction with proteins on their absorption is meaningful. For instance, vitamin A deficiency is still a major micronutrient deficiency worldwide and a major cause for child mortality [19]. It is predominant in countries where provitamin A in form of apolar carotenoids such as β-carotene is the most relevant source, and protein intake is likewise often critical in those places, though this can also be a consideration for vegetarians/vegans [20]. Other vitamins, such as vitamin D, have also been reported to be present at marginal

to deficient concentrations in some populations, especially in persons living at higher latitudes where vitamin D formation by the sun is limited [21].

Another aspect demonstrating the importance of proteins for the bioavailability of fat-soluble micronutrients is their use as encapsulation materials [22]. For instance, β-carotene incorporated into whey protein complexes by microencapsulation showed good water solubility [14] as well as bioaccessibility following *in vitro* GI digestion [23], pointing out toward increased bioavailability of such combinations. Similar positive effects on the solubility and cellular transport (through Caco-2 cell monolayers) of curcumin using β-lactoglobulin as a carrier have been reported [24].

Therefore, in this review, we aimed to focus on the use of typically consumed proteins or those employed for encapsulation purposes, either from animals or plant sources, as effective emulsifiers of apolar bioactive plant compounds during digestion. The functional properties of proteins related to their emulsification capacity, chemical interactions with digestive enzymes and/or plant bioactive compounds taking place in the stomach and small intestine, challenges and limitations of the use of different protein matrices as emulsifiers is emphasized in this review. Finally, we have highlighted the properties of proteins which are essentially linked to their emulsifying activity, and we also summarized the results from human, animal and *in vitro* trials investigating the relationship between protein intake, digestion, and influence on the absorption of carotenoids as well as liposoluble vitamins (A, D, E, K) and the apolar polyphenol, i.e. curcumin.

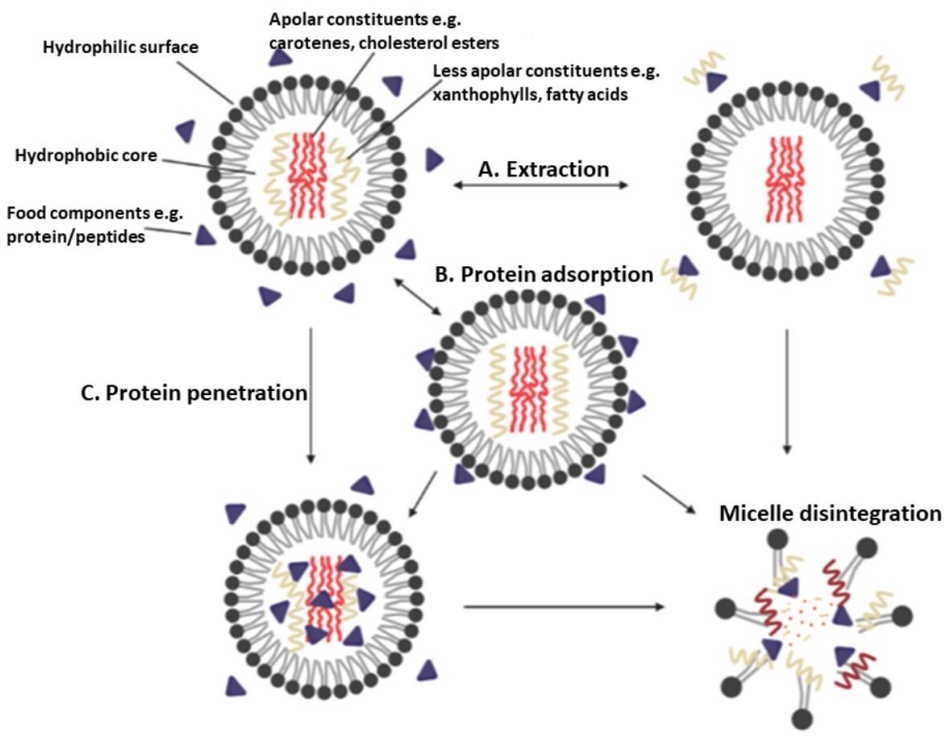


Figure 2.1. Degradation of mixed micelles depending on the presence of proteins (adapted from [18]).

2.2 General interactions of proteins with food constituents during digestion

2.2.1 Introduction

The recommended dietary allowance (RDA) of proteins for a healthy adult is 0.8 g/kg body weight per day, equivalent to about 60 g/day for a 75 kg adult [25]. Proteins consumed normally entail 20 genetically encoded amino acids, which are needed by the human body

to function. The 9 essential ones are histidine, isoleucine, leucine, valine, lysine, threonine, phenylalanine, methionine, and tryptophan [26]. When 2 to 10 amino acids are linked by peptide bonds, the term oligopeptide (or peptide) is used, including dipeptides, tripeptides, tetrapeptides and so on. Between 10 and 100, this is referred to a polypeptide (ca. >5.5-11 kDa), while above 100 amino acids, it is generally considered a protein (or as a long polypeptide).

The chemical and biological properties of proteins do vary considerably. With respect to acting as emulsifiers, molecular weight, water solubility, net charge at a given pH, shape and digestibility are among the most important attributes [27, 28]. For instance, food-derived proteins have been differentiated according to their water solubility into albumins (soluble), globulins (insoluble, but soluble in diluted salt solution), prolamins (insoluble, soluble in 70-80% ethanol) and glutelins (insoluble, soluble in diluted alkaline solution) [29]. The solubility of proteins is related to the net charge at a given pH. If the pI, where proteins have a net outer charge of zero, is close to the surrounding pH, then solubility is minimum. It has been emphasized that plant-based proteins generally exhibit a lower water solubility than animal-derived ones in the GI tract [30] (Table 2.1). For instance, the water solubility varies from e.g. 85 g/100 g (40oC, pH 3.5-7.5) for WPI [31] to only about 20 g/100 g (pH 2-7, 0.1 M NaCl) for soy protein isolate (SPI) [32], while above pH 7, solubility of the latter increases. Indeed, proteins with a low pI such as 4.5 for SPI (Table 2.1, [33]) tend to have limited solubility during the gastric phase of digestion, though other factors such as structure may affect water solubility more strongly.

Interaction of proteins with lipophilic vitamins and secondary plant metabolites during digestion

Proteins also contain typically non amino acid components, including e.g. phosphorous and calcium, and plant proteins can be tightly associated with flavonoids/isoflavonoids, lectins, saponins, and phytates [27]. These may affect protein digestibility, as has been reported for food processing [34].

Regarding their emulsifying properties, especially globular proteins (e.g. albumins, globulins), in contrast to fibrous proteins (e.g. collagen/gelatin (GEL)), have been emphasized to undergo unfolding and rearrangements in oil-water (o/w) films, forming a viscoelastic film at the interface [33], such as around lipid droplets. Their thickness may only range a little over 1 nm [33]. By doing so, they can effectively alter droplet size distribution by decreasing interfacial tension, and can stabilize emulsions by steric action and electrostatic forces [35]. This is especially the case when the pH is not too close to the pI of the protein, due to the higher net charges and repulsive electrical potential (zeta-potential) at the emulsion interface [36], while mechanical effects and a pH close to the pI can result in increased interactions between the proteins and thus produce coalescence. However, following binding to the interface, the films may thin out, resulting in the exposure of reactive protein groups, which could then lead to increased flocculation and coalescence of the lipid droplets. Such unfolding upon adsorption at the interfaces together with aggregation has clearly been shown for bovine serum albumin (BSA) and β-lactoglobulin [36].

Table 2.1. Overview of frequently consumed proteins and properties related to their emulsification properties.

Protein	Water solubility (pH 7), %	Water solubility (pH 2-4), %	Chain lengths (Da)	Iso-electric point (pH)	Digestibility (%) – global after GI	Other characteristics (Solubility in salt solution…)	Ref.
WPI	92.76 ± 0.46	87.13 ± 0.03	11 000 Da[1]	4.5	100	Major subunits: β-lactoglobulin (ca. 50%), α-lactalbumin (ca. 20%), immunoglobulins (ca. 10%), BSA (ca.6%), lactoferrin	[31, 37, 38]
SPI	80.2	80.5	9 300 Da	4.5	84.0	Major subunits: conglycinin (7S) and glycinin (11S)	[39-41]
SC	>90.0	<20.0	23 200 Da	4.6	76.25	α-casein and β-casein (4:1), k-casein	[42-44]
GEL	87.0	Ca. 70.0	220 000 Da[2]	4.86[3] 9.0[4]	98.0	α-chain, β-chain	[45-47]
Collagen[5]	95.0	~100	300 000 Da	7.0-8.0	98.0	α-chains	[48-50]
Rice	25.0-30.0	<20.0	25 000[6] Da	4.5	93.0	globulin, α-gluteolin	[51, 52]
Pea	70.0	30.0	320 000-380 000[7] Da	4.3	86.0	legumin (11S), vicilin (7s)	[53, 54]

Table 2.1. Overview of frequently consumed proteins and properties related to their emulsification properties (continued).

Protein	Water solubility (pH 7), %	Water solubility (pH 2-4), %	Chain lengths (Da)	Iso-electric point (pH)	Digestibility (%) – global after GI	Other characteristics (Solubility in salt solution…)	Ref.
Egg white	73.94[8]	34.75	45 000 Da	4.5	90.9[9]	ovoalbumin	[55, 56]
Chicken[10]	94.0	21.3	16 800 Da	5.5	90.3	α -2,3,4,6 and β2,3,5	[57, 58]
Cod	85.0	80.0	41 000 Da	5.5	86.0	gad c I, parvalbumin	[59-62]
Salmon	~10	90 (pH2) 10 (pH4)	205.000[11] Da	5.5	92.8	myosin, actin	[60, 63]

1. 1 A.A. equals 110 Da. 2. Bovine skin collagen. 3. Isoelectric point of acidic gelatin. 4. Isoelectric point of basic (alkaline) gelatin. 5. Hydrolysed collagen. 6. Brown rice protein. 7. Molecular weight of pea legumin. 8. Solubility analysed at pH 8. 9. Cooked egg-white. 10. Chicken breast. 11. Myosin.

2.2.2 Emulsifying properties of various proteins

Depending on the type and nature of the protein, the protein-coated lipid droplets may have different structure and rheological properties, which would imply a difference in the characteristics of the interfacial film implicated in the stability of the emulsions [64]. For instance, flexible proteins such as β-casein (strongly amphiphilic, constitutes up to 35% of the caseins in bovine milk [65]), have been reported to adsorb to lipid droplet surfaces and form a highly viscoelastic interfacial film, resulting in stable emulsions [66], though this may also be partly related to steric stabilization due to the thick film adsorbed to the surface of lipid droplets [67]. In contrast, monomeric globular proteins such as α-lactalbumin (14.7 kDa) and BSA (66.5 kDa) have been described to form a thin monolayer interfacial film with high elasticity, but the resulting emulsions were found susceptible to coalescence, especially when shear stress was applied [68, 69]. However, almost no flocculation was observed when the amount of protein was sufficient to completely cover the lipid droplets [70]. In the case of globulins such as β-conglycinin (a trimer, with a molecular mass of 180 kDa, comprising 30-50% of the total SPI [71]) and β-lactoglobulin (at a native dimeric form, 18.5 kDa), a dense and viscoelastic interfacial film can be formed at the surface of lipid droplets, exhibiting a high coalescence stability as a result of high viscoelasticity of the interfacial film and a relatively low tendency to aggregate, due to their highly hydrophilic nature [64]. Finally, proteins such as oligomeric glycinin (a hexamer with a molecular mass of 300-400 kDa, constituting ca. 30% of the total SPI [71]), have been reported to form very thick and inhomogeneous multilayer interfacial films with

a high tendency to aggregate, leading to entrapped lipid droplets within a network consisting of aggregated particles, especially at high concentrations of these proteins [64]. This would prevent digestive enzymes to adsorb at lipid droplet surfaces, and could also implicate potential negative influences of proteins on the micellization of lipophilic compounds such as carotenoids (Figure 2.2).

It is generally recognized that a good balance between hydrophobicity and hydrophilicity within a protein is an important factor for its emulsifying ability [72]. Given their high amphiphilic nature, such proteins exhibit a good ability to diffuse and/or adsorb to lipid droplets, while folding at o/w interfaces, by exposing rather hydrophobic groups to the lipid core of the droplets and hydrophilic groups on their surface [73], thus acting as effective emulsifiers to form and stabilize o/w emulsions [74]. For example, the structure of WPI consists of two main fractions, namely α-lactalbumin (approx. 25%) and β-lactoglobulin (approx. 50%), and their conformation (secondary structure, mainly α-helix and β-sheet, respectively) determines their properties at the o/w interface [75]. Similarly, glycinin and β-conglycinin account for about 70% of total SPI [71]. Applying an acid treatment resembling the gastric environment (pH 2-3) to SPI led to a progressive increase in the extent of denaturation, and subsequently, a progressive subunit dissociation, exposure of hydrophobic clusters, and aggregation of unfolded and/or denatured protein fractions [76, 77], due to the loss of solubility of glycinin, while β-conglycinin was much less affected by these structural changes [78].

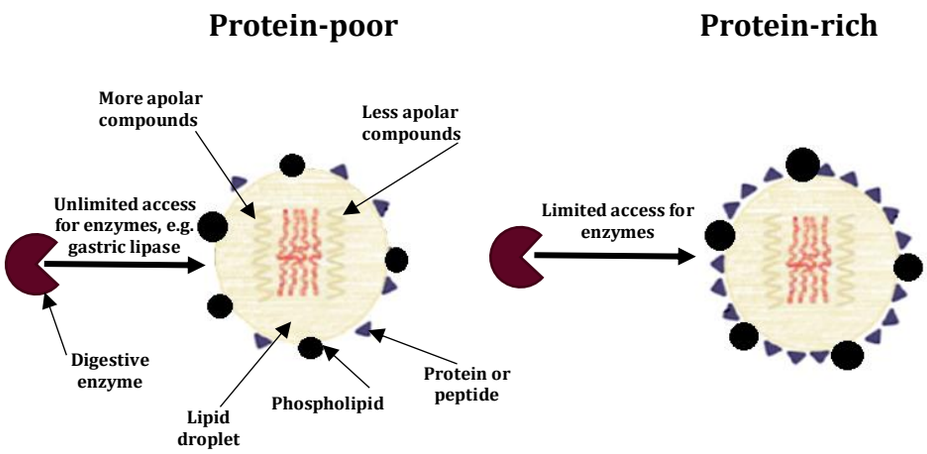


Figure 2.2. Action of proteins on enzymatic access on lipid droplets.

2.2.3 Digestion of proteins and potential interactions with lipophilic vitamins and secondary plant metabolites in the stomach

In the oral cavity, food items are mainly ground into smaller particles during chewing, and they are also wetted under the influence of saliva to prepare the food bolus by dispersing the formed particles and initiating enzymatic food breakdown. The food bolus is then passed on into the stomach, where the pH varies from about pH 1.3-2.5 under fasting conditions to pH 4.5 or even higher after the entry of meal, due to the buffering capacity of the food matrix. Upon further

digestion, due to the secretion of additional acids, the pH of the stomach drops again toward pH 2 [79].

Following the ingestion of a meal, gastric chief cells secrete pepsinogen, which is converted into the proteolytic enzyme pepsin under the influence of the hydrochloric acid of the stomach. In addition, the gastric phase contains gastric lipase, electrolytes, and mucus, which all aid in the emulsification of lipid food constituents [80]. The amount of pepsin present in the stomach has been reported to vary largely, approx. between 20 and 1000 µg/mL gastric juice [79], while its cleavage capacity has been reported to be between 200 and 7000 U/mL [79] (one unit will produce a change in absorbance of 0.001 at 280 nm at 37 °C, in 1 min, at pH 2.0, with hemoglobin as substrate). Similarly, *in vitro* digestion conditions employ between 160 and 12500 U/mL [79]; the well-established INFOGEST protocol uses 2000 U/mL [81].

Pepsin in the stomach has been reported to be active especially below pH 3, while being denatured at pH 5-6 [82]. As reviewed by Ahn et *al.*, pepsin, an endopeptidase, exhibits a broad cleavage specificity. Generally, hydrophobic amino acid residues, such as phenylalanine, methionine and leucine at the P1 position of the N-terminal, or phenylalanine, tryptophan and tyrosine at the P1' position of the C-terminal, increase cleavage probability, whereas the presence of positively charged amino acids such as histidine, lysine, and arginine at the P1 position, substantially reduce cleavage probability [82].

According to their structure, proteins differ largely in their digestibility in the stomach. While some proteins clump at the acid pH

of the stomach such as casein, and can be hydrolyzed to a large extent there, others such as WPI and SPI, with better solubility properties under these conditions, may be more rapidly passed on into the duodenum to be further hydrolyzed in the small intestine [27]. Despite the differing effects of the pH on the various types of proteins, most are denatured in the stomach and partly unfolded [83]. Another important aspect, which is typically disregarded at least during *in vitro* simulation [80, 81], is the effect of peristalsis of the stomach, which may aid in creating a more uniform mixture of the digesta, producing the chyme, aiding in the emulsification and reduction of particle size [84]. In essence, the results of protein digestion in the stomach are generally shortened and denatured polypeptides, with low amounts of free amino acids. However, it has also been stated that this step is not crucial, as persons who had their stomach removed were still able to digest and take up proteins from the diet [85]. Thus, protein digestion in the stomach is far from being considered complete, depending on the protein type, the co-digested food matrix, and the volume of the food, as a larger volume may slow down gastric passage and lead to prolonged exposure of the food matrix to the milieu of the stomach. Digestion times can vary between 15 and 20 minutes for small liquid meals up to 3-4 h for more voluminous and solid meals [86]. Particle size is reduced to typically 1-2 mm [87], which is important, as too large particles would delay further emptying of the stomach via the pylorus into the duodenum. In line with the incomplete digestion, original investigations by Garret et *al*. [88] and later by Biehler et *al*. [89] also showed that leaving out the pepsin digestion step did not largely influence carotenoid micellization *in vitro*.

Regarding physicochemical properties, proteins may reduce surface tension of the aqueous phase in the stomach [90, 91], due to their amphiphilic nature of some of them. Due to their amphiphilic properties, proteins may foster stabilization of lipid droplets in the gastric phase of digestion, fostering fatty acid release and transition of lipid droplets into smaller droplets. Conversely, proteins, perhaps especially at high concentrations (Figure 2.2) may also inhibit access of gastric lipase to lipid droplet surfaces.

2.2.4 Digestion behaviour and effects of proteins on lipophilic vitamins and secondary plant metabolites in the small intestine

The secretion of bile and pancreatic juices containing hydrogen carbonate, neutralizes the acid from the stomach. More specifically, the intraluminal pH is rapidly changed to about 5.4-7.5 in the duodenum, 5.3-8.1 in the jejunum and 7.0-7.5 in the ileum [79].

The majority of the digestive processes take place in the small intestine, including proteolysis. Indeed, additional enzymes result in the further digestion of the proteins released into the duodenum, while the jejunum is considered the major place of amino acid absorption [85]. The following secreted zymogens (inactive precursors, secreted by the pancreas) are turned into active proteases (under the influence of trypsin):

a) Trypsinogen, which is activated into trypsin (endopeptidase) following its cleavage by an enteropeptidase. Once activated,

the trypsin cleaves the peptide bond on the carboxyl side of arginine and lysine;

b) Chymotrypsinogen, which is activated into chymotrypsin (endopeptidase), favorably cleaving peptide bonds that are on the carboxyl side of tryptophan, phenylalanine, tyrosine or leucine;

c) Proelastase, which is activated into elastase (endopeptidase), cleaving especially peptide bonds on the carboxyl side of small, hydrophobic amino acid residues such as glycine, valine, leucine, isoleucine and alanine;

d) Procarboxypeptidases, which are cleaved to exopeptidases called carboxypeptidases A, B, and other aminopeptidases. In contrast to an aminopeptidases, which cleave peptide bonds at the N-terminus of proteins, carboxypeptidases A have a stronger preference for cleaving peptide bonds at the carboxyl side of hydrophobic and branched chain amino acids such as phenylalanine, tryptophan, tyrosine or leucine/isoleucine, while carboxypeptidase B preferably cleaves the peptide bonds on the carboxyl side of positively charged amino acids such as arginine and lysine.

The typically resulting di- and tripeptides are then in part further cleaved at the brush border by aminopeptidases into amino acids, prior to their absorption by the enterocytes. Thus, it is unlikely that the further action of these peptidases at the brush border and their products do play a significant role in the further emulsification of liposoluble compounds such as fat-soluble vitamins and carotenoids,

due to the very short nature of the resulting structures and limited amphiphilicity.

Protein digestibility is assumed to play an important role given the potential interactions of the resulting products with other dietary ingredients, i.e. well soluble and partially cleaved proteins may result in more interactions with lipid droplets and later with mixed micelles than with largely undigested proteins, as proposed by improved emulsification activity of certain protein hydrolysates [92]. Some proteins, e.g. those rich in proline stretches such as gluten, have been shown to be of low digestibility, due to the reduced flexibility and inaccessibility to digestive proteases [85]. Proteins with high levels of β-pleated sheet are likewise poorly accessible for digestive enzymes, such as shown for certain wheat flour doughs [93]. Also much cross-linking of proteins, i.e. via disulphide bridges, reduces the digestibility of proteins [85]. Such proteins are presumed to have low emulsification properties. While food processing such as high-pressure homogenization and ultrasound treatments [94, 95] can improve digestibility of proteins, the intake of larger amounts of proteins or the presence of external factors, earlier termed “anti-nutrients”, can prevent or drastically retard the breakdown of proteins. Many are trypsin and chymotrypsin inhibitors, such as tannins and other phenolics, phytates, hemagglutinins/lectins, as well as dietary fiber (through rather physical effects, i.e. occlusion) among other, as reviewed by Joye et *al.* [85].

Contrarily, rapid digestion starting in the gastric phase and ongoing in the intestinal phase may foster potential positive interactions of peptides with mixed micelles, which would get rather

lost under complete digestion into amino acids and short peptides. WPI did show good and consistent stabilization of lipid droplets, both during the gastric phase (with a slight increase of droplet size explained by partial proteolysis of the interface) and the intestinal passage (by reducing again droplet size to around 2 μm). The stabilized lipid droplets were smaller than those of lecithin-stabilized droplets, perhaps as the β-lactoglobulin fraction of the WPI is resistant to pepsinolysis [96], despite the fact that WPI has been proposed to have one of the highest digestibilities in the ileum, about 97-98% (vs. 94% for caseins [97]).

Which interactions are to be expected between proteins and other constituents during intestinal digestion? Most important regarding the digestion of liposoluble food compounds such as fat-soluble vitamins and secondary plant metabolites (e.g. carotenoids) would be likely their interaction with lipid droplets, which are then further processed under the influence of pancreatic lipase and bile acids into mixed micelles, a prerequisite formation for the absorption of liposoluble vitamins and other compounds.

In this respect, both positive and negative effects on the micellization processes can be envisioned, either due to enhanced stabilization of lipid droplets and faster micelle formation, or reduced access of digestive enzymes and slowed down process (Figure 2.2). In an *in vitro* study by Mun et *al.* [16], the effect of different surfactants on the resistance of lipid droplets against digestion by pancreatic lipase followed the order non-ionic surfactant (Tween20), phospholipids (lecithin) and finally proteins (caseinate or WPI), suggesting that compared to other surfactants, proteins do not

strongly hamper lipid digestion. More recent results comparing the effect of Tween20, β-lactoglobulin and lecithin showed only small differences in the release of free fatty acids (FFAs) from an o/w emulsion [98], emphasizing that differences can depend on the type of proteins digested and the specific digestive conditions.

Interesting, an impact of bile salts on protein digestion has also been found, showing drastically improved pancreatic proteolysis *in vitro* for β-lactoglobulin, BSA, myoglobin, and a commercially available dietary protein supplement [99], perhaps via the emulsifying properties of bile acids and the unfolding properties of proteins [100]. However, it is somewhat unclear whether proteins have an effect on the apparent concentration and activity of bile salts, but precipitation of bile salts during digestion has been reported [101], which may contribute to the negative effects of proteins at low bile salt concentrations.

2.3 Interactions of proteins and lipophilic constituents during digestion

A common similarity that is shared by fat-soluble vitamins and lipophilic secondary plant compounds is their low water solubility, requiring incorporation/solubilization into lipid droplets, which result in the formation of mixed micelles [102, 103]. The size of the mixed micelles is of approx. 5-8 nm in diameter, and they are composed of a hydrophilic surface made from the polar parts of mono- and di-glycerides, phospholipids, bile salts, cholesterol, xanthophylls, vitamin E, etc., and of a hydrophobic inner part with more apolar constituents

such as carotenes [103]. These micelles then diffuse to the unstirred water layer from where the liposoluble constituents can then be absorbed, either via passive diffusion (at high concentrations) or via protein carriers (e.g. CRBPII, SR-BI, CD36, and NPC1L1 [103-105]), typically at lower (dietary) concentrations. The main absorption area is the small intestine.

2.3.1 Carotenoids

Despite their non-essentiality, much attention has been dedicated to determine dietary intake, which has been estimated around 10 - 15 mg/day [106, 107]. However, due in part to their poor water-solubility (e.g. the LogP for lutein=7.9, and β-carotene=17.6 [108, 109]), the concentration of carotenoids (the most abundant ones being lycopene, β-carotene, α-carotene, β-cryptoxanthin, lutein, and zeaxanthin) in human serum is fairly low and highly variable, estimated between 0.33 and 5.78 μmol/L in healthy European adults [110]. In fact, several dietary as well as host related factors have been reported to influence carotenoid bioavailability aspects, such as their bioaccessibility and absorptive processes, including dietary lipids [111, 112], fibers [113], and divalent minerals [114, 115] (Chapter 1). However, not much is known about the potential influence of co-consumed dietary proteins on digestion processes of carotenoids, though the use of proteins as encapsulation materials of carotenoids could be a very effective strategy to improve their chemical stability, solubility, as well as bioaccessibility following *in vitro* GI digestion, pointing out toward increased bioavailability of such combinations [22].

Recently, several studies have reported the use of proteins for designing functional foods, with the aim to improve the stability of carotenoids during the storage of food items and thus shelf-life, as well as to enhance their absorption. For this purpose, emulsification of carotenoids and encapsulation techniques have been employed, as reviewed previously [22]. For instance, corn oil as the dispersed phase (5 or 10%), was homogenized with 2% SC in a microfluidizer, to prepare a delivery system of β-carotene (0.1% in corn oil) [116]. The results showed that the emulsions were stable to coalescence or flocculation over 30 days, similar as obtained in another study describing that the encapsulation of carotenoids is a very effective strategy to improve their chemical stability under common processing conditions and also storage [117]. In addition, the nanoemulsion delivery system was postulated to modulate the release kinetics from the protein-carrying system and to enhance the solubility of lipophilic compounds [116]. The incorporation of an *in vitro* digestion process of different SC-based emulsions, preceded by different homogenization pressures (10-100 mPa) produced samples of various droplet diameters (124-368 nm). The relatively small particle size of these droplets resulted in an improved bioaccessibility of the encapsulated β-carotene at the end of the GI digestion [116], compared to the conventional emulsions (>200 nm), showing that SC can be used for the design of effective delivery systems for encapsulation of carotenoids and other lipophilic bioactive components, similar to a previously reported study [118]. In another study, 1% of SC or Tween80 dissolved in water were homogenized with 10% sunflower oil containing 0.2% β-carotene. The produced emulsions were

subjected to an *in vitro* digestion followed by a Caco-2 cellular uptake. The authors reported that SC-stabilized emulsions enhanced the bioaccessibility and facilitated the uptake of β-carotene, compared to Tween80-stabilized emulsions [119]. Apparently, fat droplets remained small and stable against coalescence throughout the digestion process, and could rapidly be processed further into mixed micelles.

The impact on lipolysis changes and subsequent release of β-carotene were also investigated during *in vitro* digestion, e.g. based on an o/w emulsions stabilized with SPI [120]. The emulsion was prepared by dissolving 0.1% β-carotene in soybean oil, of which 10% were homogenized with a protein solution (1.5% of SPI dispersed in water). Significant interfacial changes were reported as indicated by the increased negative charge of the oil droplets (-40 to -70 mV), and a significant increase of lipolysis efficiency was found, resulting in a maximum lipid hydrolysis and a transfer of about 50% of β-carotene to the mixed micelles. Similarly, Zhang et *al.* have characterized the impact of SPI structure modification on the physicochemical properties of the emulsion and its oxidative stability. Carotenoids from L. barbarum (1% from boxthorn) were dissolved in medium-chain triglyceride oil, to which 1 to 9% of a protein solution were added (1% of SPI dispersed in PBS). SPI generated short peptide chains, with a better amphiphilic property, as shown by the decrease in the interfacial tension and the size of the emulsion droplets, compared to other small molecule surfactants (Tween80) [121].

A commonly investigated protein type, WPI, was used in another study to investigate the influences of interfacial structure on

the physical properties, bioaccessibility and microstructure changes of high pressure homogenized β-carotene emulsions during *in vitro* digestion [122]. Emulsions were prepared with 10% medium-chain triglyceride oil containing β-carotene (0.15% in the final emulsion), which was further homogenized with different aqueous emulsifier solutions (4% of WPI or decaglycerol monolaurate (ML750)). Size distributions of these emulsions differed from one to another. The authors stated that WPI-stabilized emulsions showed a higher absolute ζ-potential (-36.4 ± 0.65 mV) compared to those stabilized by ML750 (-15.9 ± 0.46 mV) following GI digestion, offering stronger electrostatic repulsion and thus higher stability against coagulation. In addition to these changes, WPI-stabilized emulsions resulted in an increased micellization of β-carotene, reaching approx. 86% vs. 30% for ML750-stabilized emulsions. Similar findings were reported by Lu and co-workers regarding the influence of WPI (1% dispersed in water) on the bioaccessibility of β-carotene (0.2% dissolved in sunflower oil) following *in vitro* GI digestion [119]. β-Carotene-loaded emulsions with WPI resulted in improved bioaccessibility of β-carotene, i.e. 60% vs. approx. 40% for Tween80-stabilized emulsions. The effects of WPI on the GI processing of oil droplets were also investigated. Significant differences in lipid droplet size of emulsions stabilized by WPI or Tween80 were observed, i.e. WPI-stabilized emulsions were more negatively charged (-53 mV) vs. those stabilized by Tween80 (-25 mV). This was mainly attributed to the protein, being negatively charged at pH (7.0), which is higher than its pI (pH 4.0–5.0). The authors showed a higher rate and extent of lipid digestion for WPI-

stabilized emulsions, likely attributed to an increased lipid surface area exposed to pancreatic lipase.

Although carotenoid micellization is assumed to be a key step for carotenoid bioavailability, and is a frequently measured parameter when using *in vitro* models, it needs to be proven that similar interactions occur *in vivo*, i.e. that the presence of proteins can influence the efficiency of carotenoid absorption. However, trials studying the influence of proteins on the bioavailability of carotenoids in humans appear to be rare. Nevertheless, it is noteworthy that the lipemic response, such as plasma TAGs corresponded with carotenoid absorption, as shown in previous studies [123]. As TAGs are fostering carotenoid sequestration into chylomicrons, and also later lipoprotein sequestration by the liver, it can be speculated that affecting plasma TAG level could also influence circulating carotenoid concentrations to some extent [124].

Interestingly, proteins have been studied on their impact on TAGs. Wang et *al.* examined the effect of different protein sources on TAGs in hypercholesterolemic subjects undergoing a six-week randomized cross-over trial [125]. Diets contained either SPI or common sources of animal protein (25 g/1000 kcal), adjusted for their fatty acid profile to be similar across diets. SPI reduced TAGs by 12.4%, total cholesterol by 4.4%, and LDL cholesterol by 5.7%, compared to animal protein diet, demonstrating that dietary protein type may modulate circulating TAG and cholesterol levels in hypercholesterolemic individuals. Such effects were explained either by the protein fraction itself or also by co-occurring soy-isoflavonoids. Other studies have reported the influence of reducing protein intake

on the level of plasma TAGs. In a randomized controlled clinical trial, Treviño-Villarreal and co-workers have shown that patients receiving 4-6 weeks of protein restricted diets (7-9% of energy from protein) significantly decreased plasma TAGs, compared to controls continuing their usual diet (n=19 per group) [126]. The authors speculated that protein restriction mediated the increase in APOA5 expression, which plays a role in facilitating TAG hydrolysis from lipoprotein particles by stimulating lipoprotein lipase activity in the periphery [127].

It should be mentioned that care should be taken to extend results from long-term trials on TAGs to short, i.e. postprandial bioavailability results of carotenoids. Nevertheless, such long-term protein intakes may influence also circulating carotenoid concentrations, as well as other lipophilic concentrations such as those of vitamins, also associated with circulating lipoproteins.

In summary, due to the relatively poor water-solubility of carotenoids, the use of proteins as emulsifiers and encapsulants has proven to be an effective target for their incorporation in o/w emulsions, and for enhancing several carotenoid bioavailability aspects, such as micellization and cellular uptake. However, considering the wide polarity range of carotenoids (polar xanthophylls vs. nonpolar carotenes), a special attention should be given to the structural differences between proteins and their effect on carotenoid micellization. Although theoretically smaller sizes, higher flexibility, high amphiphilicity and solubility over a wider pH-range warrant good emulsifying properties of a protein, their final effectiveness to enhance bioavailability remains to be confirmed *in vivo*. Thus, further investigations on the complex interactions of co-ingestion of proteins

and carotenoids are necessary in sight of the potential health-associated benefits of this group of dietary antioxidants.

2.3.2 Liposoluble vitamins

Liposoluble vitamins include vitamins A, D, E, and K. However, since very little information is available for vitamin K, and as it is also formed in the gut by the microbiota, it will not be discussed in this Chapter.

2.3.2.1 Vitamin D

Vitamin D is an essential vitamin, with an RDA of 15 μg for adults [128], which is fairly low compared to the intake of other lipophilic micro-constituents such as carotenoids, which are consumed in the range of several mg per day [129], possibly not raising issues of volume/concentration overload that can result in low micellization, unless taken in form of supplements. However, the unique feature of this vitamin is that it can also be formed under the skin by the influence of UV light, with cholesterol as its precursor, thus bypassing oral bioavailability. Nevertheless, the majority of the populations living in the high latitudes (about 40-50o and higher) have been reported to have low circulating vitamin D levels, as assessed by 25-hydroxyvitamin D (25-OH D, the major form of vitamin D circulating in the bloodstream and accepted status marker), in winter [21]. For instance, approx. 40% of the population in the UK shows low vitamin D status (as defined as a concentration of <25 nmol/L of serum 25-OH D) in winter [130]. As low vitamin D status has been correlated

in meta-analyses with many diseases, including cardiovascular disease mortality [131] and also prediabetes conditions [132], factors influencing its absorption have been investigated and reviewed [133-135].

The absorption of vitamin D has been reviewed by Reboul et *al.* [136] to be fairly high, varying from 55 to 99%. However, susceptibility of its absorption in persons with intestinal malabsorption, especially steatorrhea, i.e. disturbed lipid digestion, has also been highlighted, pointing out to the importance of emulsification factors present in the intestine for its bioavailability.

Vitamin D3 (cholecalciferol) is quite apolar, with a LogP (octanol/water partition coefficient) value of 10.2 [137], thus its absorption follows micellization and the lymphatic pathway. Similar considerations apply to vitamin D2 (ergocalciferol), with a logP value of 10.4. Because of the presence of double bonds, vitamin D has been shown to be sensitive to isomerization under varying conditions such as pH, oxidation, light, and temperature, all potential sources of degradation, which can be avoided to some extent by finding suitable carriers or encapsulation processes [138]. Indeed, the usefulness of e.g. milk proteins for encapsulating has been reviewed earlier, and aspects such as protection of sensitive molecules against oxidation, photo-degradation, and enhanced release and solubility were highlighted [139]. For instance, microencapsulation of vitamin D with WPI, SPI or both resulted in good release properties in simulated gastric and intestinal fluids, being highest for SPI (>90%, combined gastric+intestinal phases) [140]. Also, It has been reported in a recent

review that vitamin D encapsulated in the β-lactoglobulin fraction from WPI, is thought to have a high bioavailability [134].

In line with the positive influence of proteins, an interesting study in mice was conducted by Lindahl et *al.* [141]. Animals were receiving vitamin D3 dissolved in ethanol and then administered either as 1) a complex with WPI, 2) a complex with caseinate, or 3) in aqueous solution. For the purpose of complex formation, vitamin D was merely dissolved in WPI or caseinate-rich solutions. Plasma levels of vitamin D3, 25-OH D3 and 24,25-dihydroxyvitamin D3 over 10 h were measured, and the WPI condition showed the fastest and highest absorption (i.e. about 20% higher than caseinate), though also the caseinate complexed form was absorbed better than free vitamin D3 in water. AUC values for vitamin D3 were ca. 525, 400 and 350 ng x h/mL, respectively. The authors explained their results because vitamin D3 complexed with proteins had higher solubility and better protection against degradation during the GI passage.

In another study, vitamin D3 was encapsulated into a β-lactoglobulin complex (by mixing), and a coagulum was then created by means of glucono-δ-lactone. This was force-fed to rats. Despite that the coagulum was fairly resistant against proteases in the intestinal phase of digestion (as tested *in vitro*), the long-term bioavailability (after 3 weeks, as measured in the plasma as 25-OH D3) was about 3 times higher from the coagulum (coagulated β-lactoglobulin complex) vs. free vitamin D3 (in aqueous solution) or vitamin D3 given as a β-lactoglobulin complex alone. This higher bioavailability was attributed to the higher water solubility of the complex, though it may be assumed that also protective aspects, i.e. increased stability of vitamin

D3 in this form, could have played a role. In another study with rats, vitamin D in a pea protein isolate (PPI) nanoemulsion was most efficient to improve vitamin D status in animals, more so than vitamin D in oil [142], though it cannot be excluded that the nanoparticles generated by ultrasound treatment contributed mostly to this effect.

Haham et *al.* nanoencapsulated vitamin D3 into reassembled casein micelles [143], and showed in human volunteers that this formulation was at least as bioavailable as Tween80 emulsified vitamin D3, as measured by plasma appearance. In an earlier study by Tangpricha et *al.* [144], vitamin D2 absorption in humans (measured postprandially during 72 h as plasma appearance of 25-OH D2) was compared in a cross-over trial in 18 adults with either whole milk, skimmed milk or corn oil added on a toast. Though the AUC of the condition with whole milk appeared slightly smaller, results were not significantly different, and it can be assumed that all 3 matrices offered comparable bioavailability. However, all 3 meals contained either lipids or lipids and proteins, so a low lipid and/or low protein source as a control was missing. Such a matrix effect was also seen by Johnson et *al.* [145], when studying the bioavailability of vitamin D2 in 8 adults in a cross-over trial, delivering vitamin D either in cheese or in water, with peak serum 25-OH D2 concentrations being 8 times higher from cheese, though it was not possible to say whether the effect was due to the lipids, the proteins, or both.

In another study by Wagner et *al.* [146], vitamin D from different food sources was given for 8 weeks in a study involving 80 adults. Sources were fortified cheddar, fortified low-fat cheese, or a liquid vitamin D supplement, taken either with or without food. 25-OH

D responses in plasma were 65.3 (SD: 24.1), 69.4 (21.7), 59.3 (23.3) and 59.36 (19.6) nmol/L, respectively, thus showing in tendency lower values for the supplement, but due to the design of the study and the high SD (non-paired design), no significant differences were found.

In summary, considering the results from the human, animal and *in vitro* trials together, there is some evidence that proteins can modulate vitamin D absorption positively. However, as often proteins and lipids were administered together, the individual contribution of these components is somewhat unclear in several studies. Furthermore, as the logP value of vitamin D is somewhat lower than e.g. that of the very apolar carotenes, it can be hypothesized that the potential effects of proteins or other emulsifiers on boosting vitamin D bioaccessibility are perhaps less potent than those of carotenes.

2.3.2.2 Vitamin E

Vitamin E has received nutritional interest mostly via its role played as an antioxidant, protecting lipids from (per) oxidation in cellular membranes [147]. It tightly interplays with vitamin C by which it is regenerated following oxidation. Higher vitamin E intake has been shown in meta-analyses to be correlated with reduced risk of stroke [148] and even Alzheimer's disease [149], though supplementation trials have not clearly shown positive effects [150]. Tocopherols (4 types: α, β, γ, δ) and tocotrienols (4 types : α, β, γ, δ) have both vitamin E activity, with the latter group having 3 double bonds in their side chain, making them potentially more prone to lipid peroxidation. Tocotrienols are especially found in vegetable oils [151].

Concentrations in food items may reach up to 100 mg/100 g, such as in wheat germ [152], though typical amounts are more in the range of 0-50 mg/100 g, with an RDA of 15 mg/d for adults [153]. Therefore, vitamin E is possibly the most abundant lipophilic micronutrient present in many food items, thus its absorption may be prone to be influenced by digestive factors. The logP of 12.2 has been determined for α-tocopherol [154], and all vitamin-E active compounds are very apolar. The potential clinical relevance of vitamin E has also sparked the interest for aspects influencing vitamin E bioavailability. Reboul [155] and Borel et *al.* [156] previously reviewed factors impinging on vitamin E bioavailability with a focus on uptake and transport. These authors highlighted the impact of dietary factors including matrix disruption and the presence of dietary lipids on the micellization and thus absorption of vitamin E from the small intestine, emphasizing that micellization efficiency does appear to play a role regarding vitamin E bioavailability. The importance of dietary lipids for high bioavailability of vitamin E and α-tocopherol acetate as a common food additive (which requires cleavage by bile acid-dependent lipase prior to absorption), have also been reviewed by Schmölz et *al.* [157]. Regarding the influence of proteins, very little is known on their influence on the micellization of vitamin E.

In an *in vitro* digestion study by Werner and Böhm, pasta with eggs (rich in lipids and proteins) resulted in a decreased bioaccessibility of total vitamin E as opposed to pasta alone, which was unexpected. Similar negative findings in that study were observed for carotenoids. However, the authors reasoned that incomplete lipolysis in the egg pasta matrix could have played a role [158], possibly

emphasizing the importance of complete digestive conditions for predicting physiological results. In another *in vitro* study, testing different o/w emulsions including vitamin E in WPI, saponin (triterpene glycosides) and gum arabic [159]. Bioaccessibility was highest from WPI produced emulsions (approx. 85%) following GI digestion, as compared to saponin and gum arabic-emulsions (approx. 60% in both emulsions). The saponin emulsion had the highest absolute zeta-potential (at pH 7), an indicator of repulsive forces preventing agglomeration. However, lower lipid digestion was found in saponin-emulsions, which was explained by the higher surface activity of saponins and more difficult removal of these particles by bile acids from lipid droplets, perhaps as unlike WPI-based emulsions, which had lowest mean particle diameter (below 100 nm, at pH 7). Similar findings were earlier obtained by Öztürk et *al.* [160], investigating vitamin E encapsulated by WPI or gum arabic. These results highlight the importance of factors including small particle size, and rapid displacement from lipid droplets by lipase and bile acids, being most important parameters for high bioaccessibility in the presence of dietary proteins. WPI has earlier been highlighted for its stability at low pH and thus for its properties to incorporate vitamin E into orange-based beverages of high acidity [161]; heat stability of the emulsions was also very high.

In another *in vitro* study [162], vitamin E microcapsules were produced by spray-drying, freeze drying or spray-freeze drying, employing saponin as a surfactant together with WPI as an outer wall material, using a homogenizer and a microfluidizer. Experiments in rats demonstrated good bioavailability of vitamin E when combined

with WPI, as determined by plasma AUC appearance, especially for the spray-freeze dried formulations, which was attributed to the better dissolution behaviour, which likely aided in the micellization process.

In an earlier human postprandial study by Lodge et *al.*, vitamin E supplements (α-tocopherol) were given together with a toast and butter or a cereal with full fat milk, among other, both containing the same amount of fat. While both formulations resulted in better absorption of vitamin E, as compared to the supplement with water, the toast/butter combination (being lower in proteins than the full fat milk meal), led to higher vitamin E absorption, perhaps due to the negative effect of dietary fiber from the cereals [163]. Contrarily, in an even earlier human study by Hayes [147], consuming vitamin E within milk, independent of the fat percentage (though this was limited to 1%), resulted in an increased vitamin E plasma levels after 4 weeks of consumption, compared to vitamin E intake from capsules (α-tocopherol acetate), also suggesting that proteins appeared to have beneficial effects. The authors reasoned indeed that dietary proteins or peptides produced during digestion could have had positive effects on the absorption of vitamin E, such as via improving micellization *in vivo*. Of note, fat-free milk alone may have limited effects on vitamin E bioavailability from supplements. Indeed, vitamin E absorption drastically increased only when the supplement was consumed together with breakfast cereals [164]. This suggests that stomach emptying plays a role, as too rapid transport through the gastric phase (and perhaps intestinal phase also) would limit the time of food processing during digestion, including any potential positive influence that proteins or peptides could have on vitamin E micellization.

In summary, there are indications that proteins, such as obtained from milk, can aid in the absorption of vitamin E by improving micellization. However, factors such as gastric retention time or transit time through the GI are also expected to play a role.

2.3.2.3 Vitamin A

Vitamin A active compounds include retinol, retinal, and retinoic acid. Also pro-vitamin A carotenoids are a source of vitamin A, following their cleavage by β-carotene oxygenases (Chapter 1). Food matrices containing preformed vitamin A include only animal-derived food items, the normally predominant form being retinol and its esters [165]. However, supplements can also contain retinyl acetate or retinyl palmitate.

The logP value of retinol (5.68, [166]) is somewhat lower than that of the other fat-soluble constituents, indicating that the compound is more polar. Absorption via the lymphatic system predominates over uptake via the portal system when logP values are above approx. > 5 [165]. Preformed vitamin A has been shown to be highly absorbed, at a level around 90% [167]. Thus there may not be much additional room for further enhancing bioavailability. However, improving gastric stability could play a role, as losses of approx. 25-35% have been reported for vitamin A after incubation in gastric juices during 2 h (reflecting the transit time of more heavy meals), with losses being higher for the alcohol form compared to retinyl acetate [168].

It is known for some time that vitamin A can form complexes with proteins. For instance, retinol is incorporated in casein [169],

fixed via binding to hydrophobic amino acids (tryptophan, phenylalanine) [170]. The same has been emphasized for retinyl palmitate and casein micelles, which were acting as nano-vehicles rich in cavities and channels, whereas BSA, β-lactoglobulin and α-lactalbumin showed less binding affinity [171]. Similar positive effects have been reported for other lipophilic constituents, such as vitamin D2 and curcumin, though whether this is related to higher bioavailability is not known. Casein micelles were typically degraded in the gastric phase of digestion and the small intestine, liberation in the small intestine is to be assumed [172]. Four main types of proteins (αs1, αs2, β and κ) belong to casein family, all possessing hydrophobic and hydrophilic parts in their molecule [173]. These proteins are highly surfactant, aiding to stabilize o/w emulsions, especially when heated, they appear to show high binding affinity to lipophilic dietary constituents. Depicturing the use of this property, the review by Sadiq et *al.* described the use of casein micelles in earlier studies for the encapsulation of resveratrol, curcumin, β-carotene, and vitamin D2/3, among others [173].

Rana et *al.* [174] investigated various casein complexes with vitamin A for their cellular uptake by a Caco-2 cellular model following GI digestion with milk. These included caseinate-vitamin A complexes, modified succinylated sodium-caseinate (SC) complexes, reassembled SC complexes and reassembled succinylated SC complexes with vitamin A. It was found that the total cellular uptake was highest from the latter complex, followed by the reassembled SC complex, control milk, and then the other 2 complexes, whereas free vitamin A was least well taken up. The authors argued that the more hydrophobic regions

exposed during the processing of the reassembled caseinates caused better binding of vitamin A and improved its stability (especially against the low gastric pH), and thus resulted in higher bioaccessibility and higher cellular uptake into Caco-2 cells.

Encapsulation by more sophisticated means, such as by liposomes together with β-lactoglobulin, has also been carried out, as well as absorption tested in mice [175]. It was shown that β-lactoglobulin improved encapsulation efficiency of vitamin A and improved its digestive stability. However, bioavailability in animals was lower compared to vitamin A dissolved in oil, possibly due to slower release kinetics. For a more in-depth overview on encapsulation techniques for vitamin A, the reader is referred to more comprehensive reviews [176, 177].

Liu et *al.* [178] studied the absorption of vitamin A from various complexes in a rat gavage study. For this purpose, following washout periods, animals received either vitamin A (retinyl-palmitate form) in oil, complex of vitamin A and β-lactoglobulin, vitamin A in oil + skimmed milk, or complex of vitamin A and β-lactoglobulin combined with skimmed milk for prolonged times (up to 4 weeks). It was shown that the complexed form with β-lactoglobulin, prepared by a rather simple mixing technique, had similar bioavailability than the oil-based vitamin A form, as determined by serum and liver retinol measurements. Similar findings were encountered after heat treatment, suggesting high bioavailability of β-lactoglobulin complexes with vitamin A, even in the absence of lipids. Contrarily, negative findings for vitamin A absorption when complexed by β-lactoglobulin, were found in an animal study by Mensi et *al.* [15]. In

their study, retinol was incorporated into β-lactoglobulin by mixing, and force-fed to mice. When compared to retinol incorporated into emulsions (based on peanut oil and PBS, prepared by sonication), plasma levels were much lower. It is possible that the additional oil aided in the better absorption. Interestingly, β-carotene was also given in both formulations, and here β-carotene showed comparable absorption from the β-lactoglobulin complex vs. the emulsion, perhaps indicating that very apolar molecules (as in the study above with retinyl palmitate) may benefit more from protein-complexes than the more polar forms. The authors speculated on lower stability of retinol when bound to β-lactoglobulin than when present in the emulsions, but the reasons for these differences remain unclear.

Already several years ago, β-lactoglobulin was described to complex retinol [179], in addition to having good stability at low pH and being fairly resistant to proteolysis in the stomach. Also, its potential to increase intestinal uptake has been mentioned over 30 years ago [180]. Said et *al.* studied the effect of β-lactoglobulin added at a 2:1 molar ratio to retinol, on the intestinal uptake of the latter, by the everted sac technique in suckling rats. Significant higher absorption was found in the small intestine when β-lactoglobulin was added, while other proteins, i.e. serum albumin and lactoferrin, had no effect. It was speculated that β-lactoglobulin shares structural and conformational similarities with retinol-binding protein, which may suggest the existence of a protein-ligand receptor that could also recognize β-lactoglobulin at the brush border membrane of the enterocyte, leading to a specific improvement in the intestinal uptake of retinol.

In summary, despite the relatively high polarity and the relatively high absorption of vitamin A compared to other lipophilic constituents, emulsions and encapsulated forms of vitamin A by means of proteins, especially by β-lactoglobulin and caseinate, improve the bioaccessibility and bioavailability of vitamin A, possibly with differing degrees depending on vitamin A form (palmitate vs. non esterified retinol). These proteins may act by offering protection during the GI passage, though a more limited release in the gut may in part counteract such beneficial effects.

2.3.3 Curcumin

Curcumin (diferuloylmethane), an active component of turmeric (belonging to the ginger family), has been associated with anti-inflammatory, antioxidant, anti-cancer, as well as antiviral properties, and improves endothelial function [181]. The properties of curcumin have been investigated in many studies, and findings have suggested that curcumin suppresses the NF-kB activity and thus pro-inflammatory downstream genes [182]. Curcumins may also quench free radicals, initiate the activity of intracellular enzymes such as catalase, superoxide dismutase, and glutathione peroxidase [182], and most notably has been shown to induces apoptosis and inhibit the proliferation of cancer cells [183, 184].

In recent years, a large volume of research has been carried out on curcumin due to its functional mechanisms concerning health. In addition to studying its dietary intake, curcumin supplements have been designed and recommended in some cases. However, due to its

chemical structure, its absorption and bioavailability are typically low. Its logP value of approx. 3.3 makes it poorly water soluble [185], but absorption at least in part via mixed micelles is likely [186, 187]. However, compared to liposoluble vitamins and other secondary plant metabolites such as carotenoids, its distribution in the aqueous phase is higher. Curcumin, when administered orally (0.6 mg [3H]curcumin per rat), is to a good extent excreted in the faeces (approx. 90% of the radioactivity was excreted in the faeces)[188]. Earlier studies in rats based on stable isotopes have indicated an absorption of 60-66% (regardless of the dose administered) [189], though the relative low bioavailability is also due to extensive metabolism following absorption and re-excretion into the gut via bile [190].

Curcumin is also sensitive to the acid milieu in the stomach. For instance, in free form at pH 3.6, about 80% was degraded after 20 min [191], though losses in food matrices are possibly much lower, e.g. 11% from a yogurt-based food items [192]. Indeed, various dietary factors have been reported to affect its absorption and availability including fats, polysaccharides, and proteins [185] such as albumin, which is known to increase the stability of curcumin and prevent autoxidation [193], which could also stabilize curcumin during digestion.

An interesting study was conducted by Fu et *al.* [192], incorporating curcuminoids in a buttermilk-based yogurt (300 mg/100g), which improved bioaccessibility 15-fold more compared to that of curcuminoids in aqueous buffer, though bioaccessibility was still low with 6.2%. In another study, Qazi et *al.* examined the influence of two dairy gel matrices (i.e., containing oil and dairy protein)

prepared by either coagulation with rennet or acid, on lipid digestion and the bioaccessibility of a fortified curcumin nanoemulsion during GI digestion [194]. The emulsions were prepared by mixing for 2 h the lipid fraction that included 0.03% curcumin, and the aqueous phase containing 1% SC. Both gels had the same composition and rheological properties, but the acid gel underwent faster disintegration under gastric conditions compared to the rennet gel, which slowed the release of oil droplets and protein in the digesta, and the content of curcumin-enriched oil in the digesta of the acid gel was higher than that in the digesta of the rennet gel. This resulted in greater digestion of lipids and higher bioaccessibility of curcumin in the acid gel, emphasizing the importance of the presence of proteins toward the protection of curcumin and its role in solubilization of food bioactive compounds. Similarly, Zou et *al.* investigated the impact of SC on curcumin bioaccessibility and its stability in nanoemulsion delivery systems [195]. Adding casein to these systems did not significantly improve particle size, proposing that caseinate did not prevent inevitable droplet aggregation. However, SC did influence the electrical charge in the systems, i.e. highly negative (i.e. -23 vs. 6 mV for the control), which would be in line with more stable droplets. In addition, the presence of SC in the nanoemulsion prevented the chemical degradation of curcumin under simulated GI tract conditions, and increased curcumin bioaccessibility (78.3 ± 1.1% compared to the control 61.8 ± 9.3%) [195].

Vijayan et *al.* examined complexes of 0.5 mg/mL of PPI as well as WPI formed with curcumin (1% w/v added to protein solution), to overcome the low aqueous solubility and limited bioaccessibility of

curcumin [196]. The WPI-curcumin complex showed better aqueous solubility for curcumin than the PPI-curcumin complex at pH 7, all of which would be relevant for digestion in the GI tract. Despite this difference, both proteins showed a similar high bioaccessibility of curcumin from their respective complexes (approx. 78% for WPI and 72% for PPI) compared to control (approx. 18%). The PPI complex was regarded as a good non-dairy protein alternative for curcumin delivery, especially at higher temperature of 80 °C [196].

As another example of non-dairy alternative, Tapal and Tiku investigated the complexation of SPI with curcumin (5% w/v of protein solution) and its implications on the solubility and stability of curcumin [197], stating that SPI can form a water-soluble complex with curcumin, which increased solubility of curcumin by almost 812-fold [197]. Fluorescence spectroscopy of the SPI-curcumin complex revealed that the complex was formed through hydrophobic interactions, for which SPI seems especially suitable due to its hydrophobic nature [197, 198]. In addition, stability studies by UV-spectroscopy showed that over 80% of the curcumin was stable in the SPI-curcumin complex in gastric and intestinal fluids for 12 h, which would provide sufficient time for GI passage. The SPI-curcumin complex, using various protein concentration (5, 10, 15, 20 mg/ml) exhibited further enhanced antioxidant activity and could form foams and emulsions, indicating its possible utilization in food products [197]. Another study characterized SPI-curcumin complexes, by adding curcumin at a ratio of 1:50 to SPI solution (5% w/v). The complexation remarkably increased the solubility of curcumin in the aqueous phase, and greatly enhanced curcumin bioaccessibility,

compared to free curcumin in water (60% vs. 90%), following *in vitro* digestion experiments [199]. However, results from a recent study investigating spray-drying microenpsulation of curcumin in SPI [200], showed that spray-drying remarkably decreased the bioaccessibility of encapsulated curcumin, possibly associated with the aggregation of the nanocomplexes [200].

Kadam et *al.* examined the effect of Lepidium sativum (garden cress) protein hydrolysate (LSPH) on curcumin *in vitro* stability and bioaccessibility [201]. The complexation generated by stirring and a high-speed homogenizer improved the aqueous solubility of curcumin by almost 850 times. In addition, *in vitro* GI digestion showed that the bioaccessibility of curcumin increased from 67 to 95% post complexation. It was proposed that the LSPH-curcumin complex had a competent foam-forming capacity and emulsion stability and could act as a superior lipophilic bioactive delivery vehicle in food formulations [201].

Many studies have focused on generating more sophisticated encapsulation vehicles for curcumin. Dai et *al.* examined the stability and bioaccessibility of curcumin encapsulated by a protein-polysaccharide-surfactant ternary system [202]. The authors examined the bioavailability aspect of curcumin (Cur, containing various amounts, 25 to 250 mg) complexed with either zein (Z, 1% dissolved in 70% ethanol-water solution w/v), Z and propylene glycol alginate (P, 0.2% dissolved in 70% ethanol-water solution w/v v), Z-P and rhamnolipid (R, 1% added to the mixture w/v), or Z-P and lecithin (L, mass ratio of Z to L 5:1) [202]. Several methods of characterization were employed following *in vitro* GI digestion, as a measure of

curcumin bioaccessibility [202]. The authors reported that ternary protein-polysaccharide-surfactant complexes had a higher encapsulation efficiency than simple zein nanoparticles or binary zein-polysaccharide complexes, i.e. Z-Cur (21%), Z-P-Cur (67%), Z-P-R-Cur (92%), and Z-P-L-Cur (94%). The ternary complexes also enhanced the photo-stability and bioaccessibility of curcumin, i.e. Z-Cur (29.1%), Z-P-Cur (48%), Z-P-R-Cur (82.5%), and Z-P-L-Cur (87.6%), possibly via offering protection against degradation and improving the solubility of the complexes.

Human studies seem to be scant, but Li et *al.* investigated the absorption mechanism of curcumin delivery using Caco-2 cell monolayers [24]. In their experiments, β-lactoglobulin protein fraction and WPI-stabilized nanoemulsions (1 g of protein dispersed in 100 mL of PBS at pH 7) were used as carriers to deliver curcumin to investigate their digestion, using an *in vitro* GI model. The effect of various carriers on the permeability of curcumin was determined using the Caco-2 cell monolayer model. The results showed that pH stability of curcumin was significantly enhanced and water solubility improved by binding to β-lactoglobulin. As shown by SDS-PAGE, the β-lactoglobulin-curcumin complex and WPI-stabilized nanoemulsion were resistant to pepsin digestion, as occurring in the stomach, but sensitive to trypsin as present in the small intestine, suggesting a release in the latter phase of digestion. The permeability model revealed that the digested WPI-stabilized nanoemulsion and β-lactoglobulin-curcumin complex significantly improved the permeation rate of curcumin through the epithelium, by about 21% [24].

Similarly, Jayaprakasha et *al.* investigated curcumin nanoencapsulation with WPI *in vitro*, to enhance colon cancer chemoprevention [203]. It was shown that intracellular (SW840, colon cancer cell line) presence of curcumin was increased by up to 30% for nanoencapsulated (prepared by a desolvation method) compared to plain curcumin, suggesting that such protein-based formulations may find their way into clinical practice.

In summary, despite the relatively higher water solubility compared to the very apolar liposoluble vitamins as well as other secondary plant compounds such as carotenoids, curcumin administered together with proteins such as caseinates or β-lactoglobulin have shown to enhance bioaccessibility, mostly via increased stability, and in part higher solubility compared to the free form. Nevertheless, bioavailability, determined largely by further metabolism such as glucuronidation and sulfation, has thus far not been shown to be improved by proteins.

2.4 Conclusion

Literature indicates a growing interest in the use of proteins, especially as encapsulants materials, to improve storage aspects and stability of biologically active lipophilic compounds. These capsules offer a protection against oxidation and degradation to these sensitive molecules, enhance their solubilization throughout *in vitro* GI digestion processes, and ensure a better micellization and cellular uptake (Chapter 2).

Interaction of proteins with lipophilic vitamins and secondary plant metabolites during digestion

Considering the results from the human, animal and *in vitro* trials together (Table 2.2), the use of proteins as emulsifiers and encapsulants has proven to be an effective target to modulate positively several bioavailability aspects, such as bioaccessibility and cellular uptake of liposoluble vitamins as well as secondary plant compounds, likely mostly via increased stability during the GI passage and improved micellization.

A special attention should be given to the structural differences between proteins, as their effects on boosting bioaccessibility are relatively different in magnitude, depending on the polarity of the lipophilic food constituents.

Another aspect to consider the wide polarity of the apolar bioactive compounds. As an example, carotenoids (polar xanthophylls vs. nonpolar carotenes) could be used to further research on their complex interactions with co-ingestion proteins, the results of which could overcome various limitations regarding the use of proteins to improve the bioavailability of a wide range of other fat-soluble bioactive compounds.

Finally, considering the limited data resulting from human trial, as compared to *in vitro* data, thus, the final effectiveness of proteins to enhance bioavailability of apolar dietary compounds remains to be confirmed *in vivo*.

Table 2.2. Individual studies investigating the relationship between protein intake, digestion, and influence on lipophilic food constituent absorption.

Liposoluble compound studied	Protein type studied	Study details	Effects found	Remarks	Ref.
			***In vitro* studies**		
Carotenoids	SPI-PEP	β-Carotene was encapsulated in SPI-PEP conjugate-stabilized emulsion, and its gastrointestinal behavior and antioxidant activity were assessed *in vitro*.	Compared with β-carotene-SPI emulsion and β-carotene-mixture emulsion, the bioaccessibility (12%), absorption, and stability of β-carotene significantly increased in the β-carotene-conjugate emulsion. Therefore, β-carotene-conjugate emulsion had the highest bioavailability among the three groups.	SPI-PEP conjugate emulsion increased the bioavailability of β-carotene in the simulated gastrointestinal tract compared with β-carotene-SPI emulsion and β-carotene-mixture emulsion.	[204]
Vitamin D	WPI, SPI	VD encapsulated with different combinations of WPI and SPI were investigated *in vitro*.	5% WPI + 5% SPI mixture exhibited the highest encapsulation efficiency. All the encapsulates showed significantly higher stability and VD retention (> 93%) during storage at 4 °C as compared to free VD.	The study concluded that the complex encapsulates presented stronger interactions for matrix development than a single protein that resulted in better protection and controlled release of VD.	[140]

Table 2.2. Individual studies investigating the relationship between protein intake, digestion, and influence on lipophilic food constituent absorption (continued).

Liposoluble compound studied	Protein type studied	Study details	Effects found	Remarks	Ref.
***In vitro* studies**					
Vitamin A	Caseinate complexes	The bioavailability of VA through sodium caseinate-VA complexes was evaluated by exposing Caco-2 cells to the digesta of milk fortified with various complexes.	Up to 37% higher uptake by Caco-2 cells compared to free VA The total uptake of VA by Caco-2 cells was highest for milk fortified with RSNaCaS-VA followed by RNaCaS-VA, control milk, SNaCaS-VA, NaCaS-VA, and free VA.	During the formation of RNaCaS-VA and RSNaCaS-VA complexes, more hydrophobic sites were exposed, leading to the attachment of VA on the interior hydrophobic regions of sodium caseinate molecule. This led to higher stability of VA during GI digestion and further resulted in higher bioaccessibility and bioavailability of vitamin A in Caco-2 cells.	[174]

Table 2.2. Individual studies investigating the relationship between protein intake, digestion, and influence on lipophilic food constituent absorption (continued).

Liposoluble compound studied	Protein type studied	Study details	Effects found	Remarks	Ref.
***In vitro* studies**					
Vitamin E	WPI	The influence of plant-based (gum arabic and quillaja saponin) and animal-based (whey protein isolate, WPI) emulsifiers on the production and stability of VE-fortified emulsions was investigated *in vitro*.	85% increased bioaccessibility with WPI than in saponin- or gum arabic-emulsions.	Lesser effect for VE with saponin and arabic gum. Lipid digestion was slower in saponin-emulsions, presumably because the high surface activity of saponins inhibited their removal by bile acids and lipase.	[159]

Table 2.2. Individual studies investigating the relationship between protein intake, digestion, and influence on lipophilic food constituent absorption (continued).

Liposoluble compound studied	Protein type studied	Study details	Effects found	Remarks	Ref.
Animal studies					
Vitamin A	β-lactoglobulin (β-LG)	*In vitro* and *in vivo* assessment of VA encapsulation in a liposome–protein delivery system. Animals: 72 Male CBA/C57 mice; Group A: pure VA in soybean oil, Group B: VA + liposomes.	The % EE of VA in liposomes in absence and presence of β-LG was 50.64% (±1.29%) and 56.17% (±1.90%), respectively. The stability of incorporated VA in protein/liposome complex was significantly enhanced by protein presence the whole period of storage, compared to that of bare VA.	The most important determinant of the encapsulated VA in liposome–protein complex was the *in vivo* bioavailability evaluation. Phospholipid–sterol- protein-membrane resembling system may be one of the promising approaches to enhance the absorption of VA.	[175]

Table 2.2. Individual studies investigating the relationship between protein intake, digestion, and influence on lipophilic food constituent absorption (continued).

Liposoluble compound studied	Protein type studied	Study details	Effects found	Remarks	Ref.
Animal studies					
Carotenoids	SC	Male Sprague-Dawley rats aged 3 weeks were divided into groups receiving VA free diet and an experimental diet.	The serum retinol content in the low β-carotene diet groups was about 7–12% of that in the mid-level β-carotene diet groups. However, the liver retinol content in the low β-carotene diet groups was 0.12–0.18% of that in the mid-level β-carotene diet groups. Fat-soluble β-carotene and retinol accumulation decreased with a higher fat intake (with a 10% fat diet than with a 2% fat diet) rather than increased with an increase in fat intake. This study shows that the mid-level dietary protein groups were influential for retinol accumulation and metabolism of β-carotene.	The low β-carotene diet groups (0.006 mg β-carotene/100 g diet) showed a higher serum retinol accumulation rate, CDO activity, and liver β-carotene accumulation rate than the mid-level β-carotene diet groups. These results suggest the marked effect of protein, fat, and beta-carotene level in diets on the absorption and metabolism of β-carotene.	[205]

Table 2.2. Individual studies investigating the relationship between protein intake, digestion, and influence on lipophilic food constituent absorption (continued).

Liposoluble compound studied	Protein type studied	Study details	Effects found	Remarks	Ref.
Animal studies					
Vitamin D	Pea protein isolate (PPI)	Weaned male albino rats (n= 35) were fed either normal diet (VD 1000 IU/kg) (control group; n= 7) or a VD-deficient diet (<50 IU/kg) for six weeks (VD-deficient group; n= 28).	2.3 times higher absorption (increase in serum 25(OH) VD) compared to control without PPI emulsion. Consumption of VD dispersed in PPI nanoemulsion created by ultrasound and pH shifting improved its circulating status, which influenced the levels of bone turnover biomarkers in VD-deficient rats.	The presence of canola oil did not result in increased absorption. PPI can be used to encapsulate other fat-soluble micronutrients and bioactive needed in fortification and supplementation programs such as VA, VE, and carotenoids.	[142]

Table 2.2. Individual studies investigating the relationship between protein intake, digestion, and influence on lipophilic food constituent absorption (continued).

Liposoluble compound studied	Protein type studied	Study details	Effects found	Remarks	Ref.
Animal studies					
Vitamin D	WPI, SC	Sprague-Dawley rats (n= 78) were administered 840 IU VD3 dissolved in ethanol and either (a) complexed with whey protein isolate (protein: vitamin ration 2:1), (b) complexed with caseinate (protein: vitamin ration 2:1), or (c) provided in a water solution.	Compared to non-complexed and caseinate complexed, serum VD3 concentrations were higher across the different time points in rats that received the VD3 complexed with whey protein.	Using an *in vivo* rat model, the study demonstrated that complexation with milk proteins is an efficient strategy to enhance the bioaccessibility of VD3. Both complexation with whey protein isolate and caseinate, respectively, enhanced bioaccessibility of VD3, but complexation with whey protein isolate was found to be more potent than complexation with caseinate.	[11]

Table 2.2. Individual studies investigating the relationship between protein intake, digestion, and influence on lipophilic food constituent absorption (continued).

Liposoluble compound studied	Protein type studied	Study details	Effects found	Remarks	Ref.
Human studies					
Vitamin E	Milk protein	Included 3 experiments: a) (n= 48), compared delivery of 100 mg all-rac-α-tocopheryl acetate/d, 1%-fat milk containing soybean oil, milk fat, or both (1:1); b) (n= 24), compared delivery of RRR-α-tocopheryl acetate and all-rac-α-tocopheryl acetate in milk with the delivery of all-rac-α-tocopheryl acetate in orange juice (200 mg/d in each group), c) (n= 7), compared delivery of 30 mg all-rac-α-tocopheryl acetate/d in milk with and without added VA and VD.	Micro dispersion of VE in milk increased the molar ratio of plasma tocopherol to cholesterol by >2-fold compared with the molar ratio after consuming VE capsules. In contrast, the molar ratios were comparable after ingestion of orange juice and capsules. VA and VD did not affect vitamin E delivery by milk.	Milk augments VE transport by human lipoproteins at intakes of 100–200 but not 30 mg/d. This augmentation depends on the presence and type of fat in milk, its VA and VD contents, and whether VE is natural or synthetic.	[147]

EE= Encapsulation efficiency, VD= viatmin D, WPI= whey protein isolate, SPI= soy protein isolate, SC= sodium caseinate, GEL= gelatin, PEP= pleurotus eryngii polysaccharide, VA= vitamin A, RSNaCaS= reassembled succinylated sodium caseinate, SNaCaS= succinylated sodium caseinate, NaCaS= sodium caseinate, VE= vitamin E, PPI= pea protein isolate, CDO= Carotene 15,15'-dioxygenase.

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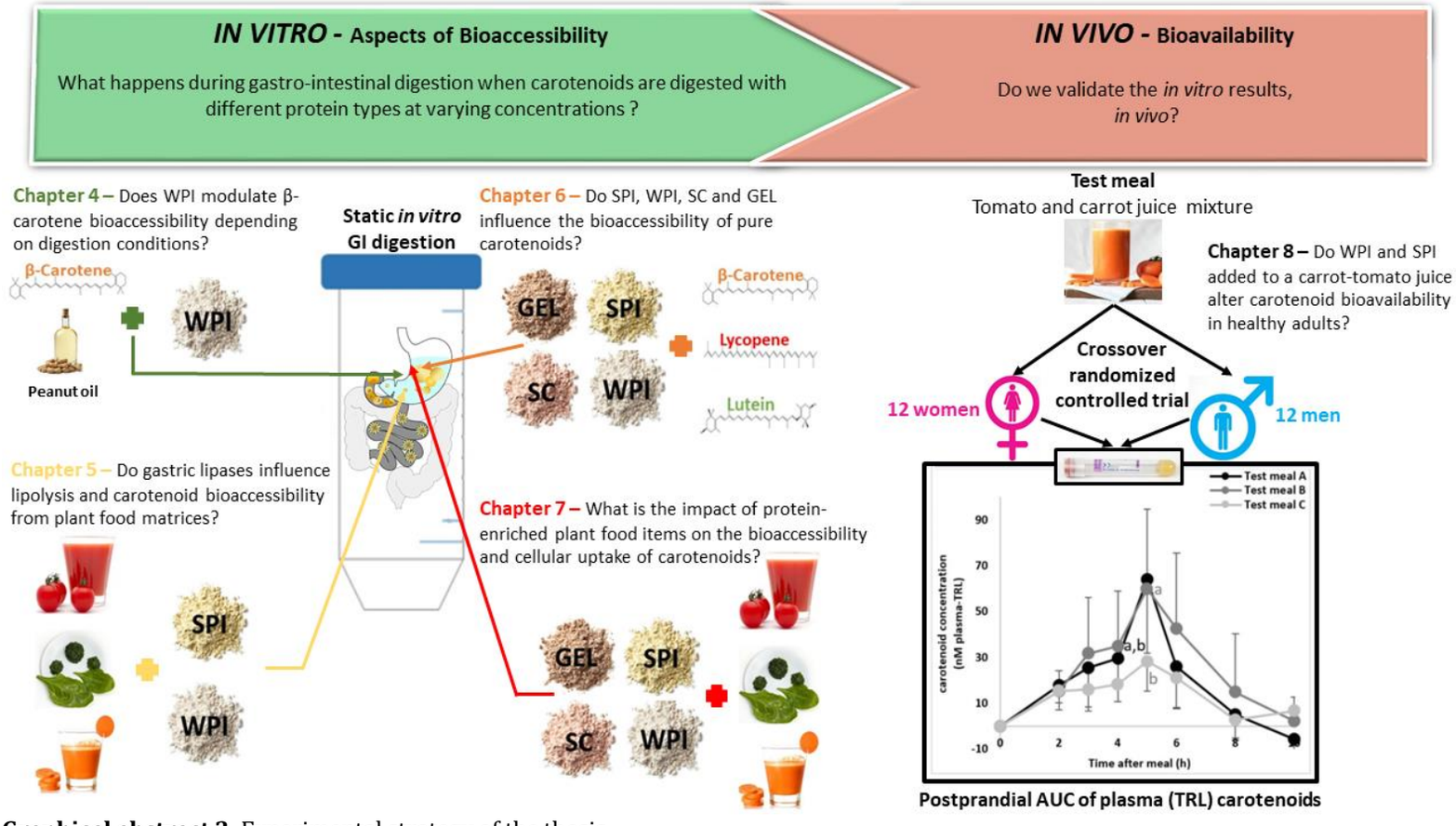
Chapter 3

Objectives and experimental strategy



Graphical abstract 3. Experimental strategy of the thesis.

Carotenoids are recognized for several health benefits, such as their potential role in preventing chronic diseases, including type 2 diabetes and cardiovascular diseases [1, 2]. In addition, a low intake of carotenoids within a mixed diet represents a major health concern in populations with low preformed vitamin A intake, leading to increased susceptibility to infections, growth disorders, skin diseases, xerophthalmia and night-blindness [3].

Though carotenoids occur in many plant-based food items, their absorption from the diet is low and variable [4]. A factor that is paramount for their absorption is micellization, and includes the release from the food matrix, transfer into lipid droplets and incorporation into mixed micelles. Several dietary factors have shown to interfere with bioaccessibility and bioavailability aspects, including fat, dietary fiber and minerals [5-7]. However, not much is known on the interaction between proteins and carotenoids during gastric and small intestinal digestion, and on the potential influence of these macromolecules on the solubilization and micellization of carotenoids.

In Chapter 2, we reviewed the state of knowledge on the interactions of proteins and liposoluble bioactive compounds in general, with a focus on carotenoids. Proteins have been used for encapsulation purposes of poorly soluble compounds, including carotenoids, to improve their chemical stability, solubility, as well as bioaccessibility of these sensitive molecules following *in vitro* GI digestion, pointing out toward increased bioavailability of such combinations [8]. However, the interaction between proteins and carotenoids in food matrices under conditions resembling the *in vivo* environment have, to our knowledge, never been studied

systematically. This is a major gap in carotenoid-related research, which is attempted to be addressed within the present thesis. Thus, the main objective of the present work is aimed to study the influence of proteins on carotenoid digestion and aspects of bioavailability.

As a first step to study interactions between proteins and carotenoids during GI digestion, we investigate, in Chapter 4, whether the presence of whey protein isolate (WPI), as a frequently consumed protein, in the test meal could alter β-carotene bioaccessibility, by using the digestion consensus protocol [9]. As the *in vitro* digestion model is employed for a wide set of purposes and with different endpoints, the first challenge was to test several digestion parameters, to better understand whether alterations of the choice of the parameters would result in significant differences regarding the interaction of WPI and β-carotene during digestion, before modifying further protein and carotenoid types and concentrations. As the use of gastric lipase is often neglected in *in vitro* studies, we tested in Chapter 5, whether gastric lipases further promote emulsification and the transition of lipid droplets to mixed micelle formation in the presence of proteins.

The ensuing research question addressed in Chapter 6, was to investigate the potential differences between various protein sources (e.g. WPI, soy protein isolate (SPI), milk casein, and gelatin) added at different concentrations to the test meals (equivalent to 0, 10, 25, and 50% of the RDA), on the influence on micelle formation, physicochemical properties of the digesta, release and bioaccessibility of individual carotenoids during GI digestion, including β-carotene, lutein and lycopene. Following the study of individually added

carotenoids, the third research question was to verify whether frequently consumed proteins of various hydrophobicity and sources (previously tested individual proteins, plus protein-rich matrices including cod and turkey) affect in a similar way the bioaccessibility and cellular uptake of carotenoids from regular food items, including solid and liquid matrices (spinach, carrot juice, tomato juice).

Finally, we aimed to prove or disprove the hypothesis that selected proteins, for which WPI and SPI were chosen based on *in vitro* findings, can impair postprandial carotenoid bioavailability in healthy human adults. This clinical trial, presented in Chapter 8, consisted of a randomized, postprandial crossover trial with 24 subjects, receiving the test meal made of a mixture of 350 g of tomato and carrot juice (1:1, v/v) that contained or not 30 g of either SPI or WPI (equivalent to ca. 50% of the RDA). Carotenoid bioavailability was assessed by HPLC in the triacyl-rich-lipoprotein fraction (TRL), representing newly absorbed carotenoids.

It is intended by this thesis to address many of these unanswered questions and to advance the state-of-the-art in the field of carotenoid bioavailability.

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Chapter 4

Whey protein isolate modulates beta-carotene bioaccessibility depending on gastro-intestinal digestion conditions

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Foreword

As mentioned in Chapter 1, the development of the static *in vitro* models simulating human digestion processes, by subjecting food samples to sequential oral, gastric and intestinal digestion, has facilitated the assessment of carotenoid bioaccessibility, which is an important component of their bioavailability. The growing interest in using such models has led to the development of the harmonized INFOGEST *in vitro* model, standardizing several key parameters, such as stomach and intestinal transit times, pH, digestive enzymes and their activity, based on available physiological data and practicality aspects.

Since *in vitro* digestion is carried out for a wide range of purposes and with different endpoints, some adaptations could be needed depending on the particular requirements of each experiment.

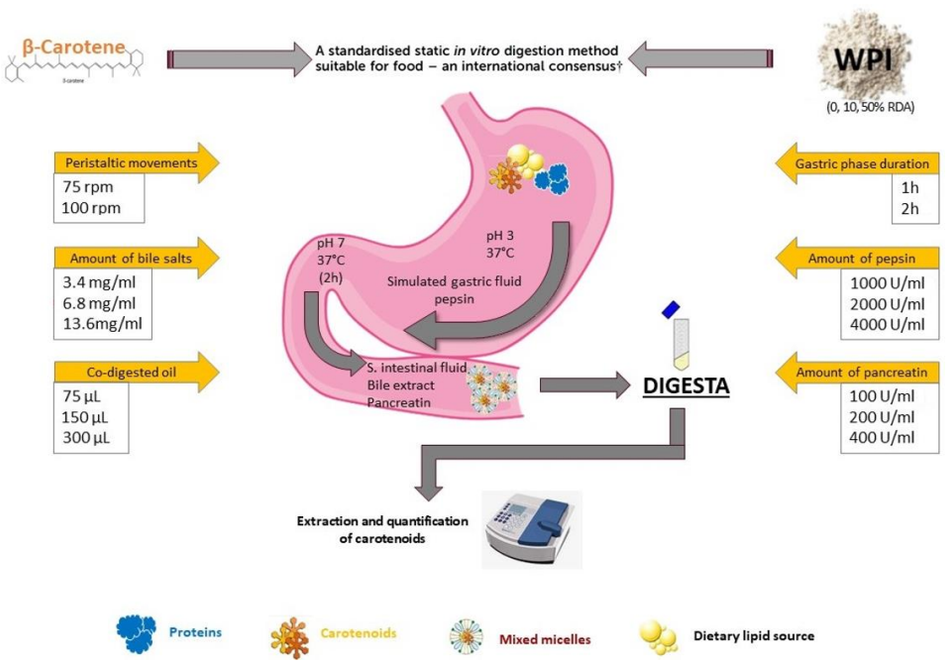
In this Chapter 4, we aim to investigate whether WPI can modulate β-carotene bioaccessibility, under various digestive conditions, such as digestive transit time, the amount of pepsin, pancreatin, bile, and kinetic energy (to simulate peristalsis) for the entire digestion process.

Whey protein isolate modulate beta-carotene bioaccessibility depending on gastro-intestinal digestion conditions

Abstract

Carotenoids are lipophilic phytochemicals; their intake has been associated with reduced chronic diseases. However, their absorption depends on emulsification during digestion and incorporation into mixed micelles, requiring digestive enzymes, gastric peristalsis, bile, and dietary lipids. In this study, we investigated whether whey protein isolate (WPI), a commonly consumed protein source, can modulate β-carotene bioaccessibility *in vitro*, especially under incomplete digestive conditions, i.e. under low digestive enzyme conditions. Thus, pepsin and pancreatin, kinetic energy, gastric digestion time, and amount bile and co-digested lipids were modified, and WPI at concentrations equivalent to 0/25/50% of the protein recommended dietary allowance (approx. 60g/d) were added to β-carotene dissolved in oil. WPI enhanced bioaccessibility by up to 20% ($p<0.001$), especially under higher simulated peristalsis or reduced dietary lipids. Conversely, they impaired bioaccessibility to one third ($p<0.001$) under incomplete digestive conditions. WPI modulated β-carotene bioaccessibility depending on digestive conditions.

Keywords: Proteins, carotenoids, micellization, emulsion, digestion, lipid droplets.



Graphical abstract 4. Whey protein isolate modulates beta-carotene bioaccessibility depending on gastro-intestinal digestion conditions.

4.1. Introduction

Carotenoids and their metabolites are recognized as playing a role in the prevention of human diseases. Based on epidemiological studies, a positive association has been suggested between dietary intake and tissue concentrations of carotenoids and lowered risk of several chronic diseases [1], such as cardiovascular diseases and type 2 diabetes [2], cancer [3], as well as reduced risk of age-related macular degeneration [4]. These correlations may be attributed to their antioxidant and anti-inflammatory activity [5], as well as their influence on the immune system [6], both possibly related to a number of transcription factors and nuclear receptors such as NF-κB [7] and RAR/RXR [8]. In addition to these health relevant aspects, certain carotenoids, including β-carotene, constitute vitamin A active compounds, following their cleavage by β-carotene oxygenase 1 (BCO-1) *in vivo* and metabolism into retinal.

Before being absorbed, carotenoids must be released from the food matrix and dissolved in lipid droplets. Then, under the influence of digestive enzymes (especially lipase), pH, gastric peristalsis, and bile, carotenoids are incorporated into mixed micelles, allowing their uptake by the mucosa of the small intestine [9]. Due to their lipophilic nature, the bioavailability of carotenoids, including β-carotene, is relatively low, typically between 10 and 20% [10, 11]. Consequently, several studies have been investigating potential dietary as well as host-related factors influencing carotenoid bioavailability aspects, including their bioaccessibility and absorptive processes.

Regarding matrix-related factors, several studies have concluded that dietary lipids can significantly enhance carotenoid absorption, fostering micellization [11, 12]. Also, it has been reported that dietary fiber can decrease the micellization of carotenoids, possibly by limiting enzymatic access and activity [13]. Recently, divalent minerals at higher concentrations were suggested to compromise carotenoid bioaccessibility, likely by precipitating fatty acids and bile salts [14, 15]. In addition to these dietary factors, it was shown that host-related factors can modulate carotenoid bioavailability [reviewed in 9], including conditions that alter the gastro-intestinal (GI) surface area or digestive enzyme secretion. For example, bariatric surgery has been associated with several physiological modifications, among them, gastric peristalsis movement, at least in part explaining the decrease of plasma carotenoid concentrations [16]. In another study, it was reported that Crohn's disease resulted in carotenoid malabsorption [17]. Other anomalies such as cholecystectomy (reducing the available amount of bile), and pancreatitis (leading to secretory insufficiency of digestive enzymes) have been proposed to compromise carotenoid bioavailability [10].

One factor that may impinge on carotenoid absorption, but has not yet received much attention, is the presence of proteins. For example, caseins have been successfully employed for micro- and nano-encapsulation of β-carotene, significantly improving its bioaccessibility [18, 19]. It was proclaimed that proteins may aid in the bioaccessibility of liposoluble dietary constituents in several ways. Following their adsorption to lipid droplet surfaces, proteins may

stabilize oil-in-water (o/w) emulsions in the GI tract, attributed to the fact that proteins can be highly surface active molecules [19, 20], and the formed particles tend to be highly negatively charged, preventing the aggregation of lipid droplets [21]. However, the same study also showed that gliadin decreased the enzymatic degradation of lipids, likely by hindering digestive enzymes to adsorb to the droplet surfaces or binding to enzymes directly, also implying potential negative influences of proteins on the micellization process. Whey protein isolate (WPI) in contrast was effective at inhibiting lipid oxidation of o/w emulsions [21], which may contribute to limit oxidative degradation of sensitive molecules, as were caseino-phosphopeptides, which were proposed to chelate iron which may trigger oxidative degradation [22]. Furthermore, WPI also facilitated the formation of smaller lipid droplets; Salvia-Trujillo and co-workers showed that reducing the diameter of these particles was related to increased β-carotene bioaccessibility [23].

It is assumed that the emulsifying properties are greatly influenced by the composition and structure of proteins. For instance, both hydrophobic and hydrophilic groups are abundant in WPI, highlighting its amphiphilic behavior [24]. The structure of WPI is comprised of two main fractions, i.e. α-lactalbumin (123 amino acids) and β-lactoglobulin (162 amino acids), together accounting for approx. 75% of the whey proteins, and their conformation (predominantly α-helix and β-pleated sheet, respectively) affect their properties at the oil/water interface [25].

In the current study, we aimed to investigate the effect of a commonly consumed protein source, WPI, on the bioaccessibility of

pure β-carotene, under a range of digestive conditions, as proteins were hypothesized to rather enhance bioaccessibility under marginal, i.e. somewhat insufficient digestive conditions. For this purpose, we employed a previously established *in vitro* GI consensus model [26], and WPI was added at different concentrations, reflecting various fractions of the recommended dietary allowance (RDA) of proteins, i.e. 0, 25 and 50%. Micellization of co-digested β-carotene thus measured also under insufficient digestion parameters (i.e. shortened gastric digestion duration, limited concentrations of digestive enzymes, gastric peristalsis, bile, and a surplus of dietary lipids), such as possible in subjects with GI disorders.

4.2. Materials and methods

4.2.1. Enzymes and chemicals

Pepsin from porcine gastric mucosa (powder, ≥250 U/mg, Art. No. P7000), pancreatin from porcine pancreas (activity equivalent to 4x USP specifications. Art. No. P1750), porcine bile extract (Art. No. B8631), as well as β-carotene standard (≥97% UV, >95% *all-trans* form according to supplier and own HPLC analysis, Art. No. 22040) were purchased from Sigma-Aldrich (Overijse, Belgium). Whey protein isolate (WPI) was obtained from Pure Nutrition USA (95% purity, California, USA) while the peanut oil, typically free of native carotenoids (according to the USDA database and own blank examinations), was purchased from a local supermarket (Delhaize, Strassen) and used without further purification.

Unless otherwise specified, all chemicals were of analytical grade or superior. Potassium chloride (≥99%, Art. No. P9541), potassium phosphate (monobasic ≥99, Art. No. P5655), sodium bicarbonate (≥99, Art. No. S5761), sodium chloride (≥99.5%, Art. No. 71376), magnesium chloride hexahydrate (Art. No. M2393), ammonium carbonate (Art. No. 68392), sodium hydroxide solution (1 M, Art. No.1.09137), calcium chloride dihydrate (≥99, Art. No. C3306), Nile red (Art. No. 19123), fluorescein isothiocyanate isomer I (FITC) (Art. No. F7250), and phenolphthalein (Art. No. 105945) were acquired from Sigma-Aldrich. Hexane (≥95%, Art. No. 24574), acetone (≥99%, Art. No. 20063), and hydrochloric acid (1 M, Art. No. 30024.290) were obtained from VWR (Leuven, Belgium).

4.2.2. β-Carotene, WPI and enzyme solutions

β-Carotene standard solution was first prepared by dissolving 1 mg of β-carotene standard in 2 mL of hexane. The solution was sonicated for 5 min. and warmed up at 30 °C for 5 min. (Ultrasonic Cleaner, VWR Symphony®, Massachusetts, USA). Then, 1 mL of peanut oil was added to foster the dissolution of β-carotene by sonication and warming at 30 °C for 5 min. The hexane was removed by evaporation under a stream of nitrogen for ± 20 min. at 30 °C (TurboVap LV from Biotage®, Uppsala, Sweden). The standard β-carotene solution was made by the addition of 4 mL of peanut oil to the remaining 1 mL to reach a concentration of 0.2 mg/mL, and two other concentrations (0.1 and 0.4 mg/mL) were also prepared for further investigation by adding 9 and 1.5 mL (instead of 4 mL) of peanut oil, respectively. The concentration of the standard solutions was later verified

spectrophotometrically as explained below (section 2.5). Aliquots of the standard solutions were pipetted into 15 mL falcon tubes, and stored at -80 °C until usage.

Assuming a total volume of 2 L of intestinal fluids during GI digestion, the amount of β-carotene added to each digesta was 30 μg, by pipetting 75, 150 or 300 μL from the standard solutions (0.4, 0.2 or 0.1 mg/mL, respectively), representing 1.2 mg/L of β-carotene and 3, 6 or 12 g/L of oil (Table 4.1).

In the same way, the amounts of WPI tested were 0, 7.5 and 15 g/L (Table 4.1), corresponding to 0, 25 and 50% of the recommended dietary allowance (RDA) (60 g/d for human adults) [27]. Practically, 0, 187.5 or 375 mg of WPI were dissolved in 6.25 mL of pure water, and together with the added β-carotene solution in oil, the final ratio of matrix (WPI+β-carotene solution) to simulated gastric fluid of 50:50 (v/v) was reached. A brief mixing of the WPI solution with the beta-carotene in peanut oil and the simulated gastric fluid (SGF) was achieved by vortexing for 15 sec.

SGF and the simulated intestinal fluid (SIF) were made as recommended by the European consensus digestion model [26]. Pepsin solution was prepared in SGF to reach a final concentration of 2000 U/mL of the final gastric mixture. In addition to the standardized concentration (Table 4.1), two other concentrations were tested (1000 and 4000 U/mL).

Regarding pancreatin and bile extract, pancreatin was weighed based on a trypsin activity of 100, 200 or 400 U/mL, while the bile extract was weighed in order to reach a concentration of 3.4, 6.8 or

13.6 mg/mL of the final digestion volume (Table 4.1); both were prepared in the same SIF solution.

It is noteworthy that the European consensus suggested a standardized amount of pancreatin based on trypsin activity at 100 U/mL, but in the present study, pancreatin amount was doubled following pre-experiments (200 U/mL of trypsin), in order to enhance the digestibility of both WPI and oil (by increasing at the same time the lipase activity). As explained above, two other concentrations of pancreatin (100 and 400 U/mL) as well as bile extract (3.4 and 13.6 mg/mL) were tested. Conditions with low pancreatin (100 U/mL) and/or low bile (3.4 mg/mL) are denoted in the following as marginal digestive conditions in this investigation.

Table 4.1. Overview of digestion conditions studied in dependence of various concentrations of whey protein isolate (WPI).

Aspect of digestion	Condition	Parameters chosen[$]
Matrix	Amount of WPI	0 mg/mL, 7.5 mg/mL, 15 mg/mL
	Amount of oil[1]	3 g/L, 6 g/L*, 12 g/L
Gastric	Peristalsis speed	75 rpm*, 100 rpm
	Gastric duration	1h, 2h*
	Amount of pepsin	1000 U/mL, 2000 U/mL*, 4000 U/mL
Small intestine	Peristalsis speed	75 rpm*, 100 rpm
	Amount of bile	3.4 mg/mL[&], 6.8 mg/mL*, 13.6 mg/mL
	Amount of pancreatin	100 U/mL[&], 200 U/mL*, 400 U/mL

[$]all concentrations are per final volume of digesta at the respective digestive step.
*Standard conditions according to the European consensus digestion model [26].
[&]considered and termed in the text as marginal digestion conditions.

[1] Contained 30µg of β-carotene, representing 1.2mg/L of β-carotene, considering a typical intake of around 4.1 mg/d of β-carotene (Böhm V et *al.* From carotenoid intake to carotenoid blood and tissue concentrations – implications for dietary intake recommendations. Nutrition Reviews 2021, 79(5):544-573).

4.2.3. Simulation of gastro-intestinal (GI) digestion

In vitro simulated GI digestion was carried out according to the harmonized INFOGEST protocol, with some modifications as explained above (section 2.2). The model was used to test the influence of proteins at three different concentrations on β-carotene bioaccessibility, including also rather marginal, i.e. insufficient digestion parameters. The oral phase was omitted as the matrix employed did not include significant amounts of carbohydrates and was liquid.

For the gastric phase, 6.25 mL of protein solution at the desired concentration (equivalent to 0, 25 or 50% of the RDA) was added to each sample, followed by the addition of 75, 150 or 300 µL of β-carotene solution in oil (corresponding to a total of 30 µg of β-carotene per digest, with amounts similar to former *in vitro* experiments (Biehler et al. 2011)). Before the addition of 4 mL of SGF (1.25 x concentrate), 1 mL of pepsin solution was added to the mixture in order to reach enzymatic activities of 1000, 2000, 4000 U/mL of the final gastric volume. Then, 31 µL of calcium dichloride (0.03 M) was added to reach a concentration of 0.075 mM in the final mixture, and finally, pH adjustment to 3 by hydrochloric acid (1 M) was carried out, bringing the volume of each sample to 12.5 mL with pure water.

Finally, the samples were incubated in a shaking water bath (GFL 1083 from VEL®, Leuven, Belgium) for 1 or 2 h at 37 °C, with a shaking speed of 75 or 100 rounds per minute (equal to a doubling of the kinetic energy, which is proportional to the square of the speed),

depending on the desired condition. In the standardized INFOGEST digestion model [26], no specific shaking speed was suggested.

At the end of the gastric incubation, 7.5 mL of SIF, and 2.5 mL of pancreatin and bile extract solution at various concentrations (100, 200 or 400 U/mL based on trypsin activity for pancreatin, and 3.4, 6.8 or 13.6 mg/mL for the bile extract) were added to the chyme. Then, 250 µL of calcium dichloride (0.03 M) was added to reach a concentration of 0.3 mM in the final mixture. Before bringing the volume to 25 mL, the pH was adjusted to 7 by the addition of sodium hydroxide solution (1 M), then the samples were incubated for 2 h at 37 °C, with a shaking speed of 75 or 100 rounds per minute (rpm).

4.2.4. β-Carotene extraction

At the end of the intestinal incubation, 12 mL were removed from the digesta and transferred into 15 mL falcon tubes. The samples were centrifuged for 1h at 3200 x g (4 °C), then 5 mL were collected from the middle aqueous phase and filtered through 0.2 µm nylon membrane syringe filter. In a new 15 mL falcon tube, 4 mL of hexane:acetone (2:1, v:v) were added to 2 mL of the filtered aqueous phase, vortexed for 30 sec. and centrifuged for 2 min. at 3200 x g (4 °C). The supernatant, which represents the hexane phase, was transferred into a new falcon tube, then the extraction process was repeated two times with 4 mL of hexane. All extracts were combined in the same tube and dried under a stream of nitrogen and stored at -80 ^{o}C until analysis.

4.2.5. Spectrophotometric analysis

Spectrophotometric analysis of β-carotene was done as described previously [28]. The dried residue was reconstituted in 1 mL of cold (4ºC) hexane and transferred to a 1 mL quartz cuvette (absorption cuvette, Hellma®, New York, USA). The absorbance was measured between 300 and 600 nm (GENESYS™ 10S UV-Vis Spectrophotometer, Thermo Fisher Scientific, Massachusetts, USA). The concentration was calculated by applying the Beer-Lambert law, taking into account the molar absorption coefficient [29], and different dilution steps:

$$C = \frac{abs_{450} * Fd}{\varepsilon_{\lambda}} x\ d\ x\ M$$

where C = concentration of the measured solution (mg/mL), abs_{max} = maximum measured absorbance of β-carotene at approx. 450 nm, Fd = dilution factor adjusting for extraction or reconstitution process, ε_{λ} = molar absorption coefficient ($Lmol^{-1}cm^{-1}$) of β-carotene in hexane (138824), d = length of light pathway through cuvette (cm) and M = β-carotene molar mass (536.89 g/mol).

Finally, the percentage of β-carotene micellization was used as a measure of bioaccessibility, and was expressed as the percentage of the solubilized amount of β-carotene present in the aqueous phase of the filtered digesta after *in vitro* GI digestion, compared to the initial amount added to the sample.

4.2.6. Further physicochemical characterization of digesta

To study further physicochemical properties of the digesta, macroviscosity and surface tension, micelle size and zeta potential, fatty acid titration and the evaluation of protein hydrolysis, as well as

the emulsion structures were investigated, as these parameters were also believed to possibly impinge on β-carotene bioaccessibility.

4.2.6.1. Rheological measurements and surface tension of the digesta

Flow curves were determined as a function of the shear rate between 0.1 s^{-1} and 130 s^{-1} on an Anton-Paar rheometer (MCR 302, WESP, Graz, Austria), equipped with a double gap cell at 5°C. Each measurement cycle did comprise a temperature equilibration phase, a pre-shear phase at 5 s^{-1} of 60 s, a flow curve with increasing shear rates, an intermediate equilibration phase at 131 s^{-1} of 60 s and a flow curve with decreasing shear rates. At the end of each cycle, a regression flow curves was calculated for the shear rate range between 0 s^{-1} and 100 s^{-1} for the shear stress respectively between 1 s^{-1} and 100 s^{-1} for the viscosity. Each measurement cycle was performed three times, because of the fluidity of the samples. The regression curves were averaged and plotted as viscosity and shear stress as a function of the shear rate.

Surface tension of digesta samples, pre-conditioned at 25 ± 0.1 °C, were determined by the weight-drop method as previously described by [30]. The air-water interfacial properties of digesta were calculated as follows:

$$\sigma_{digesta} = \frac{m_{digesta}}{m_{H2O}} \text{ x } \sigma H_2O$$

where σ_{H2O}= 71.99 dyn cm^{-1} is the surface tension of pure water.

4.2.6.2. Micelle size and zeta potential analysis

Aliquots of the aqueous micellar fraction obtained after GI digestion under standard conditions (Table 4.1) were filtered through a 0.2 μm nylon membrane syringe filter for the analysis of the micelle size and zeta potential. The intensity-weighted mean hydrodynamic radius and zeta potential were determined by dynamic light scattering and laser doppler micro-electrophoresis, respectively. Measurements were done at room temperature with at least four replicates, by using a Zetasizer Nano Zs instrument (Malvern Instruments, Malvern, UK).

4.2.6.3. Lipid and protein digestion

The amount of free fatty acids in the digesta after GI digestion was estimated as described previously [31], based on the titration against sodium hydroxide (NaOH 0.1 M), using phenolphthalein as an indicator.

Sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) was carried out to study completeness of protein digestion. Samples were denatured at 95^{o}C for 5 min. in Lämmli buffer. About 22 μg of protein per pocket was loaded to a 15% acrylamide and 0.4% bisacrylamide gel (gastro-intestinal digestion) and 45 μg of protein per pocket was loaded to a 10% acrylamide/0.3% bisacrylamide gel (gastric digestion). Marker was Invitrogen novex see blue plus 2 (Invitrogen, Carlsbad, CA). Gel was run at 80 V (30 min.) plus 100 V (90 min.). Running buffer contained SDS (0.1%), glycine (190 mM), tris-HCl (25 mM). Fixation/staining was done with methanol/acetic acid/coomassie brilliant blue R (50%, 10%, 0.1%)

(30 minutes), destaining in methanol/glacial acetic acid/water (30%, 10%, 60%, 3 h). Pictures were taken in a Kodak Gel Logic 2200 Imaging System (Kodak, Rochester, NY).

4.2.6.4. Confocal laser scanning microscopy (CLSM)

Confocal imaging of emulsion structures before and after GI digestion under standard conditions (Table 4.1) was carried out at room temperature with a confocal laser scanning microscope (Zeiss LSM 880, Airyscam SR, Jena, Germany), using a 63x objective. Nile Red dissolved in ethanol and FITC dissolved in acetone were mixed into the aliquots of digesta for fat and protein staining with final concentrations in the samples of 1 µg/mL. A small-sample aliquot was placed on a slide for visualization. The fluorescent dyes were excited by an Argon 488 nm laser and the emitted light was collected at 522 nm for proteins and 635 nm for the fat phase. The diameter of lipid droplets (n=20/group) at various concentration of WPI was measured by using a modular image-processing and analysis software for digital microscope (Zen 2.3 blue edition, Carl Zeiss Microscopy GmbH, Jena, Germany).

4.2.7. Statistical analyses and data treatment

In order to minimize day-to-day variations between experiments, bioaccessibility of β-carotene was normalized to a daily control which was assessed for each digestion. Unless otherwise stated, all values are expressed as the mean ± standard deviation. Replicates were obtained from at least 2 individual sets of analyses

obtained at different days (N≥2), and at least 4 replicates were obtained for each digestive condition during 1 set of analyses.

Statistical analysis was performed using SPSS 22 software (SPSS Inc., Chicago, IL). Normal distribution of data was verified by Q-Q -plots and equality of variance by box-plots and Levene's test. For statistical evaluation, β-carotene bioaccessibility values were log-transformed to achieve a normal distribution. Linear mixed models were developed with the effect of WPI concentration, amount of lipid source, bile extract concentration, kinetic energy and duration of gastric phase, pepsin and pancreatin concentration as fixed factors and log-β-carotene bioaccessibility as the observed dependent factor. As a model including all factors was not fully factorial, but several significant interactions were obtained, additional linear mixed models were run to keep constant certain parameters and to better allow further group-wise comparisons. P-values < 0.05 were considered statistically significant different (2-sided). Where needed, Fisher F-tests were followed by post hoc tests (Bonferroni, for comparing >3 groups) or LSD tests (for comparison of ≤ 3 groups).

4.3. Results

4.3.1. Interactions of WPI with matrix related effects affecting β-carotene bioaccessibility

4.3.1.1. Bioaccessibility under standard conditions and with escalating concentrations of WPI

Under control conditions (no addition of WPI), bioaccessibility of pure β-carotene following the GI digestion averaged approx. 0.3 μg/mL, representing around 25% of β-carotene recovered from the aqueous micellar fraction at the end of the *in vitro* digestion, compared to the initial amount added at the beginning of digestion (Figure 4.1A). Although the addition of an equivalent of 50% protein RDA of WPI slightly enhanced bioaccessibility (Figure 4.1A) compared to the control (no protein added), this effect was not significant under standard conditions [26].

4.3.1.2. Influence of WPI at different amounts of co-digested oil on β-carotene bioaccessibility

Following linear mixed models, the effect of oil ($p<0.001$) was statistically significant, while the influence of WPI had no significant effect. In addition, a significant interaction between the amount of added oil and WPI concentration was encountered ($p<0.001$), thus the effects were further studied at individual WPI levels.

When no WPI was added, increasing the volume of oil (up to 12 g/L) negatively affected the bioaccessibility ($p<0.001$) compared to the standard conditions (6 g/L of oil). No significant effect on β-

carotene bioaccessibility was observed with a lower amount of oil (3 g/L of oil, Figure 4.1B, Table 4.2).The addition of 25% RDA of WPI to samples with a reduced amount of oil further enhanced the bioaccessibility (by up to 20%) compared to the control, while the addition of WPI at 50% RDA had no significant effect (Figure 4.1B). However, adding WPI to the simulated GI digestion with a high volume of oil (12 g/L) caused an additional decrease in bioaccessibility, in a dose dependent manner ($p<0.001$). In this scenario, adding the equivalent of 25 and 50% RDA of WPI significantly decreased β-carotene bioaccessibility by 20% ($p=0.017$) and up to 35% ($p<0.001$), respectively, compared to the control (no WPI).

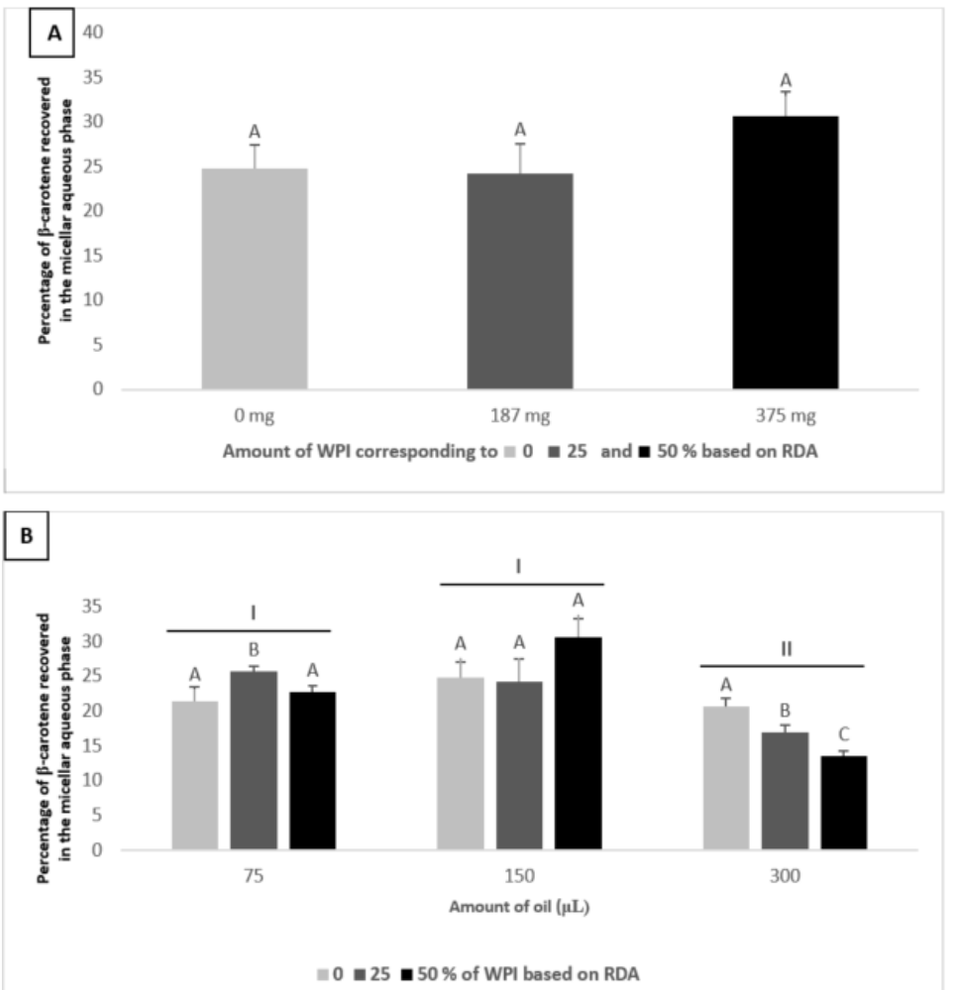


Figure 4.1. Influence of whey protein isolate (WPI) (A), and oil, co-digested with WPI (B) on β-carotene bioaccessibility at 75 rpm water bath shaking speed. β-Carotene was digested either in the presence of WPI at different amounts (0, 187 and 375 mg per final 25 mL digesta volume, representing 0, 25 and 50% of protein recommended dietary allowance (RDA)), or in the presence of different volumes of oil (75, 150 and 300 µL per final 25 mL digesta), at various amounts of WPI (0, 25 and 50% of RDA) at 75 rpm. Bioaccessibility is expressed as the percentage of β-carotene recovered from the aqueous micellar fraction at the end of the *in vitro* GI digestion, compared to the amount of β-carotene added at the beginning of digestion. Values represent means ± SD of $n = 4$ and N≥2 (sets repeated at different days). Labeled means without a common superscript (alphabetic letters or roman numbers) differ significantly, $P<0.05$.

4.3.2. Influence of WPI on β-carotene bioaccessibility at various gastric conditions

4.3.2.1. Impact of WPI at altered simulated peristalsis on β-carotene bioaccessibility

Following linear mixed models, the effect of peristalsis conditions on β-carotene bioaccessibility was statistically significant ($p<0.001$), while the influence of WPI had no significant effect. Also, the interaction between the added amount of WPI and peristalsis movements had a significant ($p=0.019$) effect on the bioaccessibility of β-carotene, thus effects were further studied at individual shaking speed.

The higher shaking water speed of 100 rpm induced an up to two times higher β-carotene micellization ($p<0.001$) compared to the lower speed of 75 rpm (Figure 4.2, Table 4.2), across all 3 levels of proteins present (0, 25 or 50% of RDA), at 2 h of gastric digestion time.

Following all-group-wise comparisons (post hoc-tests), the addition of 25% RDA of WPI significantly increased ($p<0.001$) β-carotene bioaccessibility when a higher kinetic energy was applied, relative to the control (Figure 4.2A). However, no significant influence was observed with the addition of 50% RDA of WPI, i.e. WPI did not influence β-carotene bioaccessibility in a linear dose-dependent manner.

4.3.2.2. Influence of WPI on β-carotene bioaccessibility at altered gastric phase duration

Following linear mixed models, the effect of gastric phase duration ($p<0.001$) was statistically significant, while the influence of WPI had no overall significant effect. However, the impact of gastric digestion time (2 vs. 1 h) depended significantly on the shaking speed ($p=0.0016$, Figure 4.2A), with larger differences observed at lower simulated peristalsis.

Reducing gastric transition to 1 h decreased bioaccessibility by 25% at 75 rpm (in general for all levels of WPI addition), while the negative effect was less pronounced, yet significant, with a higher shaking speed, compared to the standardized 2 h of gastric phase ($p<0.001$, Table 4.2).

At 75 rpm, no further influence was noticed on β-carotene bioaccessibility following the addition of WPI, with a reduced gastric digestion time (Figure 4.2A), while a significant ($p<0.001$) interaction was found at 100 rpm between protein concentration and gastric phase duration (Figure 4.2A). Here, the addition of WPI significantly ($p<0.001$), though only slightly, increased the bioaccessibility of β-carotene by up to 20% (for the 25% RDA protein group), compared to the control.

4.3.2.3. Effect of varying concentrations of pepsin

Following linear mixed models, the effects of both WPI ($p<0.001$) and of pepsin concentration ($p<0.001$) were statistically significant. A statistical significant interaction of pepsin x protein ($p=0.008$) was also found regarding β-carotene bioaccessibility.

Under control conditions (no WPI), no influence of varying the amount of pepsin on β-carotene micellization (Figure 4.3A) was found. Similarly to the standard conditions (2000 U/mL of pepsin), addition of WPI to the simulated GI digestion with 4000 U/mL of pepsin did not influence β-carotene bioaccessibility compared to the daily control (Table 4.2). However, the addition of WPI to the samples with a lower concentration of pepsin decreased β-carotene bioaccessibility by up to 40% ($p<0.001$), compared to the control (no WPI).

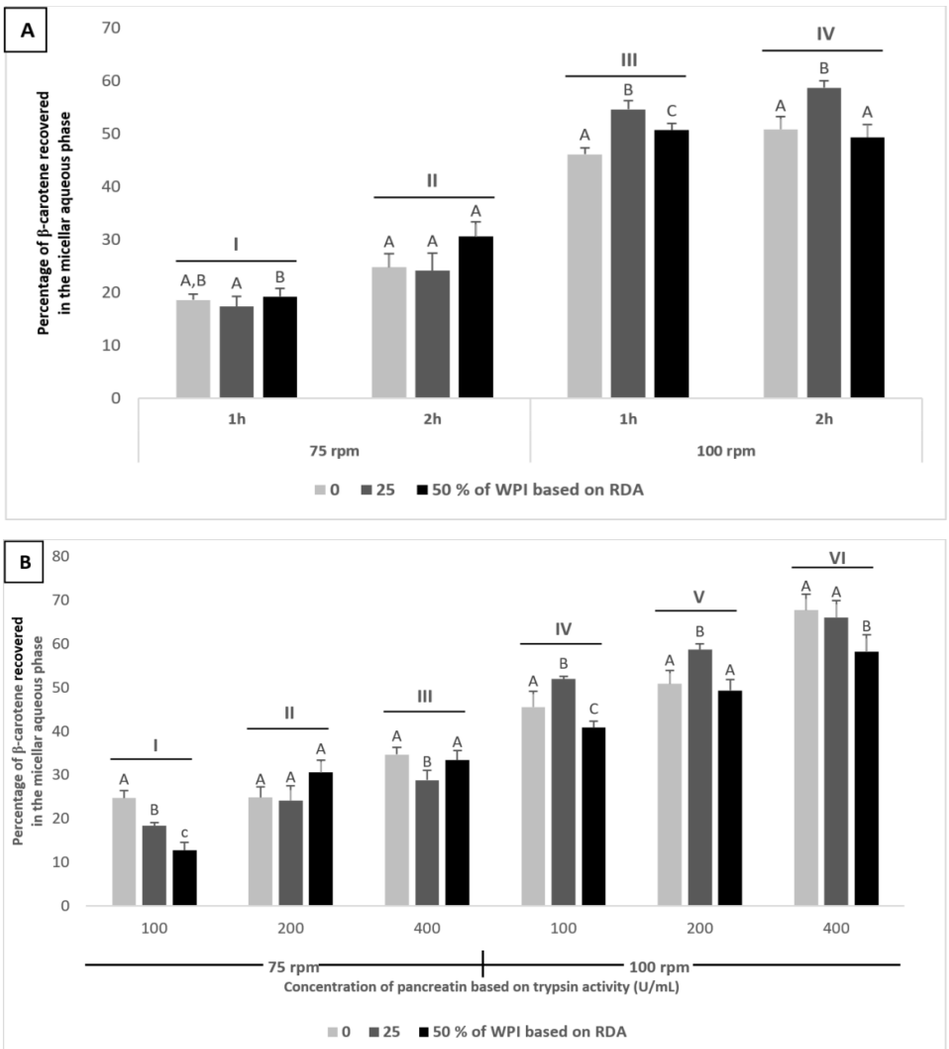


Figure 4.2. A) Influence of peristalsis energy and gastric phase duration on β-carotene bioaccessibility and the effect of additional co-digested whey protein isolate (WPI). B) Influence of pancreatin concentration on β-carotene bioaccessibility and the effect of additional co-digested whey protein isolate (WPI) at 75 (left panel) and 100 rpm (right panel) water bath shaking speed. β-Carotene was digested in the presence of different amounts of pancreatin representing 50, 100 and 200 U/mL digesta, expressed as protease activity, in the presence of various amounts of WPI (0, 187 and 375 mg per final 25 mL digesta volume, representing 0, 25 and 50% of recommended dietary allowance (RDA)) at 75 rpm and 100 rpm. Bioaccessibility is expressed as explained in Fig. 1 heading. Values represent means ± SD of n = 4 and N≥2 (sets repeated at different days). Labeled means without a common superscript (alphabetic letters or roman numbers) differ significantly, $p<0.05$.

Table 4.2. Average bioaccessibility (%)[1] of pure β-carotene digested with whey protein isolate (WPI) at varying concentrations[2]. Values represent mean ± SD of $n = 4$ and N≥2 (sets repeated at different days). Means without a common superscript differ significantly, $p<0.05$.

Condition	Bioaccessibility (%) WPI (% RDA)		
	0	25	50
Oil			
3 g/L [I]	21.4 ± 2.2[A]	25.6 ± 0.8[B]	22.7 ± 0.9[A]
6 g/L [I]	24.8 ± 2.5[A]	24.1 ± 3.3[A]	30.6 ± 2.8[A]
12 g/L [II]	20.7 ± 1.2[A]	16.8 ± 1.2[B]	13.5 ± 0.8[C]
Peristalsis energy			
75 rpm [I]	24.8 ± 2.5[A]	24.1 ± 3.3[A]	30.6 ± 2.8[A]
100 rpm [II]	50.8 ± 2.6[A]	58.7 ± 1.3[B]	49.3 ± 2.5[A]
Gastric duration			
At 75 rpm			
1h [I]	18.6 ±	17.4 ± 1.9[A]	19.2 ± 1.5[B]
2h [II]	1.1[A,B]	24.1 ± 3.3[A]	30.6 ± 2.8[A]
At 100 rpm	24.8 ± 2.5[A]		
1h [III]		54.6 ± 1.6[B]	50.7 ± 1.2[C]
2h [IV]	46.1 ± 1.2[A]	58.7 ± 1.3[B]	49.3 ± 2.5[A]
	50.8 ± 2.6[A]		
Pepsin			
1000 U/mL [I]	26.1 ± 2.1[A]	15.7 ± 1.7[B]	19.9 ± 1.1[C]
2000 U/mL [II]	24.8 ± 2.5[A]	24.1 ± 3.3[A]	30.6 ± 2.8[A]
4000 U/mL [II]	28.4 ± 0.9[A]	24.9 ± 1.9[A]	24.7 ± 1.2[A]
Pancreatin			
At 75 rpm			
100 U/mL [I]	24.7 ± 1.6[A]	18.4 ± 0.7[B]	12.7 ± 1.9[C]
200 U/mL [II]	24.8 ± 2.5[A]	24.1 ± 3.3[A]	30.6 ± 2.8[A]
400 U/mL [III]	34.7 ± 1.6[A]	28.8 ± 2.3[B]	33.3 ± 2.2[A]
At 100 rpm			
100 U/mL [IV]	45.5 ± 3.6[A]	51.9 ± 0.7[B]	40.8 ± 1.4[C]
200 U/mL [V]	50.8 ± 2.6[A]	58.7 ± 1.3[B]	49.3 ± 2.5[A]
400 U/mL [VI]	67.7 ± 3.7[A]	65.9 ± 3.9[A]	58.2 ± 3.9[B]
Bile extract			
3.4 mg/mL [I]	21.1 ± 0.9[A]	10.0 ± 0.7[B]	7.5 ± 0.9[C]
6.8 mg/mL [II]	24.8 ± 2.5[A]	24.1 ± 3.3[A]	30.6 ± 2.8[A]
13.6 mg/mL [II]	25.5 ± 0.7[A]	26.5 ± 1.1[A]	22.6 ± 0.7[B]

RDA: recommended dietary allowance. [1]Bioaccessibility is expressed as the percentage of β-carotene recovered at the end of the *in vitro* GI digestion, compared to the amount added at the beginning of digestion. [2]Concentrations given in final digestion stage (small intestine) are 0, 25 and 50% based-RDA.

4.3.3. Influence of WPI at altered intestinal conditions

4.3.3.1. Effect of varying concentrations of bile extract

Following linear mixed models, the effects of both WPI ($p=0.002$) and of bile concentration ($p<0.001$) were statistically significant. In addition, a significant interaction between added amounts of bile extract and protein concentration was found ($p<0.001$).

A maximum of β-carotene micellization (around 25%) was obtained with the higher amounts of bile extract (6.8 and 13.6 mg/mL), while 3.4 mg/mL of bile extract significantly ($p<0.001$) decreased β-carotene bioaccessibility, compared to the standard conditions (6.8 mg/mL, Figure 4.3B).

There was no significant effect of adding WPI at high amounts of bile (6.8 and 13.6 mg/mL per digesta), except for a slight decrease of β-carotene bioaccessibility when 50% RDA of WPI was added to the samples with highest amounts of bile extract ($p=0.0011$). However, the addition of WPI to the digesta with reduced amount of bile extract (3.4 mg/mL) negatively affected the bioaccessibility in a concentration dependent manner ($p<0.001$). The addition of 25% and 50% RDA of WPI decreased the bioaccessibility to half and 1/3, respectively, compared to the control ($p<0.001$, Table 4.2).

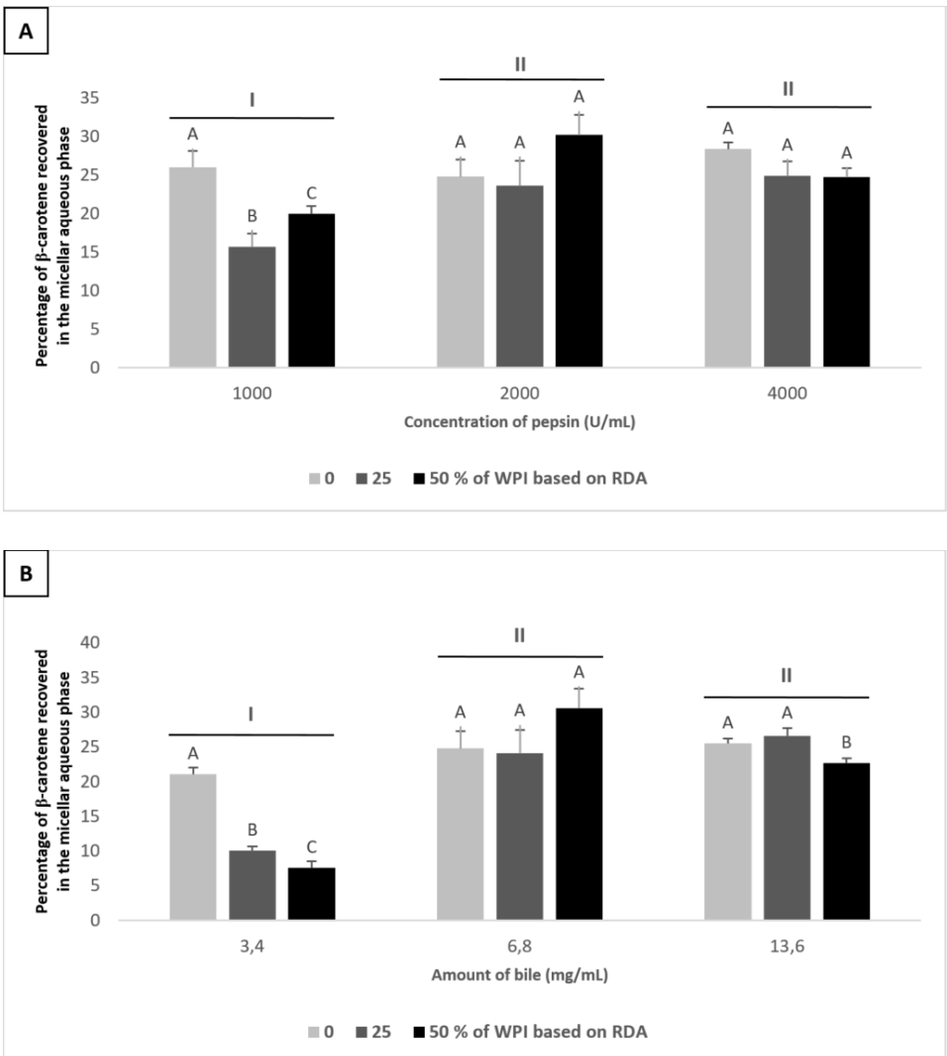


Figure 4.3. Influence of pepsin (A), and bile extract concentration (B) on β-carotene bioaccessibility and the effect of additional co-digested whey protein isolate (WPI) at 75 rpm water bath shaking speed. β-Carotene was digested either in the presence of pepsin at different concentrations (1000, 2000 and 4000 U/mL digesta), or in the presence of different concentrations of bile extract (3.4, 6.8 and 13.6 mg/mL per final 25 mL digesta); as well as in the presence of various amounts of WPI (0, 187 and 375 mg per final 25 mL digesta volume, representing 0, 25 and 50% of protein recommended dietary allowance (RDA)) at 75 rpm. Bioaccessibility is expressed as explained in fig.1 heading. Values represent means ± SD of n = 4, and N≥1 for the experiment with bile extract and N≥2 for the experiment with pepsin (sets repeated at different days). Labeled means without a common superscript (alphabetic letters or roman numbers) differ significantly, $p<0.05$.

4.3.3.2. Effect of varying concentrations of pancreatin

Following linear mixed models, the effects of both WPI amount (p=0.009) and of pancreatic enzyme concentrations (p<0.001) were statistically significant. Under control conditions (no WPI), increasing the pancreatin concentrations positively affected the bioaccessibility (p<0.001) in a concentration dependent manner (Figure 4.2B), i.e. increasing β-carotene micellization from 25% up to 34% (75 rpm), and from 45% up to 68% (100 rpm).

Furthermore, a statistical interaction (pancreatin x peristalsis x protein, p=0.004) regarding β-carotene bioaccessibility was also found. At 75 rpm, the addition of WPI to the simulated GI digestion with half the typical amount of pancreatin negatively affected (p<0.001) β-carotene bioaccessibility, by up to 50%, compared to the control (Figure 4.2B, Table 4.2), in a dose-dependent fashion. When doubling the amount of pancreatin to 400 U/mL, a significant (p<0.001) negative effect of WPI on β-carotene micellization was found only with the addition of 25% RDA, compared to the control.

A similar effect was observed for conditions resembling a stronger peristalsis effects (100 rpm). Using the double amount of pancreatin (400 U/mL) slightly decreased β-carotene micellization following the addition of 50% RDA of WPI (p=0.006, Figure 4.4). However, employing half the amount of pancreatin compared to standard digestive conditions resulted in ambivalent effects, with 25% RDA of WPI resulting in enhanced β-carotene bioaccessibility (p=0.004), while the addition of 50% RDA of WPI slightly decreased β-carotene bioaccessibility (p=0.015), compared to the control.

4.3.4. Effect of WPI on the physicochemical properties of the digesta

In general, the shear stress and viscosity of digested samples at various concentrations of WPI was found very close to that of aqueous systems (Figure S4.1A, left panel), and samples had a very small yield point (0.01 Pa or even lower), with lower yield points after digestion. Non-digested WPI solutions at 0% RDA and all digested solutions showed almost Newtonian behavior above shear rates of 5 s^{-1} (Figure S4.1A, right panel).

Surface tension for control and supplemented digesta with 25% of WPI showed no significant difference (Figure S4.2). The addition of 50% WPI slightly reduced surface tension compared to the control digesta ($p<0.001$), being in line with the higher macroviscosity data.

Regarding mixed micelle size and zeta potential, both 25% and 50% RDA of WPI additions resulted in significantly reduced micelle size of 3.3 ± 0.1 nm compared to WPI free control digesta (Figure S4.3A, $p<0.001$). However, the average absolute zeta potential of the samples digested with 25% and 50% RDA of WPI was somewhat reduced (from −46.8 ± 1 mV to -25.3 ± 6.2 and -28.8 ± 1.7 mV, respectively (Figure S4.3B, $p<0.001$).

Lipid digestion results obtained by fatty acid titration were not fully informative and rather reflected the buffering capacity of the added proteins, and/or additional protein hydrolysis, thus it was not feasible to separate their contribution to lipolysis (Figure S4.4). The size of lipid droplets and the co-location of lipids and proteins at 25% and 50% RDA samples were measured by confocal microscope. Prior

to digestion, the lipid droplets were distributed as large aggregates of lipid droplets in the mixture. With increasing concentration of WPI, the lipid droplets formed smaller aggregates in the digesta (Figure 4.4A). The higher concentration of WPI resulted in a smaller average lipid droplet size compared to the control digesta, decreasing from 13.42 ± 9.28 to 4.84 ± 1.52 and to 3.35 ± 1.76 μm for 0, 25 and 50% WPI, respectively (Table S4.1), with the first group being significantly different from the last 2 groups ($p<0.001$).

Regarding protein digestion, there was no detectable difference between the various digestive conditions on the WPI originating bands detectable by SDS-PAGE, following either gastric or gastro-intestinal digestion (Figure S4.5, upper panel). Gastro-intestinal digestion suggested the complete digestion of WPI into smaller fragments during all digestive conditions, as no major bands were visible following digestion. Only bands originating from enzyme additions were detectable after gastro-intestinal digestion. Following only gastric digestion however (Figure S4.5, lower panel), fragments around 18 kDa were clearly visible after WPI digestion, for all digestive conditions, likely representing either α-lactalbumin (14.2 kDa) and or β-lactoglobulin (18.4 kDa).

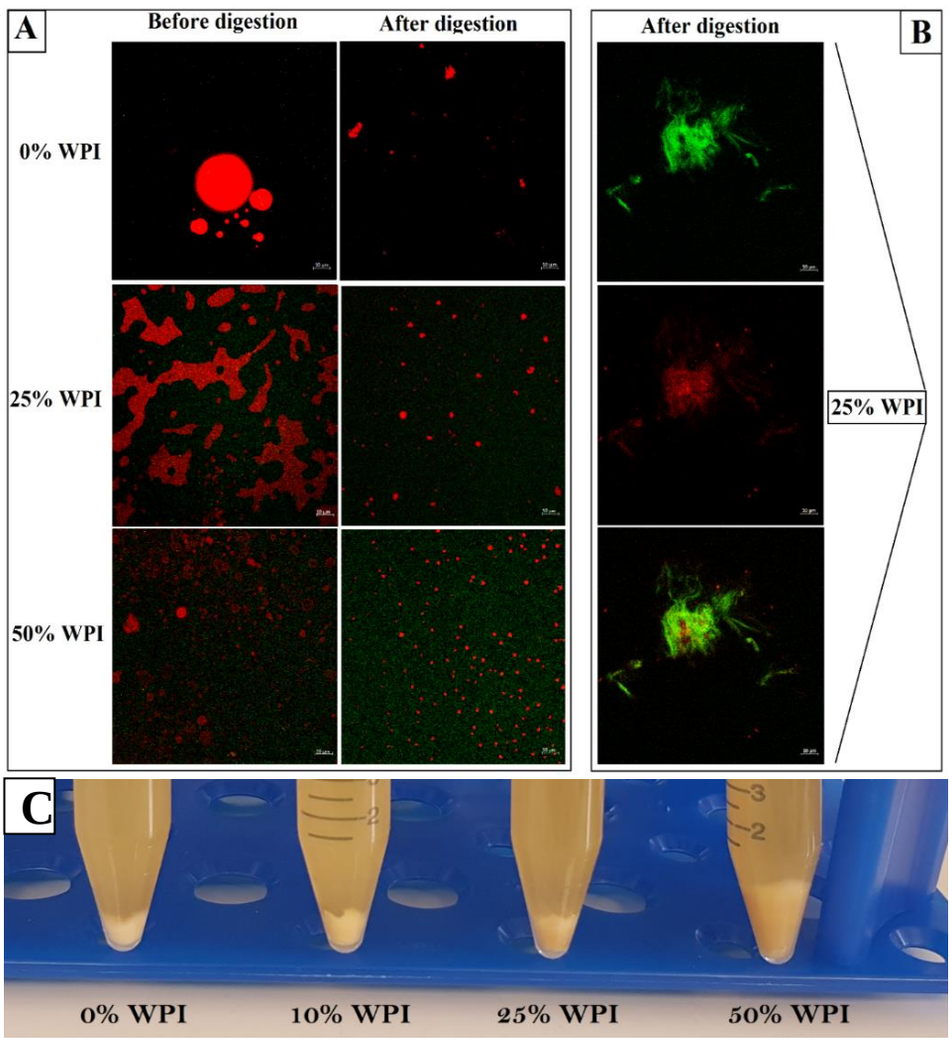


Figure 4.4. Distribution of lipid droplets in the presence and absence of whey protein isolate (WPI), before and after gastro-intestinal digestion (panel A) and colocation of lipids and proteins of samples representing a protein concentration equivalent to 25% of the recommended dietary allowance (RDA) in the digesta (panel B). The white bar indicates 10 μm. Confocal imaging of emulsion structures was carried out at room temperature with a confocal laser scanning microscope (Zeiss LSM 880, Airyscam SR, Jena, Germany), using a 63x objective. The fluorescent dyes were excited by an Argon laser (488 nm) and the emitted light was collected at 522 nm for protein (green) and 635 nm for the fat phase (red). C) Optical images of the samples following gastro-intestinal digestion.

4.4. Discussion

Micellization of β-carotene constitutes one of the major steps determining its absorption and bioavailability, and previous studies have shown that they are well correlated [32, 33]. Several reports have highlighted matrix-related as well as host-related factors influencing carotenoid bioaccessibility and absorptive process, as reviewed earlier [9, 11]. In this regard, proteins have been proclaimed to aid in emulsifying liposoluble dietary constituents [19], but their presence has also shown a negative influence on lipid degradation, which could have an impact on the micellization process [21]. In the present study, we investigated the effect of WPI, a commonly consumed protein, on β-carotene bioaccessibility under selective simulated digestion conditions to study its influence at more physiological vs. somewhat sub-optimal conditions regarding lipid and protein digestion.

The most important finding was that the influence of WPI on β-carotene depended on digestive conditions. More specifically, WPI reduced β-carotene bioaccessibility by approx. 70% when lipid digestion was rather compromised by lower concentrations of bile, enzymes, or when reducing shaking speed. However, under certain conditions, enhanced bioaccessibility, compared to standard digestion conditions (European consensus model, [26]), of up to 20%, were also encountered, but appeared less consistent. Under standard digestion conditions (Table 4.1, Figure 4.1A), adding WPI reflecting 0, 25 and 50 % of the RDA for a 70 kg adult (assuming a digestion volume of 2 L) during simulated GI digestion, had no significant effect on the micellization of β-carotene, in spite of resulting in lower lipid droplet

size after GI digestion. To our knowledge, this is the first report systematically investigating carotenoid bioaccessibility in the presence of a co-digested protein, under varying simulated digestion conditions.

Whey protein consists primarily of β-lactoglobulin (50-55%) and α-lactalbumin (20-25%). Regarding its emulsion stabilizing capability, it was emphasized that the high percentage of β-pleated sheet (~50%) conformation and the relatively low content of the α-helix form (~15%) allows β-lactoglobulin (due to its higher hydrophobicity) to adsorb and produce better conformational changes at the o/w interface, compared to α-lactalbumin (β-sheet ~ 9%, α-helix ~ 45%) [34, 35]. In addition, during the gastric phase, a complete hydrolysis of α-lactalbumin was found, while a portion of β-lactoglobulin remained resistant after 1 h [36]. Thus, in whey protein-stabilized emulsions, β-lactoglobulin may play a greater role regarding emulsion stability, compared to α-lactalbumin.

The strongest positive significant effects of WPI addition were observed when halving the amount of oil (Figure 4.1B) and doubling the peristalsis energy (Figure 4.2A), reflecting more efficient digestive conditions. The effects were an approx. 20% increase, compared to their respective controls (no WPI addition), but only at protein concentrations equivalent to 25% RDA, not 50% RDA. It was reported earlier that β-lactoglobulin can interact with β-carotene within a pocket of hydrophobic residues, with strong non-covalent interactions [37], suggesting that WPI can act as a vehicle for hydrophobic compounds during the GI digestion. This may explain the positive effect observed when reducing the amount of oil. Such carotenoid-

protein interactions are not an isolated case. For instance, orange carotenoid-protein complexes with glutathione-S-transferase and crustacyanin are examples of well characterized carotenoid-binding proteins [38]. In addition, native whey proteins are highly surface-active molecules [20]. It was demonstrated that the proteins and peptides liberated during GI digestion can affect the stability of o/w emulsions [39], as well as retarding triglyceride oxidation [21]. It was also reported that after gastric digestion, some of the protein fragments remained adsorbed to the lipid droplet surfaces. However, these appeared to be removed into the surrounding aqueous phase following further proteolysis by pepsin, the low pH, and peristalsis movements in the stomach, as well as further action of the bile salts in the small intestine [20], allowing access to lipolytic enzymes. This is in line with results from the present study, as no more adsorption of proteins to lipid droplets in fully digested samples was seen by confocal microscopy. These considerations are in line with the present outcomes (Figure 4.2A) and the positive influence of peristalsis movements on the bioaccessibility.

Contrarily, the higher dose of WPI (reflecting 50% RDA) resulted in reducing bioaccessibility to the level of the standard conditions (Figure 4.2B). We can only speculate for the reasons. It is possible that a higher amount of protein, perhaps in conjunction with a less complete digestion, resulted in liberating large peptides at the o/w interface, which may obstruct the displacement of WPI from lipid droplet surfaces and/or hindering digestive enzymes to adsorb to the droplet surfaces as reported previously [21]. In general, the negative effects of WPI were more pronounced (reducing bioaccessibility by up

to 70%) and consistent compared to the positive effects, and were observed especially at conditions reflecting incomplete lipid digestion (low amount of bile and/or pancreatin, or high content of oil).

It is proposed, as supported by the observed smaller lipid droplet size with higher protein addition (Figure 4.4), that emulsion stability is influenced, in addition to the presence of emulsifiers, by the oil droplet size, and by the formation of a viscoelastic film at the oil droplet interface, formed by peptides liberated during the GI digestion of whey proteins [35]. In general, it is accepted that smaller lipid droplet size enhances lipid digestion and thus carotenoid micellization [23], though in this study, we were unable to measure the effect of proteins on lipid digestion due to the over-layering effect of protein buffering capacity and/or hydrolysis.

Also, it was also shown earlier that the generated peptides strongly affected the stability of the o/w emulsions during the gastric phase, dictating the resulting size of lipid droplets [36]. Thereupon, WPI-coated droplets, at least during earlier stages of digestion, may hinder the access of pancreatic lipase (and pancreatic co-lipase) to the droplet surface, especially at low bile salt concentration, due to the large and/or compact peptide film adsorbed to the lipid droplet surfaces [21]. In our study, this effect was corroborated by lower amounts of pepsin (Figure 4.3A), and perhaps a less complete protein digestion, though this could not be differentiated in the present SDS-page (Figure S4.5). However, protein digestion following gastric phase was found to be incomplete, with remaining bands of α-lactalbumin and/or β-lactoglobulin, which may have restricted the subsequent adsorption of lipase required for lipid digestion. This could have

resulted in the observed decrease in β-carotene bioaccessibility (by 40%), which was not observed with regular or elevated amounts of pepsin (2000 or 4000 U/mL, respectively).

Likewise in line, reducing the amount of pancreatin (Figure 4.2B) or doubling the amount of oil (Figure 4.1B) resulted in negative effects on β-carotene bioaccessibility, by up to 50%. Pancreatin contains lipase, allowing for fat hydrolysis, and either doubling the amount of oil, or halving the amount of pancreatin could cause a relative lack of lipolytic enzymes, thereby promoting incomplete droplet processing, flocculation and coalescence [21]. The addition of WPI further impeded the bioaccessibility in a dose-dependent manner, decreasing β-carotene bioaccessibility by half with a higher dose of WPI, compared to the control conditions.

Finally, halving the amount of bile (Figure 4.3B) resulted in one of the most drastic negative effects on β-carotene bioaccessibility, with the addition of WPI reducing bioaccessibility of β-carotene to one third (Figure 4.3B). The results obtained by Nik et *al.* showed that WPI undergoes further displacement from lipid droplet surfaces in the presence of other surface-active compounds, such as bile salts during the intestinal phase, offering finally access to lipolytic enzymes [36]. In their study, this induced a higher degree of lipolysis and led to the formation of a smaller lipid droplets. This suggests that, in addition to proteolytic enzymes and peristalsis movements, the presence of bile at the interfacial composition of the original emulsion plays a major role in determining the extent of lipolysis.

The additional physicochemical properties investigated following GI digestion under standard conditions yielded additional

insights regarding potential effects of WPI on digestive processes and carotenoid bioaccessibility. Firstly, the addition of proteins did not have a marked effect on the macro-viscosity or surface tension of the digesta, precluding that changes in these parameters effected bioaccessibility. This is further corroborated by the small changes in sheer-forces, and in line with a rather complete hydrolysis of added proteins following GI digestion to smaller, non SDS-PAGE detectable fragments, under all digestive conditions (Figure S4.5). In the future, it would be interesting to follow the fate of smaller protein fragments, e.g. by LC-MS-MS, which was not carried out in the present study. However and secondly, protein addition, via their emulsifying properties, reduced lipid droplet size after digestion, and also resulted in lower mixed micelle size, which may have contributed to the positive effect on carotenoid bioaccessibility. It is unclear why the absolute zeta-potential was somewhat decreased with higher protein addition, suggesting a less stable emulsion, though effects could have contributed to the mixed effects encountered.

4.5. Conclusion

The addition of WPI during simulated GI digestion influenced the bioaccessibility of β-carotene positively as well as negatively, depending on the digestion conditions. The results obtained in this study are in line with previous findings regarding the ability of WPI to adsorb to lipid droplet surfaces and playing a major role in stabilizing o/w emulsions, dictating the fate of lipophilic compounds. In this study, the ability of WPI to improve β-carotene bioaccessibility was

somewhat limited (around 20%), and found when applying a higher peristalsis energy (rather more suggestive of a healthy situation), or when reducing dietary lipids. The negative effects resulted following halving the amount of enzymes (pancreatin and pepsin) or doubling the amount of oil, and halving the amount of bile, decreasing the bioaccessibility of β-carotene up to one third after the addition of WPI. These results suggest that WPI and possibly other proteins may modulate carotenoid bioaccessibility, likely via their interaction during the processing of lipid droplets into mixed micelles during digestion as suggested by the altered droplet sizes found. When hypothetically transferring results to humans, a negative influence of WPI on β-carotene may be expected when digestion is less complete as characterized by lower enzyme secretion, while under rather normal and healthy conditions no negative effects may occur. Finally, a potential influence of WPI on other carotenoids (such as xanthophylls), and/or the influence of other proteins on β-carotene remains to be shown, as well as the possible influence of the food-matrix on protein-carotenoid interactions, and importantly, a confirmation of such *in vitro* results by *in vivo* studies.

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4.7. Supplementary Materials

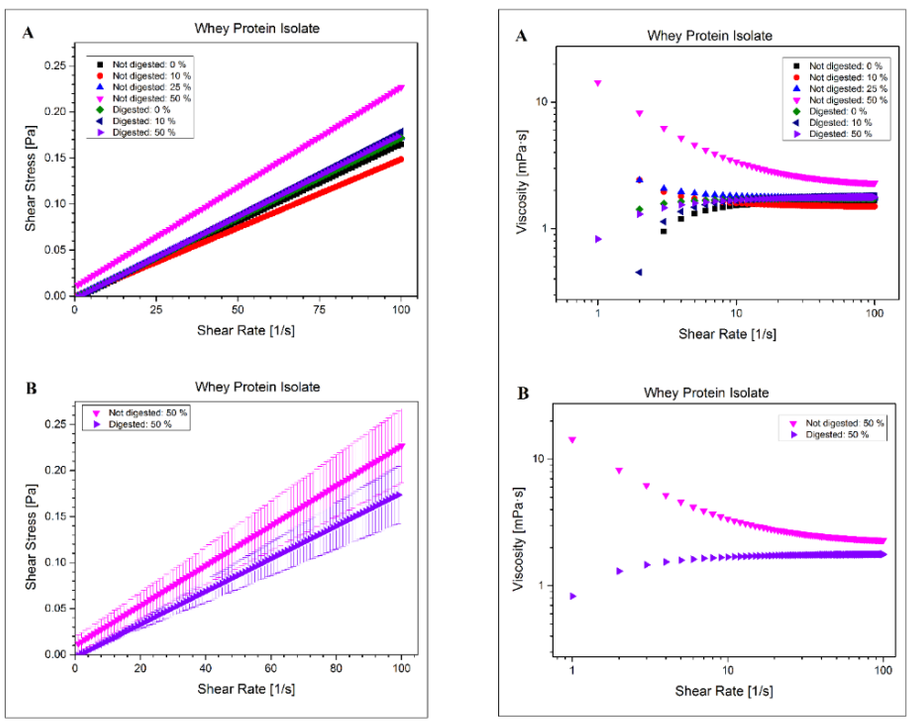

Figure S4.1. Effect of WPI at various concentrations on the shear stress (left panel) and macroviscosity (right panel) of the digesta following gastro-intestinal digestion. Left panel represents the averaged Bingham regressions (n=3) of the shear stress (0 – 0.30 Pa) as a function of the shear (1 – 100 s^{-1}) rate for (A) non-digested and digested whey protein isolate (WPI) at different concentrations representing 0 and 50% of the recommended dietary allowance (RDA), and with error bars for (B) compating non-digested and digested WPI at 50% of RDA. Right panel represents the averaged viscosity curves (n=3) as a function of the shear (1 – 100 s^{-1}) rate for (A) non-digested and digested WPI at different concentrations between 0 and 50% of RDA, and for (B) non-digested and digested WPI at 50% of RDA.

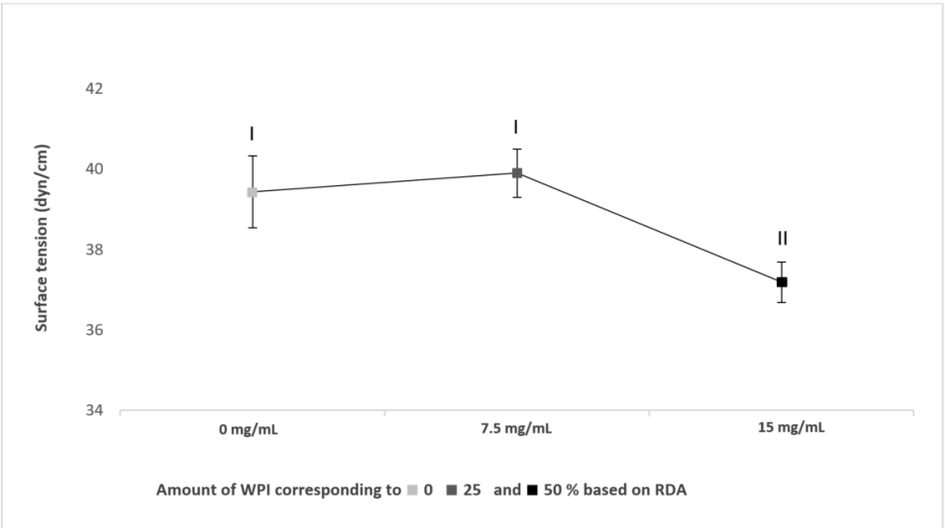

Figure S4.2. Effect of whey protein isolate (WPI) at various concentrations on the surface tension of the digesta (value of pure water: 71.99 dyn/cm), following gastro-intestinal digestion. β-Carotene was digested in the presence of WPI at different amounts (0, 7.5 and 15 mg/mL, representing 0, 25 and 50% of the recommended dietary allowance (RDA). The surface tension of digesta samples, pre-conditioned at 25 ± 0.1 °C, was determined by the weight-drop method. Values represent means ± SD of n = 4 replicates. Means without a common superscript (roman numbers) differ significantly, $p<0.05$.

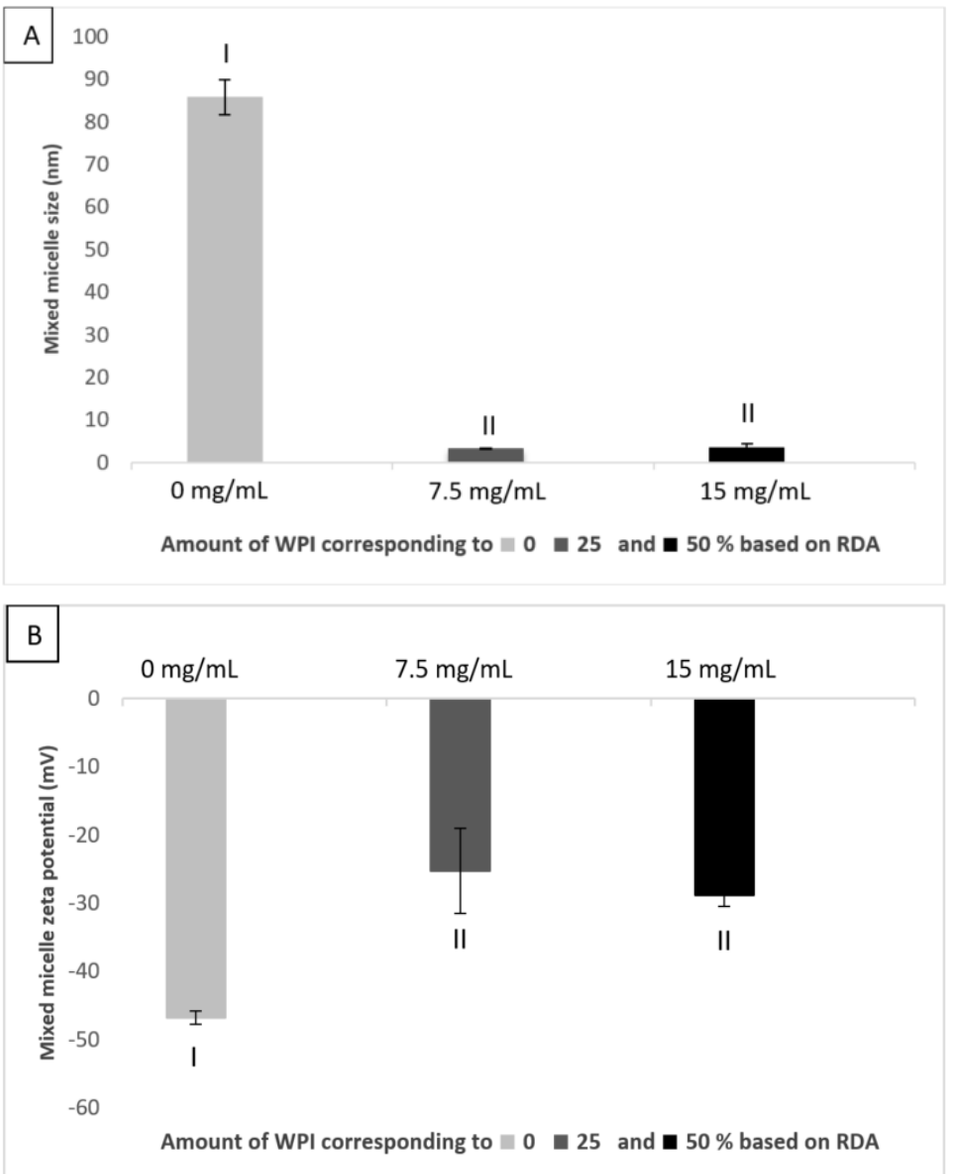

Figure S4.3. Effect of whey protein isolate (WPI) at various concentrations on physicochemical parameters of mixed micelles, produced following gastro-intestinal *in vitro* digestions. (a) Mixed micelle size, and (b) mixed micelle zeta potential. β-Carotene was digested in the presence of WPI at different amounts (0, 7.5 and 15 mg/mL, representing 0, 25 and 50% of the recommended dietary allowance (RDA)). Filtered aliquots of the aqueous micellar fraction were used to determine these two parameters by using photon correlation spectroscopy (Zetasizer Nano Zs, Malvern Instruments), at room temperature. Values represent means ± SD of n = 4. Labelled means without a common superscript (roman numbers) differ significantly, $p<0.05$.

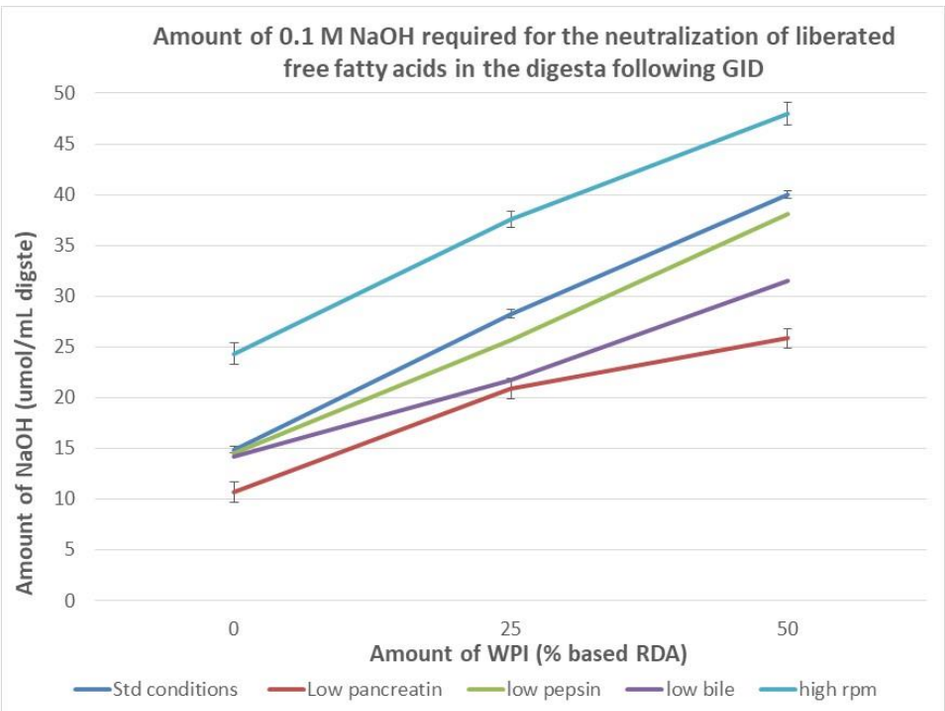

Figure S4.4. Lipid digestion as measured by titrimetric determination with NaOH. Aliquots of gastro-intestinally digested (GID) samples (4.5 mL), at various digestion conditions and with addition of WPI equivalent to 0, 25 or 50% of RDA for proteins were titrated against NaOH (0.1 M), until a color change to pink. Phenolphthalein was used as a pH indicator. Amount given per mL of digesta. N=4 replicates/measure.

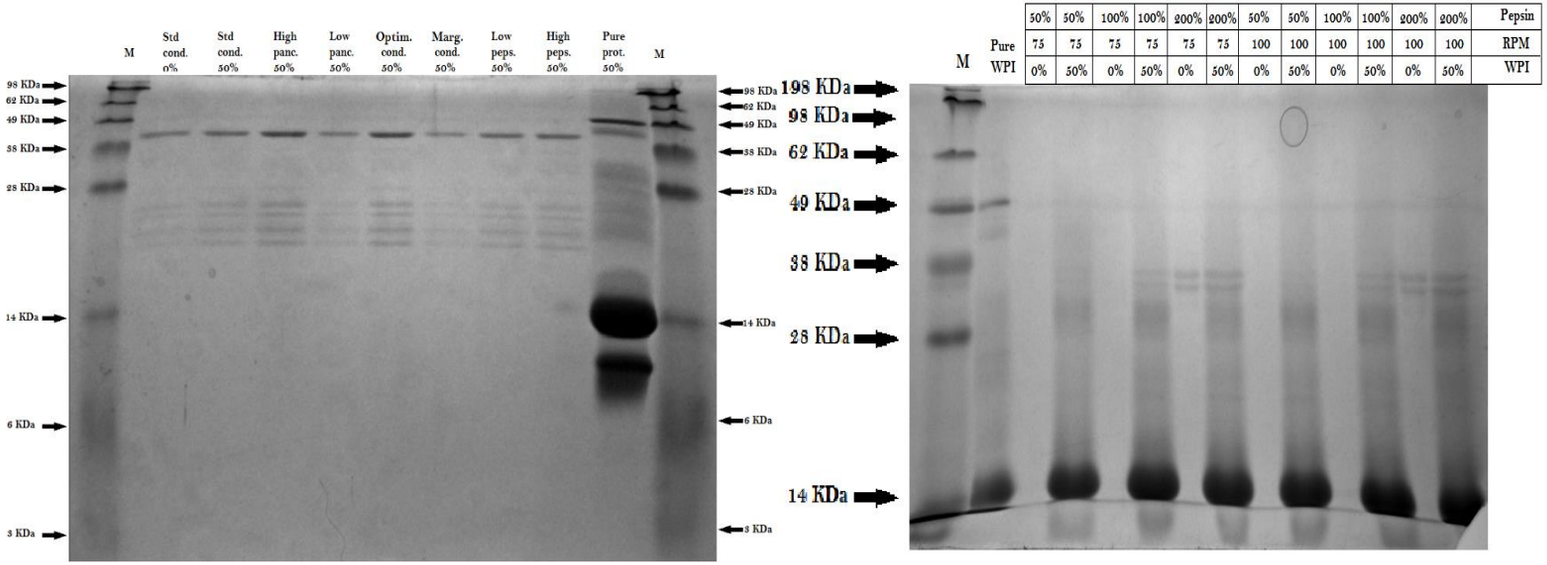

Figure S4.5. SDS-PAGE gel showing the protein bands of pure whey protein isolate (WPI), compared to gastro-intestinally (right panel, 15% acrylamide gel) and gastric digestion only (left panel, 10% acrylamide gel) digested WPI at concentrations equivalent to 0 and 50% of the RDA of proteins. Marker bands are shown on each side. Std. conc. 0%: no WPI isolate added (protein bands originate from enzymes). Std. cond. 50%: WPI isolate added at concentration equivalent to 50% of the RDA; High panc.: high pancreatin concentration during digestion: 400 U/mL; Low panc.: low pancreatin conc. during digestion: 100 U/mL; Low peps: Low conc. of pepsin during digestion, i.e. 1000 U/mL; High peps. conc. during digestion, i.e. 4000 U/mL. Optim. cond.: optimal digestive conditions, i.e. high protein and pancreatin conc., together with high bile (13.6 mg/mL), at 100 rpm of shaking water bath. Marg. cond.: Marginal digestion conditions, i.e. low pancreatin and low protein cond., together with low bile (3.4 mg/mL) and 75 rpm of shaking water bath.

Table S4.1. Lipid droplet size distribution following gastro-intestinal digestion. The lipid droplets size was evaluated at the end of the gastro-intestinal digestion, either in the absence or presence of whey protein isolate (WPI) at a concentration equivalent to 25 and 50% of the RDA[1]. Visualization was carried out by confocal imaging of emulsion structures with a confocal laser scanning microscope and further image-processing and analysis by using the Zen 2 software (Zeiss, Darmstadt, Germany). Values represent means ± SD of n = 20 (per group). Means without a common superscript differ significantly, $p<0.05$.

	WPI concentration based on RDA		
	0%	25%	50%*
Average diameter (µm) of lipid droplets of (n=20)	13.42 ± 9.28 ^{I}	4.84 ± 1.52 ^{II}	3.35 ± 1.76 ^{II}

[1] Recommended dietary allowance.

Chapter 5

Gastric lipase can significantly increase lipolysis and carotenoid bioaccessibility from plant food matrices in the harmonized INFOGEST static *in vitro* digestion model

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Foreword

Following on from Chapter 4, there is another aspect that has been much neglected. This is the use of gastric lipase. To date, most published literature employing the INFOGEST method did not include gastric lipase due to the lack of commercially available, acceptable substitutes for human gastric lipase.

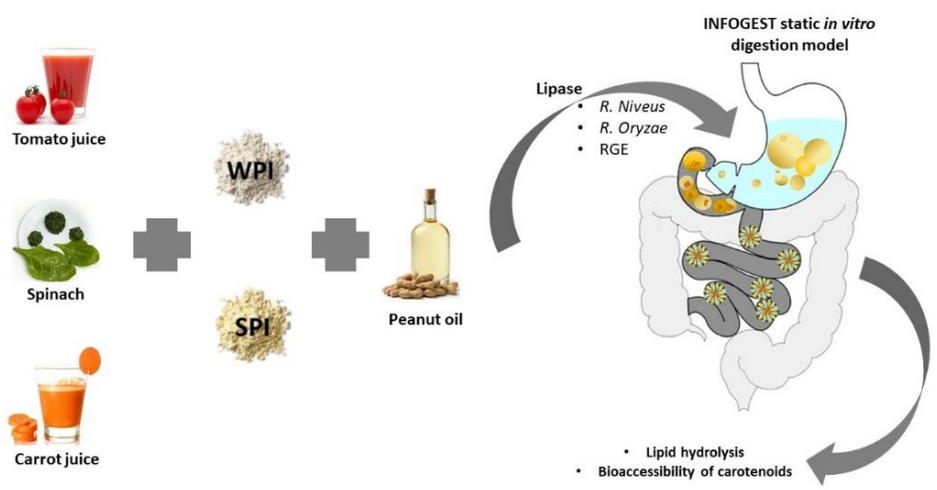
In this chapter, we compare the effect of various lipases from *R. niveus*, *R. oryzae*, and rabbit gastric extracts, at different concentrations, on carotenoid bioaccessibility from several food matrices. Their inclusion to GI digestion is expected to foster emulsification of lipophilic constituents prior to their incorporation into mixed micelles, or alter early micellization processes due to higher availability of released fatty acids.

Abstract

Gastrointestinal digestion of carotenoids has received much attention, as these lipophilic compounds have been related to several health benefits. Most commonly, static digestion models such as the consensus INFOGEST model are employed to study their bioaccessibility from test matrices. However, an aspect that has been much neglected is the use of gastric lipase. Its inclusion to gastro-intestinal (GI) digestion is expected to foster emulsification of lipophilic constituents prior to their incorporation into mixed micelles.In this study, we compared the effect of various lipases from *R. niveus*, *R. oryzae*, and rabbit gastric extracts (RGE), at different concentrations (0, 30, and 60 U mL^{-1}), on carotenoid bioaccessibility from several food matrices (tomato juice, spinach, and carrot juice). We also investigated whether co-digestion of pure proteins (whey and soy protein isolates) at 0, 25, and 50% of the equivalent recommended dietary allowance, would interact with carotenoid bioaccessibility in presence or absence of RGE. Lipolysis was also studied. Considering all matrices combined, lipases significantly improved the bioaccessibility of carotenoids ($p < 0.001$). Compared to other lipases, RGE consistently increased carotenoid bioaccessibility in all tested matrices, by up to 182% ($p < 0.001$), this effect was partly maintained in the presence of co-digested proteins. Unexpectedly, all 3 lipases improved gastric lipolysis in all matrices, by an average of 10-fold ($p < 0.001$). In conclusion, only RGE contributed significantly to improving both lipolysis extent and carotenoid bioaccessibility in all tested matrices, while the presence

of proteins mitigated the positive effect of lipases on carotenoid bioaccessibility.

Keywords: mixed micelles; oil-in-water emulsion; proteins; lipid hydrolysis; pH-optimum; fatty acid release; rabbit gastric extract, *Rhizopus niveus*; *Rhizopus oryzae*.



Graphical abstract 5. Gastric lipase can significantly increase lipolysis and carotenoid bioaccessibility from plant food matrices in the harmonized INFOGEST static *in vitro* digestion model.

5.1. Introduction

Carotenoids are mostly tetraterpenoid pigments, which typically exhibit a color spectrum ranging from yellow to red. They are lipophilic molecules and are naturally synthetized in plants, fungi, bacteria and algae [1, 2]. In general, animals and humans only obtain these natural pigments from their diets. These secondary plant compounds can be classified into two groups; xanthophylls, the least apolar pigments (comprising oxygen within their structure), and carotenes, the most apolar (no oxygen in their molecule). Both classes have antioxidant properties [3], and some carotenoids such as β-carotene, α-carotene, and β-cryptoxanthin, can be cleaved and converted into vitamin A, an essential micronutrient for human growth, development and visual function [4]. Also, the presence of certain carotenoids (.i.e. lutein and zeaxanthin) in eye tissues reduce the risk of developing ocular diseases, such as age-related macular degeneration and cataract [5, 6]. Epidemiologic research has shown an inverse relationship between carotenoid intake and their plasma concentrations and chronic diseases, such as cardiovascular diseases [7], type 2 diabetes [8], as well as certain types of cancer such as of the prostate [9, 10].

Due to the beneficial health effects of carotenoids, their bioavailability (i.e. the fraction that is absorbed and is available for physiological function or storage) has been the subject of numerous studies, as it is generally relatively low and influenced by various factors, namely host related-factors such as single nucleotide polymorphisms (SNPs), as well as food matrix-related factors, especially the presence of lipids [11].

The development of tools to study carotenoid digestion such as *in vitro* digestion models has facilitated the assessment of carotenoid bioaccessibility (i.e. the fraction that is released from the food matrix and is available for absorption), which is an important prerequisite for their bioavailability [12]. *In vitro* simulated digestion has been employed in several studies to investigate the effect of various dietary factors on carotenoid bioaccessibility such as lipids [13], fiber [14], divalent minerals [15] and proteins [16]. The growing interest in nutrient digestion has led to the development of the harmonized INFOGEST *in vitro* model, simulating gastrointestinal (GI) digestion, to standardize several key parameters, such as stomach and intestinal transit times, pH, digestive enzymes and their activity, based on available physiological data and practicability aspects [17]. In addition, an amended and improved digestion protocol (INFOGEST 2.0) has clarified some uncertainties associated with the original method. One of these aspects is the use of gastric lipase, which is typically not integrated in practice, due to a lack of available human gastric lipase (HGL). However, the use of gastric lipase would contribute to the physiological appropriateness of the model [18]. It is acknowledged that lipid digestion starts in the stomach, with the action of HGL on the conversion of dietary triacylglycerides (TAGs) into diglycerides (DAGs) and free fatty acids (FFAs) [19]. The overall TAG hydrolysis has been estimated to be 10% in a solid meal and up to 25% in a liquid ingested matrix [20]. It is assumed that lipid digestion constitutes a key step in the micellization process of lipophilic constituents such as carotenoids, and therefore their bioavailability [21]. In fact, carotenoids follow the fate of dietary lipids during digestion, as they are first released from the food matrix, then solubilized in lipid

droplets before being finally incorporated into mixed micelles for subsequent intestinal uptake. Thus, influencing lipid digestion, especially at a rather early stage during digestion, may alter carotenoid amount and distribution in lipid droplets and thus the ensuing formation of emulsions containing carotenoids. Therefore, gastric lipase may contribute to improved micellization and bioaccessibility.

To date, most published literature employing the INFOGEST method did not include gastric lipase due to the lack of commercially available, acceptable substitutes for HGL. Instead, lipases from alternative sources such as from fungi have been used [22]. However, these HGL replacements do not exhibit the same biochemical properties as HGL regarding for instance pH optimum and fatty acid (FA) cleavage properties (Table 5.1) [22]. A recent alternative that has become available is gastric lipase from rabbits (RGE), a type that has also been recommended by the amended INFOGEST protocol, at a concentration of 60 U/mL of the final gastric digestion mixture [18]. Despite the inclusion of gastric lipase to the *in vitro* digestion model being expected to play an important role toward the emulsification of lipophilic constituents, its importance toward the bioaccessibility of carotenoids has not been tested so far and remains unclear.

A recent study has investigated the effect of additional RGE at a concentration of 60 U/mL of gastric mixture on the hydrolysis extent of β-cryptoxanthin esters from mandarin and peach pulps, compared to the addition of porcine cholesterol esterase (CEL at 1 U/mL) [23]. Compared to RGL, the addition of CEL produced a lower hydrolysis extent in both matrices. The bioaccessibility of overall carotenoids was also assessed. While no significant effect of additional CEL and RGE on

carotenoid bioaccessibility from mandarin was encountered, a slight increase for peach was found. It was speculated that these positive effects are likely due to binding carotenoid esters as substrate and/or triggering pancreatic lipase activity toward these esters.

A study by Corte-Real et *al*. is one of the few that have investigated the effect of adding gastric lipase, in the presence of co-digested lipids, on the bioaccessibility of individual carotenoids [24]. However, the addition of fungal lipase from *R. oryzae* at 340 U/ml did not significantly alter carotenoid bioaccessibility, again probably due to different pH optimum and different cleavage kinetics compared to HGL (Table 5.1).

In order to shed more light on the role of gastric lipase on the bioaccessibility of lipophilic compounds, we compared the effect of various gastric lipases on the bioaccessibility of carotenoids from several food matrices rich in carotenoids (tomato juice, spinach, and carrot juice), which provide a wide range of carotenoids, i.e. from the less apolar xanthophylls to the most apolar carotenes. Several lipases (from *R. niveus, R. oryzae,* and RGE), displaying distinct biochemical properties, were added at different concentrations (0, 30, and 60 U/mL) to the proposed INFOGEST consensus digestion model [17, 18]. Following simulated GI digestion, the overall carotenoid bioaccessibility was compared, as well as the extent of gastric lipolysis. Finally, as protein digestion co-occurs in the stomach and could potentially interact with lipid droplet formation [25, 26], pure proteins (whey protein isolate and soy protein isolate) were co-digested at different concentrations (at 0, 25, and 50% of the equivalent

recommended dietary allowance (RDA) for proteins [16]) with the plant food matrices.

5.2. Experimental

5.2.1. Enzymes, chemicals and carotenoid standards

Digestive enzymes, i.e. pepsin from porcine gastric mucosa (powder, ≥250 U/mg, measured as trichloroacetic acid-soluble products using hemoglobin as substrate, Art. No. P7000), lipase from *R. niveus* (powder, ≥1.5 U/mg, measured as FA release from triglycerides, Art. No. 62310), lipase from *R. oryzae* (powder, ≥30 U/mg, measured as FA release from triglycerides, Art. No. 80612), pancreatin from porcine pancreas (activity equivalent to 4x USP specifications, Art. No. P1750), as well as porcine bile extract (Art. No. B8631) were ordered from Sigma-Aldrich (Overijse, Belgium), except for rabbit gastric lipase (powder, ≥70 U/mg, measured as FA release using tributyrin as substrate, Art. No. RGE70) was purchased from Lipolytech® (Marseille, France, Table 5.1). Chemicals including calcium chloride dihydrate, ammonium carbonate, sodium chloride, magnesium chloride hexahydrate, sodium bicarbonate, potassium phosphate, potassium chloride, and sodium hydroxide solution (1 M) were acquired from Sigma-Aldrich, and hydrochloric acid solution (1 M) was obtained from VWR (Leuven, Belgium). The solvents methanol, acetone, and hexane were obtained from VWR, while diethyl ether was from Sigma-Aldrich; these solvents were supplemented with 0,1% of butylated hydroxytoluene (BHT, Sigma-Aldrich). Unless otherwise specified, all products were of analytical grade or higher. 18 MΩ water

was used throughout the study and was procured from B. Braun Medical SA (Sempach, Switzerland).

Table 5.1. Comparison of main biochemical properties of gastric and microbial lipases, which were employed in the *in vitro* digestion experiments. Adapted from [22, 27].

Lipase source	Stereo-specificity[a,b]	Optimum temperature (°C)[b]	Optimum pH [b]	Ref. [c]
Human gastric lipase	S_N3	37	4-5.4	[22, 27]
Rhizopus oryzae	S_N1	37-40	6.5-7.5	[22, 24, 27]
Rhizopus niveus	S_N1	30-45	5-7	[28-30]
Rabbit extract lipase	S_N3	37	4-6	[22, 27]

[a]According to a stereochemical numbering system, S_N1, S_N2 and S_N3 define the hydroxyl groups occupying the three esterification positions on the glycerol backbone. S_N1 would indicate a cleavage of the fatty acid located in the first esterification position on the glycerol backbone / [b]Relative to triacylglyceride hydrolysis / [c]Including experiments of this lipase with lipophilic constituents and descriptions of biochemical properties.

5.2.2. Food matrices

Peanut oil (typically free of native carotenoids [31] and according to own blank examinations [32]), the plant food matrices, i.e. 100% tomato juice (other ingredients: 0.5% salt), frozen spinach (Iglo, other ingredients: 0.04% salt), and organic 100% carrot juice (other ingredients: 1% concentrate from lemon juice) were purchased from a local supermarket (Delhaize, Strassen, Luxembourg). All tested matrices were aliquoted into portions of approx. 35 g in 50 mL Falcon tubes, flushed with argon and stored at -80°C until further analysis. Regarding spinach, grinding was necessary, using a kitchen blender (Moulinette chopper, Moulinex) before storage.

Whey protein isolate (WPI) was acquired from Pure Nutrition USA (95% purity, Oxnard, CA), while soy protein isolate (SPI) was obtained from Self Omninutrition® (≥90% purity, Stockholm, Sweden). The amounts of proteins tested were 0 or 15 g/L of the final digestion volume (26 mL), corresponding to approx. 0 or 50% of the recommended dietary allowance, respectively (RDA, ≈ 60 g/d for human adults), assuming that this would be consumed within 2 meals for a total volume of 2 L of gastrointestinal fluids per day [16].

5.2.3. Simulation of gastro-intestinal digestion of carotenoids

In vitro simulated GI digestion was carried out according to the harmonized INFOGEST protocol [17, 18]. The model was chosen due to the physiological relevance of the protocol, and it was strived to further verify its physiological relevance by testing the impact of

added gastric lipases on the bioaccessibility of carotenoids from different plant food matrices. Various commercially available lipases (from *R. niveus*, *R. oryzae*, and RGE) were added to the proposed digestion model at various concentrations (0, 30, and 60 U/mL gastric digesta), and their influence on the bioaccessibility of total carotenoids from tomato juice, spinach and carrot juice was investigated. The oral phase was omitted as the food matrices were either liquid (tomato and carrot juices) or turned into fine pieces by milling with a kitchen blender (spinach), and were also rather low in complex carbohydrate content.

5.2.3.1. Simulated digestion fluids and enzyme solutions

The simulated fluids, as well as the enzyme solutions were prepared as recommended earlier [17], with some modification regarding pancreatin concentration (200 U/mL based on trypsin activity), similar as carried out previously [32]. Gastric lipase solutions were prepared at a concentration of 0, 30, and 60 U/mL of the final gastric mixture. Since RGE contains gastric lipase and pepsin (70 U lipase/mg and 300 U pepsin/mg, respectively), the pepsin concentration in the gastric phase was accordingly adjusted to the recommended value.

5.2.3.2. Gastric phase

Each sample consisted of 4 g of plant food matrix (tomato juice, spinach, or carrot juice) plus 300 µL of peanut oil as a lipid source,

corresponding to approx. 12 g/L of the final digestion volume. In addition to the recommended enzymes in the INFOGEST protocol [17], various gastric lipases were added to the digestion to achieve the desired concentrations (i.e. 0, 30, or 60 U/mL) for a final gastric volume of 13 mL. When testing the effect of co-digested proteins, pure protein solutions were added at concentrations equivalent to 0 or 50% of the RDA for total proteins. The remainder of the gastric digestion procedure was carried out as previously described [16].

5.2.3.3. Intestinal phase

The intestinal digestion procedure was performed following the recommended ratio of chyme to simulated intestinal fluids of 50:50 (v/v). The final volume of each digested sample was 26 mL. At the end of the GI digestion, aliquots were prepared for carotenoid extraction following filtration of the middle aqueous micellar fraction obtained after centrifugation, as reported in our previous work [16]. Aliquots of complete digesta were kept for further analysis. All aliquots were flushed with argon and stored at -80 °C.

5.2.4. Extraction and quantification of carotenoids

5.2.4.1. Extraction of carotenoids from food matrices

Carotenoid extraction from plant food matrices was carried out as descried previously [33], with slight modifications. The extraction was similar for all tested matrices, except for spinach, for which the first step involved a saponification process before starting the extraction. Briefly, aliquots of 4 g of each matrix were extracted with

hexane:acetone (2/1, v/v), followed by a brief centrifugation and then collection of the supernatant hexane. The extraction process was repeated multiple times with either hexane or diethyl ether, and final resulting combined supernatants were evaporated to dryness. The dried extracts were flushed with argon and stored at -80°C until analysis.

5.2.4.2. Extraction of carotenoids from the bioaccessible fraction

The filtered aqueous phase was thawed and used for extraction of carotenoids as described previously [16]. In brief, 6 mL of hexane:acetone (2/1, v/v) were added to each sample, a short centrifugation was performed and the supernatant hexane phase was collected. The extraction process was repeated twice with pure hexane. All extracts were combined in the same tube, dried under a stream of nitrogen, and stored under argon at -80 °C until analysis.

5.2.4.3. Quantification of carotenoids

The percentage of carotenoid micellization was used as a measure of bioaccessibility. The bioaccessibility was expressed as the percentage of carotenoid concentration measured in the extracted digesta following GI *in vitro* digestion, compared to the initial concentration measured in the original matrix. Spectrophotometric analysis was carried out to measure the concentration of total carotenoids in the bioaccessible fraction and in the original matrix. Total carotenoid concentration was calculated by using a method based on the mean absorption coefficients and mean absorption

wavelength [34], taking into account carotenoid patterns for each matrix, with molar absorption coefficients in hexane and respective absorption wavelengths as detailed previously [35].

5.2.5. Free fatty acid determination

Complete digesta of samples stored at -80°C after gastric digestion were used to evaluate lipid hydrolysis by measuring the concentration of FFAs in the final gastric volume (13 mL). This was determined by Cayman's Free Fatty Acid Fluorometric Assay (Cayman Chemical, Art. No. 700310, Ann Arbor, MI), according to the manufacturer's protocol.

5.2.6. Statistical analyses and data treatment

Unless otherwise specified, all data is presented as mean ±SD. Linear mixed models were created to study the effects of added various gastric lipases to the digestion model on the bioaccessibility of carotenoids from plant food matrices, and independently to study the effect of additional proteins in presence and absence of gastric lipase on carotenoid bioaccessibility. For this purpose, non-normally distributed data or data with unequal variances was log-transformed.

Linear mixed models thus contained the amount of gastric lipase (0, 30, or 60 U/mL), type of lipase (from *R. niveus, R.s oryzae,* and RGE), type of matrix (tomato juice, carrot juice, spinach), as well as FFA release as fixed independent factors, while bioaccessibility was the observed dependent factor. Similarly, in a second model, protein type (WPI and SPI), amount of protein (0, 25 or 50 % RDA), food matrix

(tomato juice, and carrot juice) and gastric lipase level (0 and 60 U/mL) were included as fixed factors, while bioaccessibility was the observed parameter. P-vales below 0.05 (2-sided) were considered significant. Following Fisher-F-tests, all group wise comparisons were carried out by Fisher-protected LSD tests. Following significant interactions, models were re-run keeping one of the factors in the interaction constant in order to redo all group-wise comparisons. All analyses were carried out by SPSS (vs. 25, IBM statistics, Chicago, IL).

5.3. Results

5.3.1. Influence of additional peanut oil on the bioaccessibility of overall carotenoids from plant food matrices

Under control conditions (no addition of oil), the bioaccessibility of total carotenoids following *in vitro* GI digestion was significantly higher in spinach (43.8 ± 0.9%) compared to that of carrot juice (7.8 ± 0.4%, $p > 0.001$), while the lowest carotenoid bioaccessibility was obtained from tomato juice (2.8 ± 0.1%, $p < 0.001$) (Figure 5.1).

Addition of peanut oil (12 g/L) to the simulated digestion considerably improved the bioaccessibility of total carotenoids in all tested matrices ($p < 0.001$), compared to their controls (without addition of oil). The overall bioaccessibility of carotenoids increased to 74.1 ± 2.8% in spinach, followed by an increase to 10.7 ± 0.2% in carrot juice, and finally to 7.3 ± 0.3% in tomato juice, relative to respective controls ($p < 0.001$, no oil added).

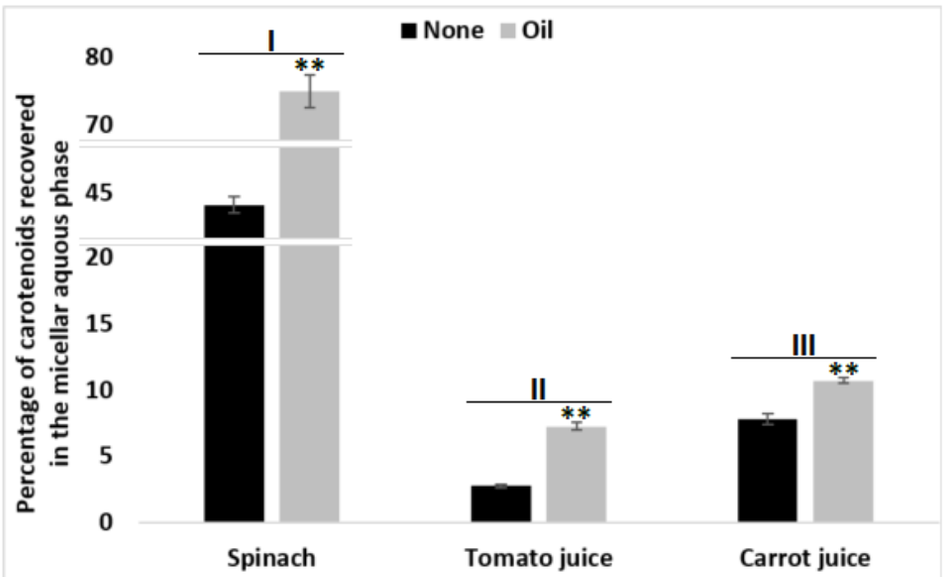


Figure 5.1. Influence of additional peanut oil on the bioaccessibility of carotenoids from tomato juice, spinach, and carrot juice. The matrices were digested either in the absence or in the presence of peanut oil (300 µL per final 26 mL digesta volume). Bioaccessibility is expressed as the percentage of total carotenoids recovered from the aqueous micellar fraction at the end of the *in vitro* gastro-intestinal digestion, compared to the initial amount present in each matrix at the beginning of digestion. Bars represent means ± SD of $n \geq 4$ and $N \geq 2$ (sets repeated at different days). Bars labelled with ** or without a common superscript (roman numbers) differ significantly, $P < 0.001$.

5.3.2. Influence of gastric lipases on the bioaccessibility of total carotenoids

Comparing gastric lipases, RGE resulted in greater overall carotenoid bioaccessibility than *R. niveus* lipase ($p < 0.001$), while the addition of *R. oryzae* lipase resulted in lower bioaccessibility of carotenoids compared to other gastric lipases ($p < 0.001$, when considering all matrices and gastric lipase concentrations applied, i.e. evaluated together statistically (Figure 5.2).

5.3.2.1. Tomato juice

Considering all lipase types, the addition of 30 U/mL caused a slight but significant decrease of the overall carotenoid bioaccessibility following simulated GI digestion ($p < 0.001$, Figure 5.2A), while 60 U/mL enhanced significantly the bioaccessibility ($p < 0.001$, all gastric lipase types considered) compared to the control (no gastric lipase added, 7.3 ± 0.3%). This pattern was found following the addition of *R. niveus* lipase and RGE at a concentration of 30 U/mL, with a decrease to 6.2% ($p < 0.05$). The addition of 60 U/mL of both *R. niveus* lipase and RGE increased significantly the overall bioaccessibility to 10.4% and 12.8%, respectively ($p < 0.001$, Figure 5.2A).

Contrary to the two other gastric lipases, *R. oryzae* lipase did not influence the bioaccessibility of carotenoids at 30 U/mL, but decreased significantly total carotenoid bioaccessibility at a concentration of 60 U/mL to 5.0% ($p < 0.001$).

5.3.2.2. Spinach

Considering all gastric lipase types, the addition of gastric lipases at a concentration of 30 and 60 U/mL to the *in vitro* digestion negatively influenced total carotenoid bioaccessibility ($p < 0.001$, Figure 5.2B), compared to the control (no gastric lipase added, 74.1 ± 2.8%). This decrease in carotenoid bioaccessibility was found following the addition of 30 U/mL of *R. niveus* and *R. oryzae* lipases, resulting in 41.3% ($p < 0.001$) and 40.3% ($p < 0.001$), respectively, while a concentration of 60 U/mL resulted in a less pronounced but significant drop in bioaccessibility to 56.6% ($p < 0.05$) and 43.3% ($p <$

0.001), respectively (Figure 5.2B). However, the addition of RGE significantly improved the bioaccessibility of total carotenoids in spinach in a concentration-dependent manner, with an increase up to 86.0% at a concentration of 30 U/mL, and up to 92.1% at a concentration of 60 U/mL ($p < 0.05$).

5.3.2.3. Carrot juice

Evaluating together statistically the influence of gastric lipases on the bioaccessibility of carotenoids from carrot juice during simulated digestion, the concentrations of 30 and 60 U/mL significantly improved total carotenoid bioaccessibility ($p < 0.05$ and $p < 0.001$, respectively), compared to the control (no gastric lipase added, 10.7 ± 0.2%). The addition of RGE and *R. niveus* lipase enhanced overall carotenoid bioaccessibility in a concentration-dependent manner, with an increase of up to 14.1% ($p < 0.001$) and 11.6% ($p < 0.05$), respectively, at a concentration of 30 U/mL, and up to 19.5 and 12.9% ($p < 0.001$), respectively, with a concentration of 60 U/mL (Figure 5.2C). Regarding the addition of *R. oryzae* lipase to the simulated GI digestion, a concentration of 30 U/mL resulted in a significant decrease of total carotenoid bioaccessibility to 8.3% ($p < 0.001$), while the addition of 60 U/mL had no significant influence on their bioaccessibility.

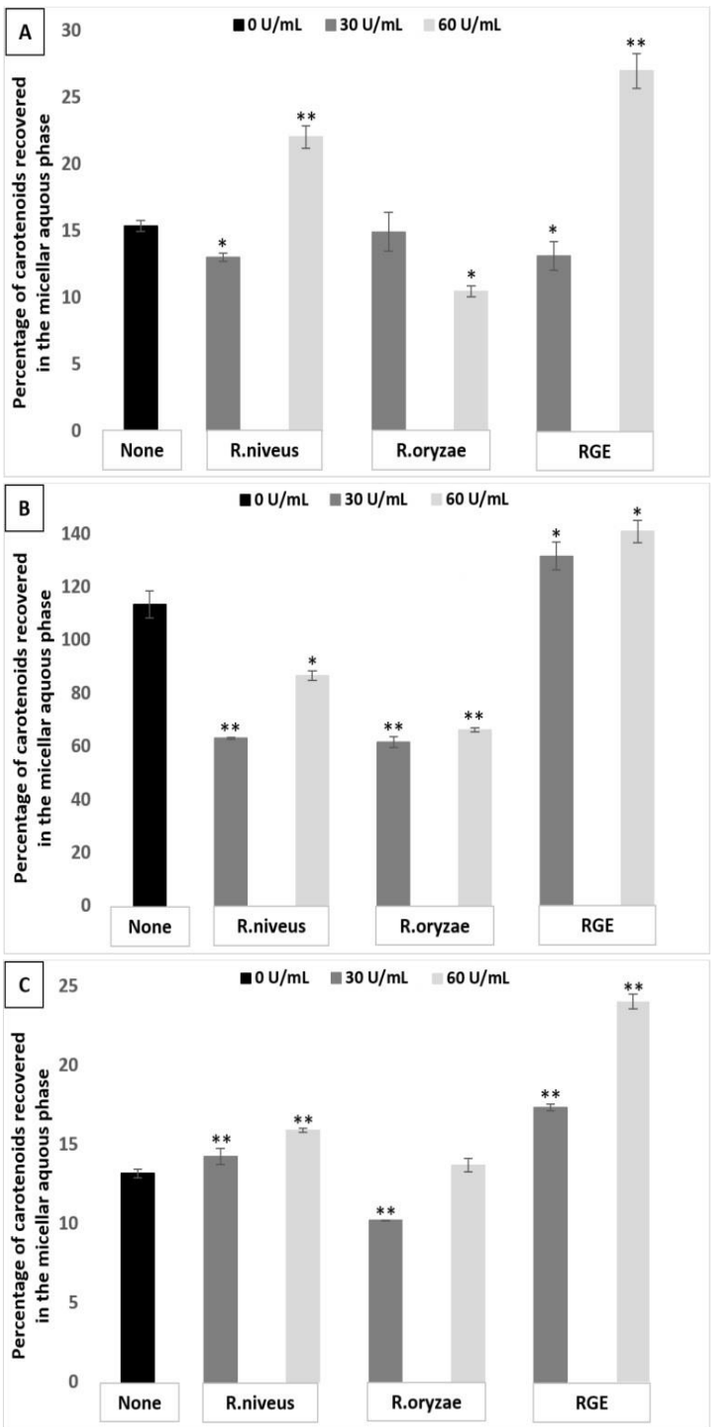


Figure 5.2. Influence of gastric lipases on the bioaccessibility of total carotenoids from tomato juice (A), spinach (B), and carrot juice (C). Carotenoid-rich matrices were digested in the absence or presence of various gastric lipases (from *R. oryzae*, *R. niveus*, and RGE) at 0, 30, and 60 U/mL of the final gastric volume. Bioaccessibility is expressed as explained in Figure 5.1 heading. Values represent means ± SD of $n \geq 3$ and $N \geq 2$ (sets repeated at different days). Bars labelled with either *($P < 0.05$) or **($P < 0.001$) were significantly different from the control condition (no added lipase).

5.3.3. Influence of gastric lipases on the release of free fatty acids

Considering all employed matrices, the addition of various gastric lipases to the simulated digestion increased the concentration of FFAs in a concentration-dependent manner ($p < 0.001$), without a significant difference between the three gastric lipases tested (Figure 5.3). Higher FFA production was found in spinach compared to tomato and carrot juices ($p < 0.001$), without a significant difference between the two juice matrices tested. Interestingly, with all tested gastric lipases, the addition at a concentration of 60 U/mL to the simulated digestion of spinach was not significantly different from the addition of 30 U/mL, regarding the release of FFAs.

However, considering all the gastric lipase concentrations and evaluating individually each plant food matrices, *R.niveus* lipase had a stronger impact on FFA release during the gastric digestion of tomato juice, compared to the two other gastric lipase types ($p < 0.001$, Figure 5.3A). In spinach, the highest release of FFAs was obtained in the presence of RGE, followed by *R. oryzae* lipase, while *R. niveus* lipase showed the least pronounced influence ($p < 0.001$, Figure 5.3B). Following the gastric digestion of carrot juice, *R. niveus* lipase had a greater influence on the release of FFAs, followed by *R. oryzae* lipase, and finally RGE with the least pronounced influence compared to the other two gastric lipases ($p < 0.001$, Figure 5.3C).

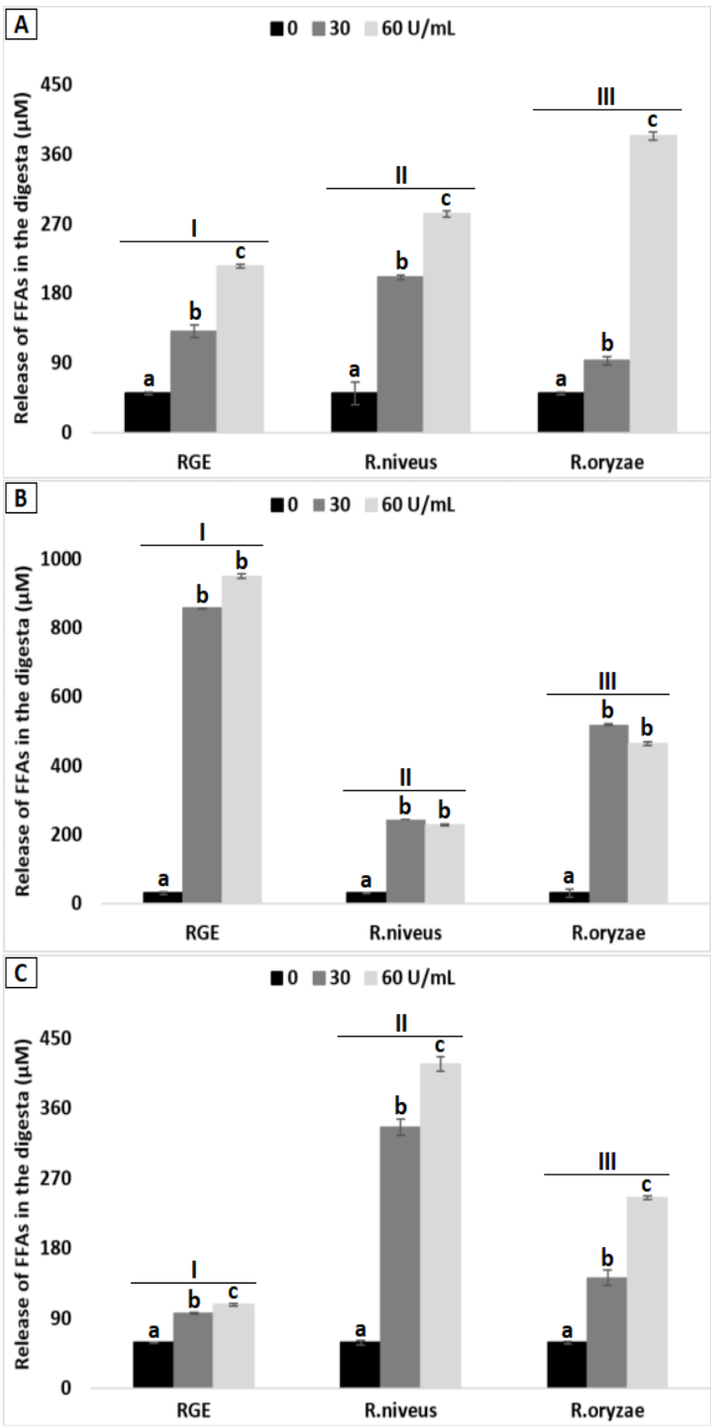


Figure 5.3. Influence of gastric lipases on the release of free fatty acids (FFAs), following the simulated gastric digestion of tomato juice (A), spinach (B), and carrot juice (C). Carotenoid-rich matrices were digested in the absence or presence of various gastric lipases (from *R. oryzae*, *R. niveus*, and RGE) at 0, 30, and 60 U/mL of the final gastric volume. At the end of the gastric digestion, FFA concentration (µM) was determined in an aliquot of the gastric phase, using Cayman's Free Fatty Acid Fluorometric Assay. Values represent means ± SD of *n*=3. Bars labelled without a common superscript (alphabetic letters or roman numbers) differ significantly, $P < 0.001$.

5.3.4. Effect of co-digested proteins in presence or absence of RGE on the bioaccessibility of total carotenoids

In the presence of proteins, the effect of RGE addition seemed to result in a less strong increase in carotenoid bioaccessibility compared to no protein addition (Figure 5.4). Nevertheless, the addition of 60 U/mL of RGE to the simulated digestion increased the bioaccessibility of total carotenoids, compared to the controls without RGE ($p < 0.001$, considering all matrices, protein types and concentrations applied).

The addition of RGE to the *in vitro* digestions containing 25% RDA of WPI did not influence the bioaccessibility of total carotenoids from tomato and carrot juices, compared to their controls (overall carotenoid bioaccessibility of 7.2 and 9.3%, respectively). However, the addition of RGE in the presence of 50% RDA of WPI increased the bioaccessibility of overall carotenoids to 10.6% in tomato juice ($p < 0.05$, Figure 5.4A) and to 18.9% in carrot juice ($p < 0.05$, Figure 5.4B), compared to the controls (overall carotenoid bioaccessibility of 6.6 and 15.5% , respectively).

The addition of RGE to the simulated digestions in the presence of 25% RDA of SPI significantly improved the bioaccessibility of carotenoids from tomato juice (9.6%, $p < 0.05$) and carrot juice (17.8%, $p < 0.05$), as compared to their controls (7.5% and 13.4%, respectively). In the presence of 50% RDA of SPI, the additional RGE did not influence the bioaccessibility of total carotenoids from tomato juice as well as from carrot juice, compared to the controls (7.4% and 12.4%, respectively).

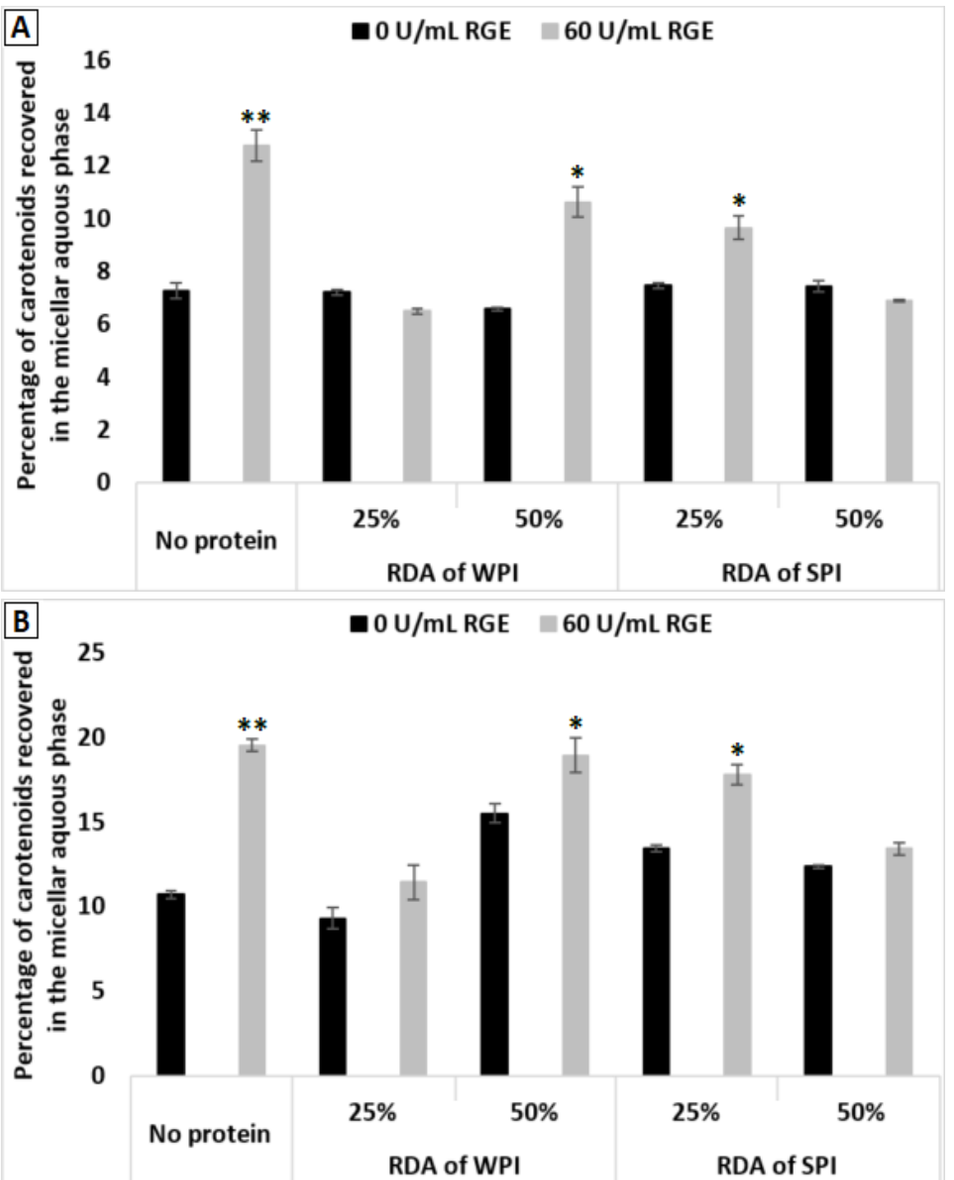


Figure 5.4. Effect of co-digested proteins at different concentrations in presence or absence of RGE on the bioaccessibility of total carotenoids from tomato juice (A) and carrot juice (B). Tomato and carrot juices were co-digested with various amounts of WPI and SPI (0, 25 and 50% of recommended dietary allowance (RDA) per final 25 mL digesta volume), in the absence or presence of added RGE at a concentration of 60 U/mL. Bioaccessibility is expressed as explained in Figure 5.1 heading. Values represent means ± SD of $n \geq 3$ and $N \geq 2$ (sets repeated at different days). Bars labelled with either *($P < 0.05$) or **($P < 0.001$) were significantly different from the corresponding control condition (no RGE).

5.4. Discussion

In the present study, we compared the effect of various gastric lipases on the bioaccessibility of carotenoids from several carotenoid-rich food matrices, using the INFOGEST consensus of static *in vitro* digestion model [17, 18], with the hypothesis that this would improve intestinal bioaccessibility and lipid release in the stomach. Indeed, it was found that all 3 lipases significantly improved carotenoid bioaccessibility, while only RGE consistently enhanced the bioaccessibility of overall carotenoids in all tested matrices, by up to 182% (Figure 5.2). The presence of gastric lipases increased FFA release, as a marker of lipid digestion, by up to 10-fold for 60 U/ml of lipase in all tested matrices (Figure 5.3). Another important finding is that the addition of proteins, co-digested in the stomach, appeared to hamper the positive effect that RGE had on carotenoid bioaccessibility (Figure 5.4).

As the presence of dietary lipids is believed to be an important factor in the micellization process of lipophilic compounds such as carotenoids, a small amount of oil was added to the proposed *in vitro* digestion. As anticipated, the addition of peanut oil considerably improved the bioaccessibility of total carotenoids in all tested matrices, especially in tomato juice, resulting in an increase of up to 264%, followed by spinach and carrot juice with an approx. up to 170 and 140% increase, compared to no oil addition (Figure 5.1). Earlier studies using the *in vitro* digestion method have reported similar findings in studying the effect of added dietary lipids on the bioaccessibility of individual carotenoids, such as following the addition of olive oil on the bioaccessibility of lycopene from tomato

cubes and puree [36], or its effect on lutein bioaccessibility from spinach [13]. However, the differences in bioaccessibility improvement between the matrices in the present study are likely explained by the carotenoid species contained in each matrix. Carotenes represent approx. 99% of total carotenoids in tomato juice (of which are over 96% of lycopene), and over 98% in carrot juice (including approx. 70% of β-carotene). In spinach however, xanthophylls represent approx. 70% of total carotenoids, among which 40% of zeaxanthin and lutein (data not shown). Earlier studies have reported that the digestion of spinach supplemented with dietary lipids enhanced the bioaccessibility of hydrophobic carotenes such as β-carotene, but not those of xanthophylls such as lutein [13], which was mainly attributed to the low solubility of xanthophylls in oil compared to carotenes [37], though high original bioaccessibility of xanthophylls is also likely playing a role.

All three lipases improved measured lipolysis. Lipolysis starts in the stomach with the action of preduodenal lipases on TAGs, with an estimated contribution between 10 and 30% of the overall lipid digestion [38]. The released FFA and the mono- or diacylglcerides are important components of the mixed micelles, formed in the intestine [39]. However, due to differences in their biochemical properties (Table 5.1), the investigated lipases differentially influenced lipid hydrolysis, as demonstrated by the FFA release at the end of simulated gastric digestion (Figure 5.3). Among the lipases that were used in our investigation, only RGE was expected to be (gastric-) acid-resistant, and according to the proposed INFOGEST protocol [18], RGE is active down to a pH of 3, compared to the investigated fungal lipases [22].

However, unexpectedly, no difference in FFA release was observed between the 3 lipases when considering all matrices combined. In contrast with *in vitro* data reported earlier (conducted at pH 3) [22, 40], we assume that the two tested fungal lipases were active within the acidic environment of the simulated gastric digestion. A recent study tested both *R. oryzae* lipase and RGE as substitutes for HGL, to catalyze the *in vitro* digestion of two infant formulas (a medium-chain triacylglyceride enriched formula (MC-IF) and a long-chain triacylglyceride formula (LC-IF)). The addition of *R. oryzae* lipase showed an unexpectedly higher lipolysis extent of gastric digestion of both formulas, independent of FA chain length [41]. However, RGE preferred the release of MC-FAs in both formulas, similar when compared to HGL. Due to the lack of specificity of fungal lipases with regard to FA chain length, the authors stated that they were therefore not an appropriate replacement for HGL in gastric human *in vitro* lipolysis models [41].

With this at least comparable if not higher FFA release by lipases from *R. niveus* and *R. oryzae* when considering individual matrices, it was expected that both lipases would significantly contribute to fostering the formation of emulsions, and therefore to improved micellization of carotenoids that were released from the tested food matrices. However, only RGE consistently improved the overall carotenoid bioaccessibility in all tested matrices, compared to *R. niveus* and *R. oryzae* lipases.

One of the crucial elements potentially contributing to the difference between RGE and the two other lipases during intestinal digestion is that the fungal lipases are inhibited by bile salts [22, 42,

43]. The resistance to bile extracts has been reported to be among the biochemical properties that makes HGL rather unique among lipases [22]. In fact, bile salts are strong surfactants that compete with lipases for adsorption at the lipid-water interface, and can prevent the enzyme from reaching its substrate. Due to its amphiphilic structure [44] and high tensioactivity [22], HGL is able to adsorb at the lipid-water interface in the presence of bile salts. On the other hand, RGE has received great interest in *in vitr*o digestion, particularly showing promising results when compared to HGL. For instance, Sassene et *al.* showed that RGE had similar surface properties compared to HGL, while the lack of specificity of *R. oryzae* lipase with regard to FA chain length does not seem to replicate the digestive properties of HGL *in vivo* [41]. The same group reported that *R. oryzae* lipase completely disrupted the spherical shape of the oil droplets, leading to droplet aggregation, while the formation of oil droplets by HGL was reproduced by RGE during *in vitro* digestion. The authors concluded that the fungal lipases are less tensioactive enzymes than HGL and RGE [22, 42, 43]. In addition, Capolino et *al*. assessed the lipolytic potential of RGE combined with porcine pancreatic extracts, and the rates of lipolysis appeared to be a good surrogate for HGL and pancreatic lipases under the same *in vitro* conditions [45]. The tensioactivity of RGE is an important biochemical priority to mimic the biological function of HGL, which was reported to be stable and active in the duodenum [19, 46], and can initiate the lipolysis of TAG emulsions covered by phospholipids that are resistant to pancreatic lipase [47]. Similarly, RGE could play an essential role in the lipolysis during the simulated intestinal passage due to its high tensioactivity [48, 49] that allows resistance to bile salts and penetration into phospholipid

layers, covering TAG droplets during digestion [22], triggering the activity of pancreatic lipase [47, 50]. Thus, we assume that the higher overall carotenoid bioaccessibility obtained in the presence of RGE is likely through fostering the formation of mixed micelles in the intestine, thereby facilitating the bioaccessibility of carotenoids.

In addition, when comparing the effect of the two fungal lipases on the bioaccessibility of overall carotenoids, the presence of *R. oryzae* lipase had a negative effect on the bioaccessibility, compared to the control. However, the addition of *R. niveus* lipase to the simulated GI digestion resulted in a positive influence on total carotenoid bioaccessibility in tomato and carrot juices. We assume that the pH optimum range could explain the observed differences between the two fungal lipases, as the pH optimum of *R. niveus* lipase (pH optimum 5-7) is closer to that of the gastric phase compared to that of *R. oryzae* lipase (pH optimum 6.5-7.5), even though lipolysis was only slightly and not significantly higher for *R. niveus*.

Since gastric lipolysis and proteolysis processes are tightly entwined during the gastric digestion, two types of protein were added to the simulated GI digestion, in presence or absence of RGE. It was found that the addition of the gastric lipase had a diminished positive effect when proteins were added, and only resulted in a positive effect on carotenoid bioaccessibility in the presence of 50% RDA of WPI and at 25% RDA of SPI (Figure 5.4). We have earlier reported earlier that WPI and SPI have contributed differently, via their emulsifying properties, to the bioaccessibility of carotenoids, depending on their type and concentration [16]. In the same study, the low solubility of SPI [51, 52] resulted in the formation of aggregates in the aqueous

phase, which reduced proteolysis and increased the surface tension. Weak repulsive forces and less stable micelles were also observed [16]. In the current study, we hypothesize that RGE in the presence of partially digested SPI (i.e. at low concentration), counterbalanced this negative influence on carotenoid bioaccessibility by enhancing lipid digestion, similar as reported earlier [16]. On the other hand, the adsorbed bile salts and resulted short peptides at the interface enhanced micelle stability [53], emulsified lipid droplets and prevented their aggregation [54]. However, at high SPI concentration (50% RDA), the reduced protein digestibility could cause the accumulation of long peptide chains on lipid droplet surfaces, which may hamper the access to lipolytic enzymes [55] and affect the transfer of carotenoids into mixed micelles, explaining the neutral effect of RGE on overall bioaccessibility of carotenoids, compared to the control (no RGE added).

Regarding the simulated digestion in presence of WPI, its major protein fraction, i.e. β-lactoglobulin, has been reported to have high surface hydrophobicity [54] and is resistant to pepsinolysis during the gastric digestion [32]. We have highlighted previously that large peptides adsorbed at droplet surfaces could form a biological barrier to digestive enzymes [16], rendering WPI-stabilized emulsions more resistant to lipolysis [56], interfering with mixed micelle formation. In the current study, the presence of RGE at low concentration of WPI (i.e. 25% RDA) did not influence the bioaccessibility of carotenoids, compared to the control (no RGE added). Here, we assume that the presence of RGE did therefore not interfere significantly with the subsequent lipolysis and mixed micelle formation, since the β-

lactoglobulin-stabilized emulsions remained resistant to hydrolysis during the gastric digestion [54] were completely digested by the pancreatic enzymes at the early stage of intestinal digestion [16]. At high WPI concentration (i.e. 50% RDA), the presence of RGE remarkably increased overall carotenoid bioaccessibility, compared to the control. We hypothesize that RGE, in synergy with pancreatic lipase, may foster the lipolysis of the previously inaccessible lipid fractions due to the remaining long peptide chains adsorbed at the interface, leading to an improved transfer and incorporation of carotenoids into mixed micelles, and therefore increasing the bioaccessibility of overall carotenoids.

5.5. Conclusion

The most important finding was that lipases added to the proposed INFOGEST static *in vitro* digestion model had a differential effect on the bioaccessibility of carotenoids from plant food matrices. A significantly improved gastric lipolysis was found by all three lipases, contrary to what was reported earlier [22, 40]. Most importantly, only RGE improved the overall carotenoid bioaccessibility compared to the fungal lipases, in line with earlier findings regarding the inhibition of *R. niveus* and *R. oryzae* lipases by the bile salts [22, 42, 43], and the high tensioactivity of RGE that allows a better resistance to bile salts and penetration into phospholipid layers covering TAG droplets and triggering therefore the activity of pancreatic lipase during intestinal passage [47-50]. Finally, the presence of rabbit preduodenal lipase compensated the negative effect that WPI and SPI showed on carotenoid bioaccessibility, resulting in a

positive influence on total carotenoid bioaccessibility in tomato and carrot juices.

5.6. References

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Chapter 6

Influence of soy and whey protein, gelatin and sodium caseinate on carotenoid bioaccessibility

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Foreword

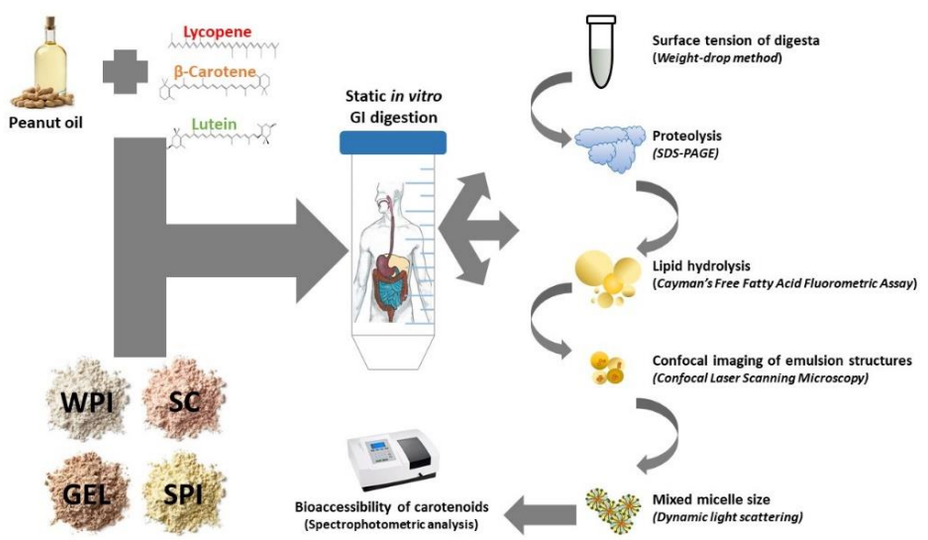
In Chapter 6, by means of the European consensus model (INFOGEST, with the adaptations outlined in Chapter 4) for static *in vitro* digestion, we aime to investigate the effect of several frequently consumed proteins of various hydrophobicity and dietary sources on the bioaccessibility of commonly ingested carotenoids. More precisely, we focuse on the influence of WPI, SPI, SC and GEL, on the bioaccessibility of individual carotenoids, i.e. β-carotene, lutein and lycopene. In order to provide additional insights about the interaction between proteins and carotenoids during digestion, we also investigate lipid and protein digestion efficiency, as well as physico-chemical properties of the digesta, including surface tension, viscosity, micelle size and ζ-potential.

Abstract

Proteins could alter carotenoid bioaccessibility through altering their fate during digestion, due to emulsifying properties of resulting peptides, or influencing access of digestion enzymes to lipid droplets. In this investigation, we studied whether whey protein isolate (WPI), soy protein isolate (SPI), sodium caseinate (SC) and gelatin (GEL), added at various concentrations (expressed as percentage of recommended dietary allowance (RDA): 0, 10, 25 and 50%) would influence the bioaccessibility of lycopene, β-carotene or lutein, added as pure carotenoids solubilized in oil, during simulated gastro-intestinal (GI) digestion. Protein and lipid digestion as well as selected physicochemical parameters including surface tension, ζ-potential and micelle size were evaluated. Adding proteins influenced positively the bioaccessibility of β-carotene, by up to 189% ($p<0.001$), but it resulted in generally decreased bioaccessibility of lutein, by up to 50% ($p<0.001$), while for lycopene, the presence of proteins did not influence its bioaccessibility, except for a slight increase with WPI, by up to 135% ($p<0.001$). However, the effect depended significantly on the type of protein ($p<0.001$) and its concentration ($p<0.001$). While β-carotene bioaccessibility was greatly enhanced in the presence of SC, compared to WPI and GEL, the presence of SPI strongly decreased carotenoid bioaccessibility. Neglecting individual carotenoids, higher protein concentration correlated positively with carotenoid bioaccessibility ($R=0.57$, $p<0.01$), smaller micelle size ($R=-0.83$, $p<0.01$), decreased repulsive forces (ζ-potential, $R=-0.72$, $p<0.01$), and higher surface tension ($R=0.44$, $p<0.01$). In conclusion, proteins differentially affected carotenoid bioaccessibility during digestion

depending on carotenoid and protein species, with both positive and negative interactions occurring.

Key-words: Interfacial properties, physicochemical characteristics; mixed micelles; oil-in-water emulsion; in vitro digestion; emulsifiers; protein intake.



Graphical abstract 6. Influence of soy and whey protein, gelatin and sodium caseinate on carotenoid bioaccessibility

6.1. Introduction

Carotenoids are naturally occurring tetraterpenoid pigments synthesized by plants, algae, and several bacteria and fungi. Despite the fact that humans cannot produce them, and that these secondary plant compounds are not essential for humans, they have always been part of the food chain, as carotenoid-rich food sources include a large variety of fruits and vegetables, as well as certain animal-derived food items such as salmon and eggs [1]. Furthermore, several carotenoids are included in fortified foods or are available as supplements.

Despite their non-essentiality, these phytochemicals contribute to important biochemical and physiological functions in the human body [2]. Some carotenoids are provitamin A compounds, such as β-carotene, α-carotene and β-cryptoxanthin, and in many countries with low animal derived food intake and for vegetarians, carotenoids are the main source of dietary vitamin A. Furthermore, many of these pigments have been associated in epidemiological studies with reduced incidence of chronic diseases, such as lowered risk of coronary heart disease [3], certain types of cancer [4], type 2 diabetes [5], and eye-health related diseases [6, 7]. Though the exact mechanisms are often unclear, this is attributed to, at least in part, their influence on the cellular level, acting on transcription factors and nuclear factors [8], which may also contribute to their anti-inflammatory and antioxidant activities [9-11].

Given these potential health benefits for humans, the bioavailability of carotenoids, i.e. the fraction that is absorbed and is available for physiological function or storage, has been high on the

agenda of carotenoid-oriented research. In fact, following their dietary intake, carotenoids are liberated from food matrices, solubilized in lipid droplets[12] and are finally incorporated into mixed micelles. The micellization of carotenoids is crucial for their uptake by enterocytes, which is a prerequisite for their intestinal absorption and further biodistribution [13].

However, in part due to their poor water-solubility, the bioavailability of carotenoids is low and variable [14, 15], and many dietary as well as host-related factors are known to interfere with their absorption [13, 16]. Regarding dietary factors, it has been acknowledged that lipids can enhance carotenoid absorption efficiency, by improving their micellization and fostering chylomicron sequestration [17, 18]. In contrast, dietary fibers negatively affect their bioavailability, likely by hampering the transition of lipid droplets into mixed micelles, also affecting the activity of digestion enzymes [19, 20].

One factor that has not yet received much attention is the influence of co-digested proteins on carotenoid solubility and/or absorption. Indeed, several proteins have exhibited emulsifying properties during digestion, being highly surface-active, stabilizing emulsions by forming a viscoelastic phase, and adsorb to the oil droplets, forming a physical barrier [12] and preventing coalescence [21]. Thus, depending on digestive conditions, proteins, via their interaction with lipid droplets during the digestion process, may influence carotenoid bioaccessibility by modulating their transfer from lipid droplets into mixed micelles [12]. Such interactions may be

positive due to the stabilization of emulsions or negative, due to hampered enzymatic access to lipid droplets [22].

For instance, it has been reported that sodium caseinates (SC) stabilized oil-in-water (o/w) emulsions and facilitated β-carotene uptake [23, 24]. In another study employing milk-protein stabilized fat droplets, these droplets remained small throughout the digestion process, suggesting that they were stable against coalescence, facilitating the formation of smaller lipid droplets [25], likely enhancing carotenoid bioaccessibility as demonstrated earlier [26]. In our previous study, the addition of whey protein isolate (WPI) during simulated GI digestion influenced the bioaccessibility of β-carotene positively as well as negatively, depending on the digestion conditions [22]. While positive effects appeared to dominate under more complete GI conditions, additional WPI reduced β-carotene bioaccessibility at lower enzymatic activity of pancreatin, lower concentration of bile and reduced peristalsis. However, most studies have focused only on the very apolar β-carotene and especially on WPI.

Thus, in the current study, we aimed to investigate the effect of several frequently consumed proteins of various hydrophobicity on the bioaccessibility of commonly ingested carotenoids. By means of a European consensus model for static *in vitro* digestion [27, 28], we focused on the influence of WPI, soy protein isolate, sodium caseinate and gelatin on the bioaccessibility of individual carotenoids, i.e. β-carotene, lutein and lycopene (Table 6.1). In order to provide additional insights about the interaction of proteins and carotenoids during digestion, we also investigated lipid and protein digestion efficiency as well as physicochemical properties of the digesta,

including surface tension, viscosity, and micelle size. The insights obtained can be valuable for fortifying/enriching food items with these health associated pigments and to scrutinize the best vehicles to support their bioavailability.

Table 6.1. Overview of the applied conditions and parameters investigated following the simulated gastro-intestinal (GI) digestion.

Conditions of the simulated *in vitro* GI digestion				**Aspects investigated following the *in vitro* GI digestion**				
					Characterization of the digesta			
Digestion steps	Protein type digested	Protein concentration (% RDA*)	Carotenoid type studied	Micellar phase Carotenoids	Macromolecules		Physicochemical characteristics	
					Proteins	Lipids	Solution	Particles
Gastric & intestinal phases	Whey protein isolate Soy protein isolate Sodium caseinate Gelatin	0 10 25 50	Lycopene β-Carotene Lutein	Carotenoid bioaccessibility	Protein degradation	Free fatty acid release	Macro-viscosity Surface tension	Z-Potential Micelle size Confocal microscopy visualization

*RDA: Recommended dietary allowance.

6.2. Experimental

6.2.1. Chemicals and carotenoid standards

Standards of *all-trans*-β-carotene (≥97% purity) and *all-trans*-lycopene (≥85%) were purchased from Sigma-Aldrich (Overijse, Belgium), while lutein (≥95%) was obtained from Extrasynthese (Genay, France). Pepsin from porcine gastric mucosa (≥250 U/mg, Art. No. P7000), pancreatin from porcine pancreas (activity equivalent to 4x USP specifications, Art. No. P1750) and porcine bile extract (Art. No. B8631) were obtained from Sigma-Aldrich (Overijse, Belgium).

Unless otherwise specified, all chemicals were of analytical grade or superior. Potassium chloride (≥99%), potassium dihydrogen phosphate (≥99%), sodium hydrogen carbonate (≥99%), sodium chloride (≥99.5%), magnesium chloride hexahydrate, ammonium carbonate, sodium hydroxide solution (1 M), calcium chloride dihydrate (≥99%), Nile red, fluorescein isothiocyanate isomer I (FITC), and phenolphthalein were acquired from Sigma-Aldrich. Hexane (≥95%), acetone (≥99%), and hydrochloric acid (1 M) were obtained from VWR (Leuven, Belgium).

6.2.2. Pure proteins and dietary lipid sources

Whey protein isolate (WPI) was acquired from Pure Nutrition USA® (95% purity, California, USA), and soy protein isolate (SPI) was obtained from Self Omninutrition® (≥90% purity, Stockholm, Sweden), whereas casein sodium salt (SC) from bovine milk (Art. No. C8654) and

gelatin (GEL) from bovine skin (Art. No. G9391) were both purchased from Sigma-Aldrich.

Peanut oil, typically free of native carotenoids [29] and own blank examinations, was purchased from a local supermarket (Delhaize, Strassen, Luxembourg).

6.2.3. Solubilization of carotenoid standards & preparation of protein and enzyme solutions

All solutions were prepared as previously described [22]. Briefly, carotenoid standard solutions were produced at a final concentration of 0.2 mg/mL. For this, β-carotene, lycopene and lutein were first dissolved in hexane. Peanut oil was added to foster the dissolution of pure carotenoids. Then, the solutions were sonicated at 50/60 Hz for 5 min. (Ultrasonic Cleaner, VWR Symphony®, Massachusetts, USA) and warmed up at 30 °C until complete dissolution. The hexane was finally removed by evaporation (TurboVap LV from Biotage®, Uppsala, Sweden) and the carotenoid standard solutions were made by the further addition of peanut oil to reach the targeted concentration of 0.2 mg/mL.

Regarding the protein solutions, the amounts of proteins tested were 0, 3, 7.5, 15 g/L, corresponding to 0, 10, 25 and 50% of the recommended dietary allowance (RDA) within 1 test meal, being 0.83 g per kg of body weight per day for adults (≈ 60g/d) [30], by dissolving/emulsifying each protein type in pure water.

The simulated gastric fluid (SGF), the simulated intestinal fluid (SIF), as well as the enzyme solutions were prepared as recommended

earlier [27]. Pepsin solution was prepared in SGF at a concentration of 2000 U/mL of the final gastric mixture, pancreatin and bile extract were prepared in the same SIF solution, at a concentration of 200 U/mL and 6.8 mg/mL, respectively. Based on our previous study [22], pancreatin amount was doubled (200 U/mL based on trypsin activity) in order to enhance protein digestion.

6.2.4. Simulation of gastro-intestinal digestion and extraction of carotenoids

In vitro simulated GI digestion was carried out according to the harmonized INFOGEST protocol, with slight modifications for pancreatin concentrations [22]. The model was used to test the influence of proteins at four different concentrations on the bioaccessibility of pure carotenoids. The oral phase was omitted as the matrix employed did not include significant amounts of carbohydrates and was liquid.

6.2.4.1. Gastric phase

Each sample comprised a total of 6.5 mL of gastric phase solution containing the desired amounts of proteins, i.e. 0, 78, 195 or 390 mg (equivalent to 0, 10, 25 or 50% of the RDA), also including 150 μL of oil containing carotenoid standards (approx. 30 μg of carotenoid standard per digestion), similar to former *in vitro* experiments [31]. The oil containing carotenoids were mixed with the gastric phase by brief sonication for 5 min at 50 Hz to obtain oil in

water emulsions. Prior to the addition of SGF (1.25 x concentrate), 2000 U/mL of pepsin was added to the sample. Then, 0.075 mM of calcium dichloride was added in the final mixture, and pH was adjusted to 3 by hydrochloric acid (1 M), bringing the volume of each sample to 13 mL with pure water in order to reach a final ratio of matrix (protein + carotenoid solutions) to simulated gastric fluid of 50:50 (v/v). Samples were then incubated in a shaking water bath (GFL 1083 from VEL®, Leuven, Belgium) for 2 h at 37 °C, with a shaking speed of 100 rounds per minute (rpm).

6.2.4.2. Intestinal phase

At the end of the gastric incubation, SIF, pancreatin (200 U/mL) and bile extract (6.8 mg/mL) were added to the chyme. Then, calcium dichloride was added at a concentration of 0.3 mM in the final mixture. The ratio of gastric chyme to SIF of 50:50 (v/v) was obtained by filling up the sample to 26 mL with pure water, the pH was adjusted to 7 by the addition of sodium hydroxide solution (1 M), then the samples were incubated for 2 h at 37 °C, maintaining a shaking speed of 100 rpm.

6.2.5. Analyses of final products of digestion

6.2.5.1. Extraction and analysis of carotenoids of the bioaccessible fraction

Carotenoid extraction and analyses were carried out as described previously [22]. Briefly, at the end of the intestinal incubation, the intestinal digestion was stopped by immediately

transferring the digests on ice. 12 mL of the digesta were centrifuged for 1 h at 3200×g (4 °C), and then 5 mL were collected from the middle aqueous phase and filtered through 0.2 μm nylon membrane syringe filters (Corning Incorporated® Life Sciences, Tewksbury, Massachusetts, USA). The extraction started by adding 6 mL of hexane:acetone (2:1, v:v) to 2 mL of the filtered aqueous phase. After brief centrifugation, the supernatant hexane phase was collected, and the extraction process was repeated two times with pure hexane. All extracts were combined in the same tube, dried under a stream of nitrogen, and stored under argon at -80 ^{o}C until the spectrophotometric analyses.

The absorbance spectrum was measured between 300 and 600 nm (GENESYS™ 10S UV-Vis Spectrophotometer, Thermo Fisher Scientific, Massachusetts, USA). Carotenoid concentration was calculated by applying the Beer-Lambert law. The percentage of carotenoid micellization was used as a measure of bioaccessibility, and was expressed as the percentage of amount of carotenoids present in the micellar phase of the filtered digesta after *in vitro* GI digestion, compared to the initial amount added to the sample.

6.2.5.2. SDS-PAGE electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) was carried out as previously described [22] to study the extent of protein digestion. Samples were denatured at 95^{o}C for 5 min. and were then loaded to a 15% acrylamide and 0.4% bisacrylamide gel for the samples obtained after complete GI digestion, or to a 10%

acrylamide/0.3% bisacrylamide gel for the digesta obtained following only gastric digestion. The running time was 30 min. at 80 V followed by 90 min. at 100 V. Protein marker was Invitrogen novex see blue plus 2 (Invitrogen, Carlsbad, CA). After each run, fixation/staining was done with methanol/acetic acid/Coomassie brilliant blue R (50%, 10%, 0.1%) for 30 minutes, and destained in methanol/glacial acetic acid/water (30%, 10%, 60%, 3 h). The gels were scanned by a Kodak Gel Logic 2200 imaging system (Kodak, Rochester, NY).

6.2.5.3. Free fatty acid determination

Lipid hydrolysis was evaluated by measuring the amount of free fatty acids (FFAs) released in the digesta after GI digestion. This was determined by Cayman's Free Fatty Acid Fluorometric Assay (Cayman Chemical, Art. No. 700310, Ann Arbor, MI) according to the manufacturer's protocol.

6.2.6. Macroviscosity and surface tension analysis

Flow curves were determined as previously described [22], using an Anton-Paar rheometer (MCR 302, WESP, Graz, Austria), equipped with a double gap cell at 5°C. A regression flow curve was calculated for the shear rate range between 0 s^{-1} and 100 s^{-1} for the shear stress respectively between 1 s^{-1} and 100 s^{-1} for the viscosity. The regression curves were plotted as viscosity and shear stress as a function of the shear rate.

Surface tension of digesta samples, pre-conditioned at 25 ± 0.1 °C, were determined by the weight-drop method as previously

described [32]. The air-water interfacial properties of the digesta were calculated as follows:

$$\sigma_{digesta} = \frac{m_{digesta}}{m_{H2O}} \text{ x } \sigma H_2O$$

where σ_{H2O}= 71.99 dyn cm^{-1} is the surface tension of pure water

6.2.7. Confocal laser scanning microscopy

Confocal imaging of emulsion structures after GI digestion was carried out at room temperature with a confocal laser scanning microscope (Zeiss LSM 880, Airyscam SR, Jena, Germany), using a 63x objective. The aliquots were dyed with Nile red dissolved in ethanol, and FITC dissolved in acetone. Both were used for fat and protein staining, respectively, at a concentration of 1 μg/mL. An Argon 488 nm laser excited the fluorescent dyes. The emitted light was collected at 500-540 nm for proteins and 590-650 nm for the fat phase. The resulting images were processed and de-noised by using a modular image-processing and analysis software for digital microscopy (Zen 2.3 blue edition, Carl Zeiss Microscopy GmbH, Jena, Germany).

6.2.8. Micelle size and ζ-potential analysis

Aliquots of the filtered aqueous micellar fraction were used for the analysis of the micelle size and ζ-potential, and the measurements were done at room temperature with at least four replicates. The intensity-weighted mean hydrodynamic radius and ζ-potential were

determined by dynamic light scattering and laser Doppler micro-electrophoresis, respectively, by using a Zetasizer Nano Zs instrument (Malvern Instruments, Malvern, UK).

6.2.9. Statistical analyses and data treatment

In order to minimize day-to-day variations between experiments, bioaccessibility of pure carotenoids was normalized to a daily control which was assessed for each digestion. Unless otherwise stated, all values are expressed as the mean ± standard deviation. Replicates were obtained from at least 2 individual sets of analyses obtained at different days ($N \geq 2$), and at least 4 replicates ($n > 4$) were obtained for each digestive condition during 1 set of analyses.

Statistical analysis was performed using SPSS 22 software (SPSS Inc., Chicago, IL). Normal distribution of data was checked by Q-Q -plots and equality of variance by box-plots and Levene's test. For statistical evaluation, carotenoid bioaccessibility values were log-transformed to achieve a normal distribution. Linear mixed models were developed with the effect of type and concentration of proteins (quantitative variable) and carotenoid species as fixed independent factors and log-carotenoid bioaccessibility as the observed dependent factor. When significant interactions were obtained, additional linear mixed models were run to keep certain of these parameters constant and to better allow for all further group-wise comparisons. P-values < 0.05 were considered statistically significant different (2-sided). Where needed, ANOVA Fisher F-tests were followed by post hoc tests (Tukey's test, for comparing >3 groups) or LSD tests (for comparison

of ≤ 3 groups). For correlation analyses, Pearson correlation coefficients were calculated between major outcomes.

6.3. Results

6.3.1. General influence of proteins on the bioaccessibility of carotenoids

Bioaccessibility differed significantly between carotenoid species. In fact, under control conditions (no protein added), the bioaccessibility of lutein was significantly higher (81.3% ±1.2) compared to that of β-carotene (26.4% ±0.7) ($p>0.001$), while the lowest bioaccessibility ($p<0.001$) was obtained for lycopene (20.2% ±0.9).

The addition of various proteins to the simulated GI digestion impacted the bioaccessibility differently. All carotenoids and all protein concentrations applied considered, i.e. evaluated together statistically (without applying post-hoc tests), the addition of SC resulted overall in slightly higher bioaccessibility (43.7%) compared to WPI and GEL (41.9% and 41.7%, respectively) ($p<0.001$), while the lowest bioaccessibility was clearly engendered by SPI (35.1%) ($p<0.001$).

6.3.2. Influence of individual proteins on the bioaccessibility of carotenoids

6.3.2.1. Whey protein isolate

Following simulated GI digestion, the overall bioaccessibility of lycopene and β-carotene was positively influenced by the presence of WPI (all concentrations considered), showing an average increase to 24.1% ($p<0.001$) and 30.3% ($p<0.001$), respectively, compared to the respective controls (see average control values above), while the bioaccessibility of lutein dropped to 71.5% ($p<0.001$). More specifically, the addition of WPI at 10 and 25% RDA decreased the bioaccessibility of lutein to 63.3% ±0.5 and 61.2% ±1.1, respectively ($p<0.001$, Figure 6.1A), while the same concentrations enhanced the bioaccessibility of lycopene up to 27.4% ±1.1 and 25.0% ±1.2 ($p<0.001$ and $p<0.05$, respectively). β-Carotene bioaccessibility was only enhanced by 50% RDA of WPI, to 39.0% ± 1.7 ($p<0.001$), while the other concentrations had no significant effect compared to the control.

6.3.2.2. Soy protein isolate

The addition of SPI (all concentrations considered) to the simulated GI digestion enhanced the bioaccessibility of β-carotene (to 31.3%, $p<0.001$), while it decreased the bioaccessibility of lycopene (to 15.8%, $p<0.001$) and lutein (to 58.2%, $p<0.001$) (Figure 6.1B). In detail, at 10 and 25% RDA of SPI, the bioaccessibility of β-carotene increased to 36.9% ±2.5 and 34.6% ±1.6, respectively ($p<0.05$). Conversely, the addition of SPI drastically decreased the bioaccessibility of lutein in a dose-dependent manner, to 41.0% ±1.2

($p<0.001$). Lycopene was less drastically influenced, with a reduced bioaccessibility to 14.3% ±0.8 at 50% RDA ($p<0.001$).

6.3.2.3. Sodium caseinate

No significant effect engendered by the presence of SC (all concentrations considered) regarding the bioaccessibility of lycopene (Figure 6.1C), while an average increase up to 39.8% of β-carotene bioaccessibility was observed ($p<0.001$). As with other proteins, the addition of SC decreased the bioaccessibility of lutein to an average of 70.2% ($p<0.001$). More specifically, at high protein concentration (50% RDA), SC reduced lutein bioaccessibility to 62.9% ±1.2 ($p<0.001$). For β-carotene, a drastic increase in bioaccessibility to 49.8% ±0.9 was observed ($p<0.001$).

6.3.2.4. Gelatin

The co-digestion of pure carotenoids with GEL resulted in very similar effects regarding the bioaccessibility as observed with SC. Briefly, when considering all protein concentrations, the presence of GEL in the digested matrix (all concentrations applied) enhanced the bioaccessibility of β-carotene to an average of 32% ($p<0.001$). However, the bioaccessibility of lutein was brought down to an average of 72.3% ($p<0.001$). Again, there was no significant change regarding the bioaccessibility of lycopene compared to the control (Figure 6.1D). The negative influence of GEL regarding the bioaccessibility of lutein appeared to be dose-dependent, reaching a minimum bioaccessibility of 63.4% ±1.2, while the bioaccessibility of

β-carotene was improved in turn in a dose-dependent manner, with an increase to 36.6% ±2.4 of bioaccessibility ($p<0.001$).

6.3.3. Protein and lipid analysis

The images of the gels revealed that all bands disappeared after complete GI digestion (Figure 6.2), except for SPI, where some low-size polypeptide fragments remained. By way of comparison, the bands observed after the gastric phase were of variable size, but always corresponded to lower molecular weights compared to their native proteins. Nevertheless, more marked bands were observed in case of WPI (Figure 6.2A), corresponding mostly to β-lactoglobulin according to the native undigested WPI. Comparatively, SPI was found less digestible (Figure 6.2B), while SC (Figure 6.2C) and GEL (Figure 6.2D) were apparently more readily digested. We observed that there were no protein bands present under control condition (0% RDA), only those corresponding to the enzymes.

Regarding lipid digestion (Figure 6.3), it is obvious that proteins influenced differentially the release of FFAs. At 10% RDA of WPI and SC, the release of FFAs was found higher compared to the other concentrations (Figure 6.3A, $p<0.05$; Figure 6.3C, $p<0.001$, respectively), i.e. the presence of these proteins at higher concentrations negatively influenced lipid digestion, resulting in a significant negative correlation ($R=-0.857$, -0.867, respectively, $p<0.001$). On the other hand, SPI had a positive influence, in a dose-dependent manner ($R=0.952$, $p<0.001$, Figure 6.3B), enhancing FFAs release more than twice compared to the control ($p<0.001$).

Conversely, the addition of GEL at different concentrations did not show any significant influence on FFAs release (Figure 6.3D).

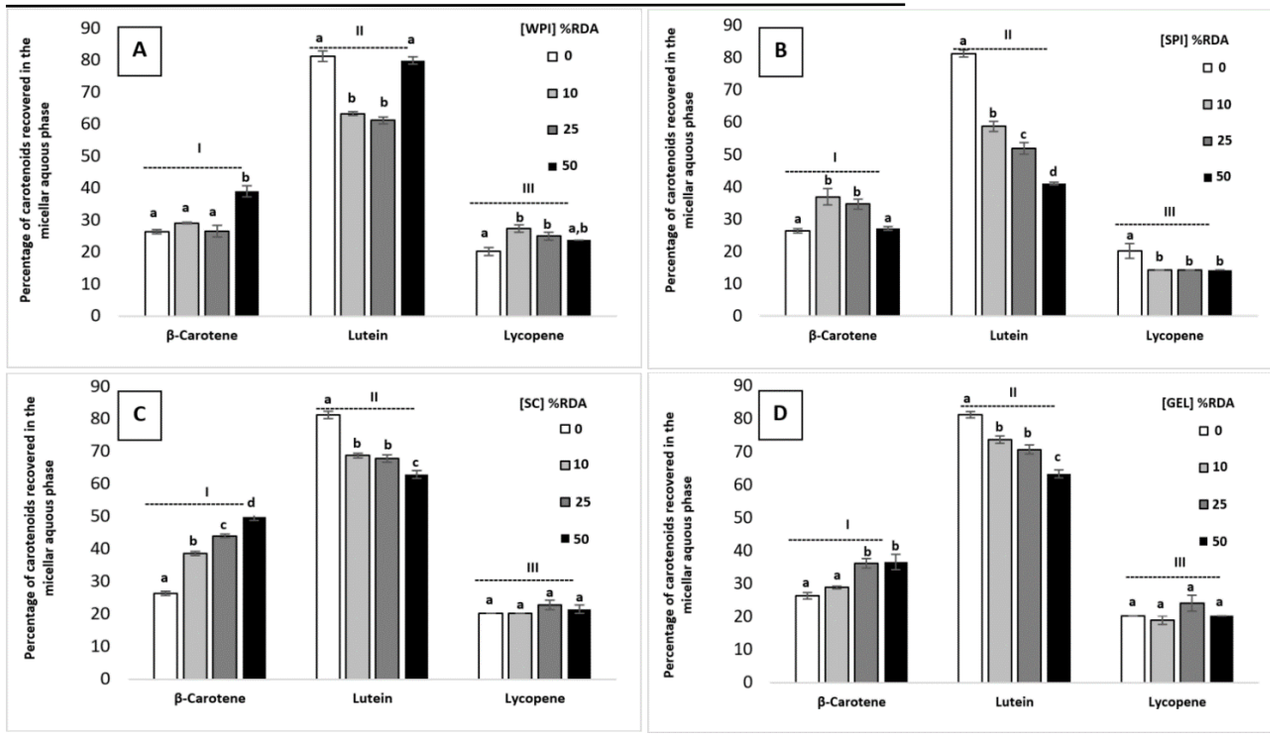


Figure 6.1. Influence of whey protein isolate (panel A), soy protein isolate (panel B), sodium caseinate (panel C) and gelatin (panel D) at various concentrations on the bioaccessibility of pure carotenoids following simulated gastro-intestinal digestions. β-Carotene, lycopene and lutein were examined in a digestion system either in the absence or presence of WPI, SPI, SC or GEL at different concentrations (0, 10, 25 and 50% of protein recommended dietary allowance (RDA)). Bioaccessibility is expressed as the percentage of pure carotenoids recovered from the aqueous micellar fraction at the end of the *in vitro* GI digestion, compared to the amount of the individual carotenoid added at the beginning of digestion. Values represent means ± SD of $n \geq 4$. Labeled means without a common superscript (alphabetic letters or roman numbers) differ significantly, $P < 0.001$.

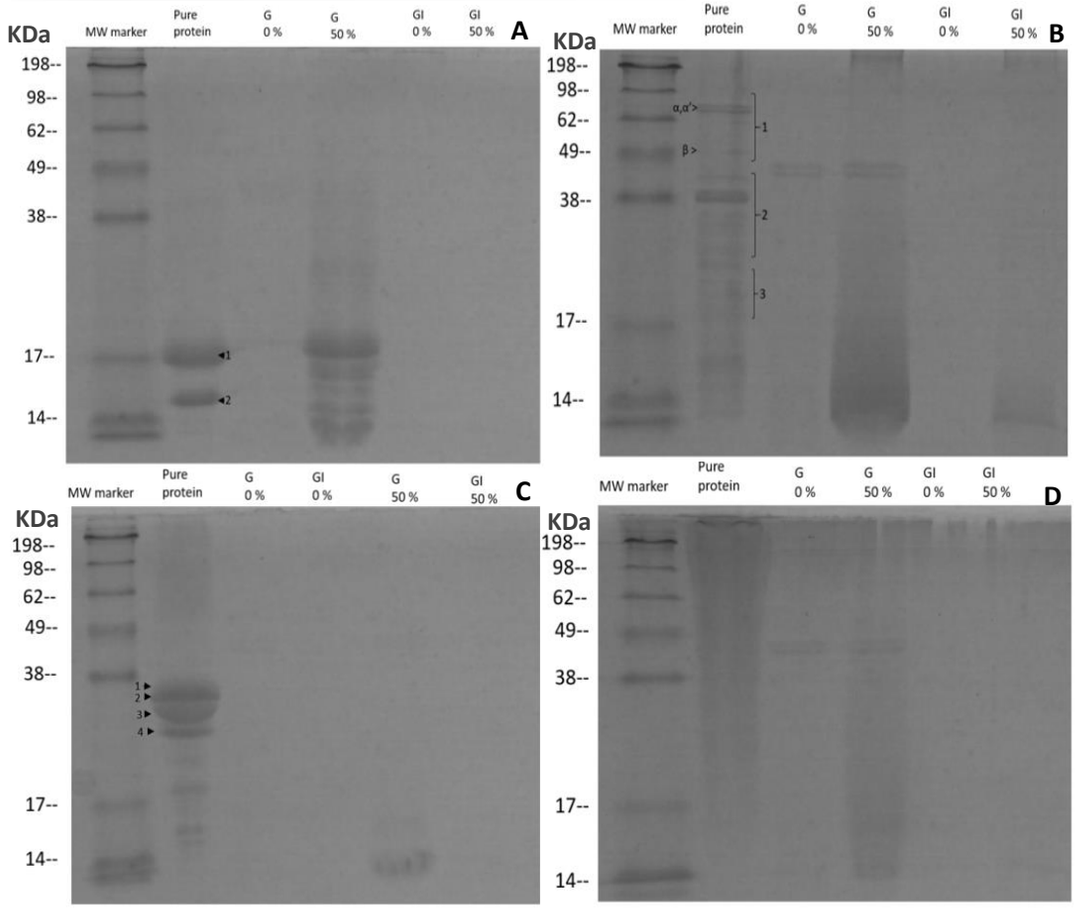


Figure 6.2. SDS-PAGE results showing the protein bands of pure whey protein isolate (panel A), soy protein isolate (panel B), sodium caseinate (panel C) and gelatin (panel D), compared to gastro-intestinal digestions and gastric digestions only in either presence or absence of proteins. Marker bands are shown on each side. G: gastric digestion of the matrix in the absence (0%) or presence of proteins at a concentration of 50% RDA. GI: gastrointestinal digestion of the matrix in the absence (0%) or presence of proteins at a concentration of 50% RDA. Pure protein represents native undigested protein. Some known polypeptides have been identified to confirm the integrity and type of the proteins. Panel A) 1: β-Lactoglobulin; 2: α-Lactalbumin. Panel B) 1: β-Conglycinin polypeptides (α, α', β subunits); 2: Acid glycinin subunit polypeptides; 3: Basic glycinin subunit polypeptides. Panel C) 1: α_{S2}-Casein; 2: α_{S1}-Casein; 3: β-Casein; 4: κ-Casein.

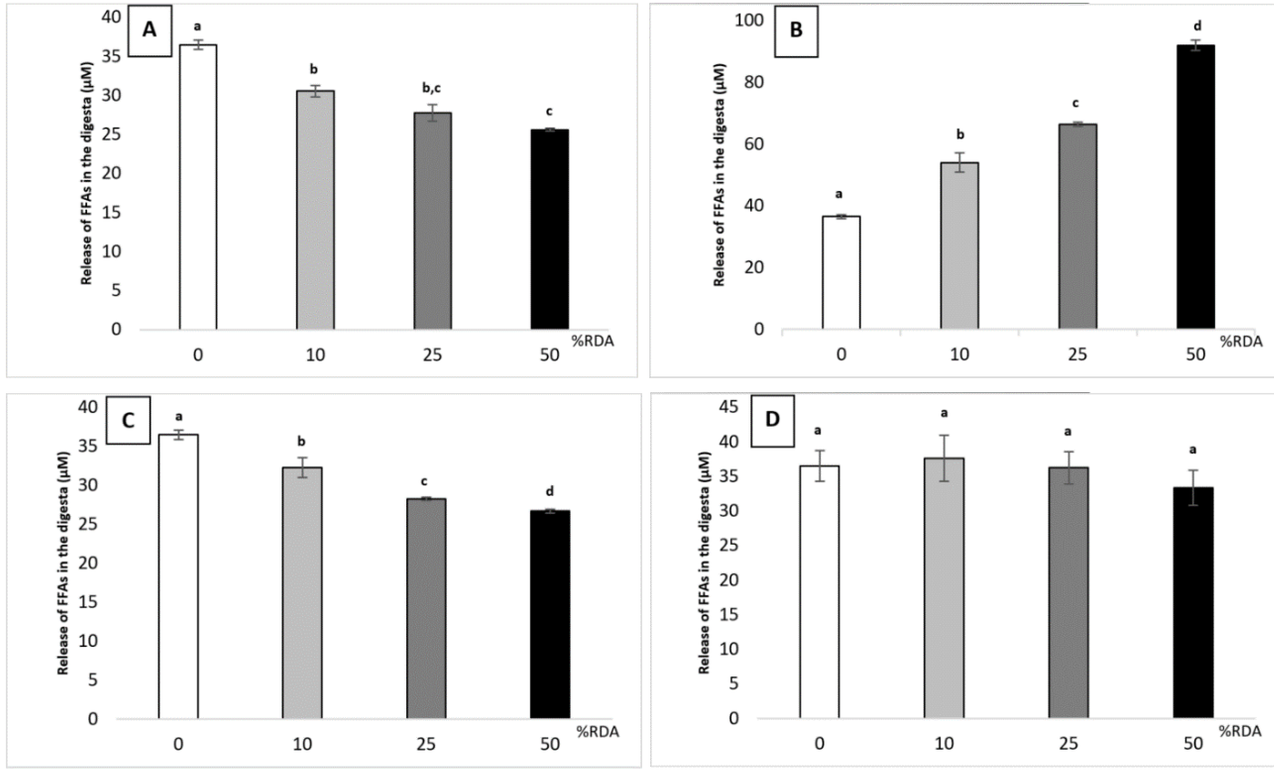


Figure 6.3. Influence of proteins on the release of free fatty acids (FFAs) during simulated gastro-intestinal digestion. Whey protein isolate (panel A), soy protein isolate (panel B), sodium caseinate (panel C) and gelatin (panel D) were digested at various concentrations (0, 10, 25 and 50% of the recommended dietary allowance (RDA)), and the release of FFAs was evaluated at the end of GI digestion by means of Cayman's Free Fatty Acid Fluorometric Assay. Values represent means ± SD of *n*=3. Labeled means without a common superscript (alphabetic letters) differ significantly, *P*<0.05.

6.3.4. Physicochemical characteristics of the digesta

Surface tension is a key indicator of the emulsifying capacity of proteins in the digestion process, since they have been proposed to reduce the surface tension of lipids in the gastrointestinal tract [21]. Overall, the addition of proteins to the simulated GI digestion influenced differentially the surface tension of the digesta, depending on the type of protein, even though absolute changes in surface tension remained rather small. The presence of SPI in the digesta (all concentrations considered) led to the highest surface tension (39.5 dyn/cm), followed by SC and GEL (38.7 dyn/cm), and finally WPI (38 dyn/cm). A significant difference was found between the three protein groups (SPI > SC=GEL > WPI, $p<0.001$) (Figure S6.1). Regarding the effect of protein concentration (individual or all proteins considered), we observed a significant increase on the surface tension at 50% RDA for all proteins (except for WPI), compared to the control ($p<0.001$).

The viscosity, as another important characteristic of the digesta was measured. In fact, viscosity is defined as the friction occurring between individual molecules in a liquid, and is a measure of the resistance of a fluid to deformation under shear stress [33]. An enhanced viscosity has been shown to reduce the emulsification of dietary lipids, resulting in lower extent of lipid hydrolysis/assimilation [34]. As proteins could change the viscosity of solutions when interacting with aqueous phase, the transport of carotenoids within lipid droplets as well as their incorporation into mixed micelles could be directly affected by viscosity. In the present study, the viscosity of digested and non-digested SC and WPI solutions did not change with the different concentrations (Figure 6.4A). The same effect was

observed in digested GEL solutions, which remained in the viscosity range of the protein-free solution. In contrary, the viscosity of undigested GEL solutions increased exponentially with its concentration. Such a viscosity response was expected due to the presence of a gel-like structure in the solution (Figure 6.4A). The shear stress of samples digested without additional proteins (0% RDA) showed a very small yield point of approximatively 1 mPa (Figure 6.4B). However, the digested samples containing WPI and SC showed a higher yield point of about 10 mPa, while digested samples with GEL had a higher yield point reaching 10 Pa, in line with the polymeric property of GEL at low temperature (Figure 6.4B). Due to sedimentation and the fact that a double gap cell was used for this experiment, we preferred not to analyze the samples digested with SPI, as this would involve changing parameters that were used during digestion of the proteins (such as requiring additional heating, ultrasonication), in order to obtain a homogeneous solution.

The lipid digestibility may vary depending of the emulsion characteristics, such as interfacial composition and oil droplet size, thus influencing micelle formation and the associated carotenoid bioaccessibility [35]. Here, the presence of proteins in the simulated GI digestion resulted in different emulsion structures, though all proteins remarkably reduced the size of the emulsion (Figure 6.5). Overall, at 10% RDA, SPI, SC and GEL showed a higher degree of aggregation compared to WPI, while the addition of these proteins at a concentration of 25% RDA resulted in a clear decrease of microstructure sizes, with a smaller emulsion in case of WPI. However, increasing the concentration to 50% RDA resulted in very

homogeneous emulsions, for all protein types, compared to the other concentrations (Figure 6.5). With all proteins, we observed a co-localization of the labelled compounds (proteins and lipids) present in the matrix, possibly indicating an interaction between these during the simulated GI digestion.

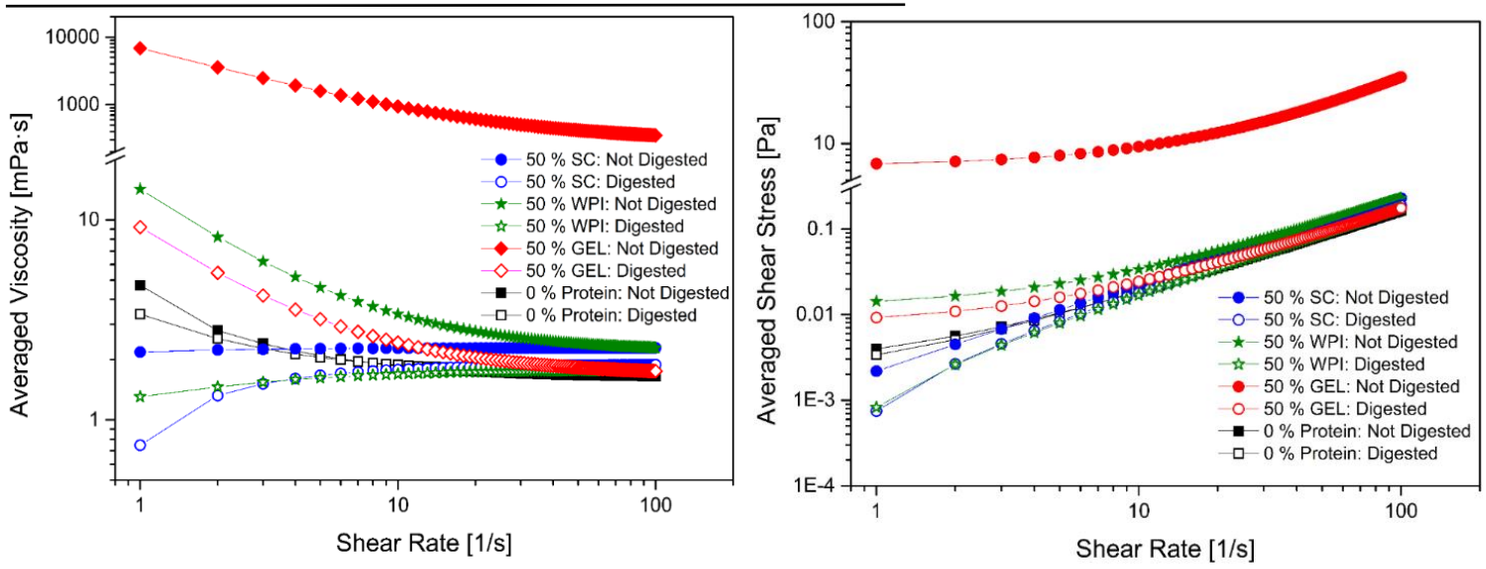


Figure 6.4. Effect of pure proteins at various concentrations on the macroviscosity (panel A) and shear stress (panel B) of the digesta following gastro-intestinal digestion. Panel A represents the averaged viscosity curves ($n \geq 3$) as a function of the shear (1 – 100 s^{-1}) rate for non-digested and digested whey protein isolate (WPI), sodium caseinate (SC) or gelatin (GEL), at different concentrations representing 0 and 50% of the recommended dietary allowance (RDA). Panel B represents the averaged Bingham regressions ($n \geq 3$) of the shear stress (0 – 100 Pa) as a function of the shear (1 – 100 s^{-1}) rate for non-digested and digested WPISC or GEL at different concentrations, representing 0 and 50% of the RDA.

The produced mixed micelles following the simulated GI digestion were significantly affected by the presence of proteins ($p<0.001$). WPI and SPI had a stronger influence on the size of mixed micelles (all concentrations considered), compared to GEL and SC ($p<0.001$). In fact, the addition of WPI and SPI (Figure S6.2A and Figure S6.2B, respectively) to the simulated digestion at a concentration of 25% RDA reduced significantly the size of mixed micelles, compared to the control ($p<0.001$), while no effect was observed following the addition of SC and GEL at this concentration. However, increasing the concentration to 50% RDA, all proteins, including GEL and SC, decreased significantly the size of the micelles, compared to the control ($p<0.001$).

An important parameter that directly affects the stability of emulsions was measured [36]. Indeed, ζ-potential determines the effective electric charge on the particle's surface by measuring the degree of electrostatic repulsion between adjacent particles, similarly charged in the emulsion [37]. Interestingly, the average of the absolute ζ-potential (all proteins considered) was reduced in a dose-dependent manner (mean $R = 0.581$, $p<0.001$); the results were similar for the different proteins. At 10% RDA of proteins, a significant difference was seen only for WPI and SPI ($P<0.001$), while at concentrations of 25 and 50% RDA, a significant difference was found in the presence of each individual protein, compared to the control ($p>0.001$) (Figure 6.6).

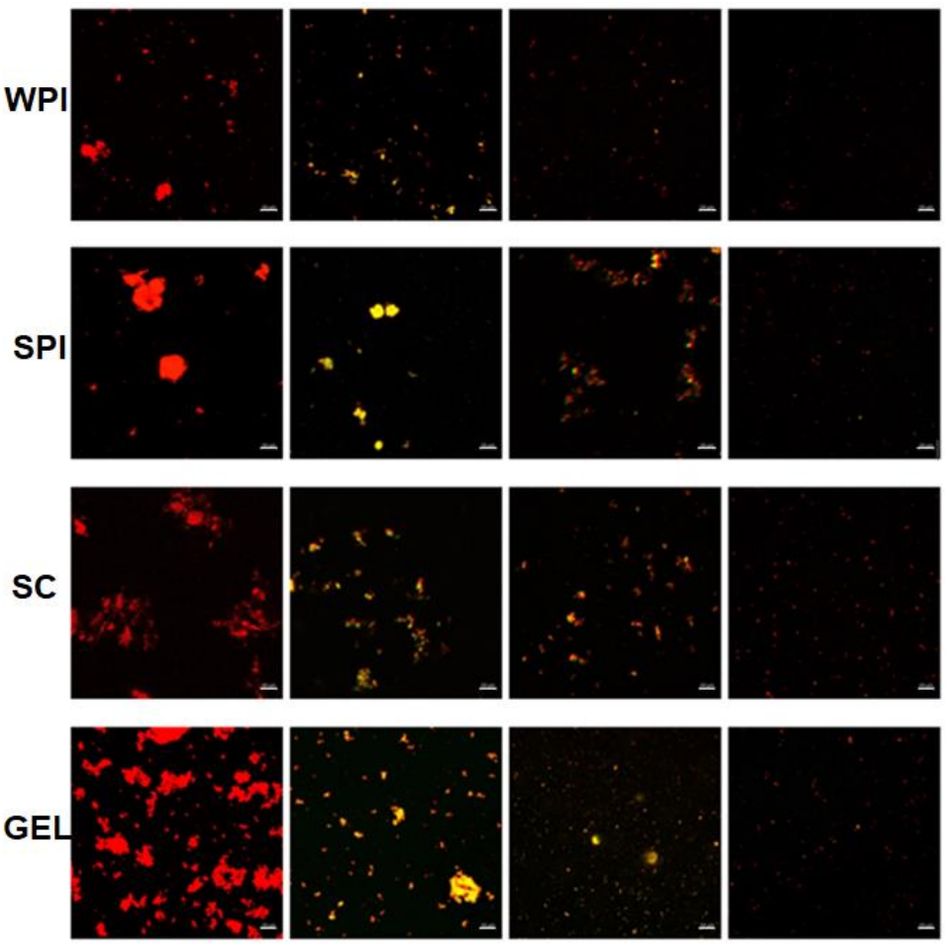


Figure 6.5. Confocal microscopy images taken after gastro-intestinal digestion with whey protein isolate (WPI), soy protein isolate (SPI), sodium caseinate (SC), and gelatin (GEL). Confocal imaging of emulsion structures was carried out at room temperature with a confocal laser scanning microscope (Zeiss LSM 880, Airyscam SR, Jena, Germany), using a 63x objective. The fluorescent dyes were excited by an Argon laser (488 nm) and the emitted light was collected at 522 nm for protein and 635 nm for the fat phase. Lipids were labelled with Nile red (red color) and proteins were labelled with fluorescent isothiocyanate (FITC) (green color). Of note, the combination of red and green is yellow in additive color configurations. The white-grey bar indicates 10 μm.

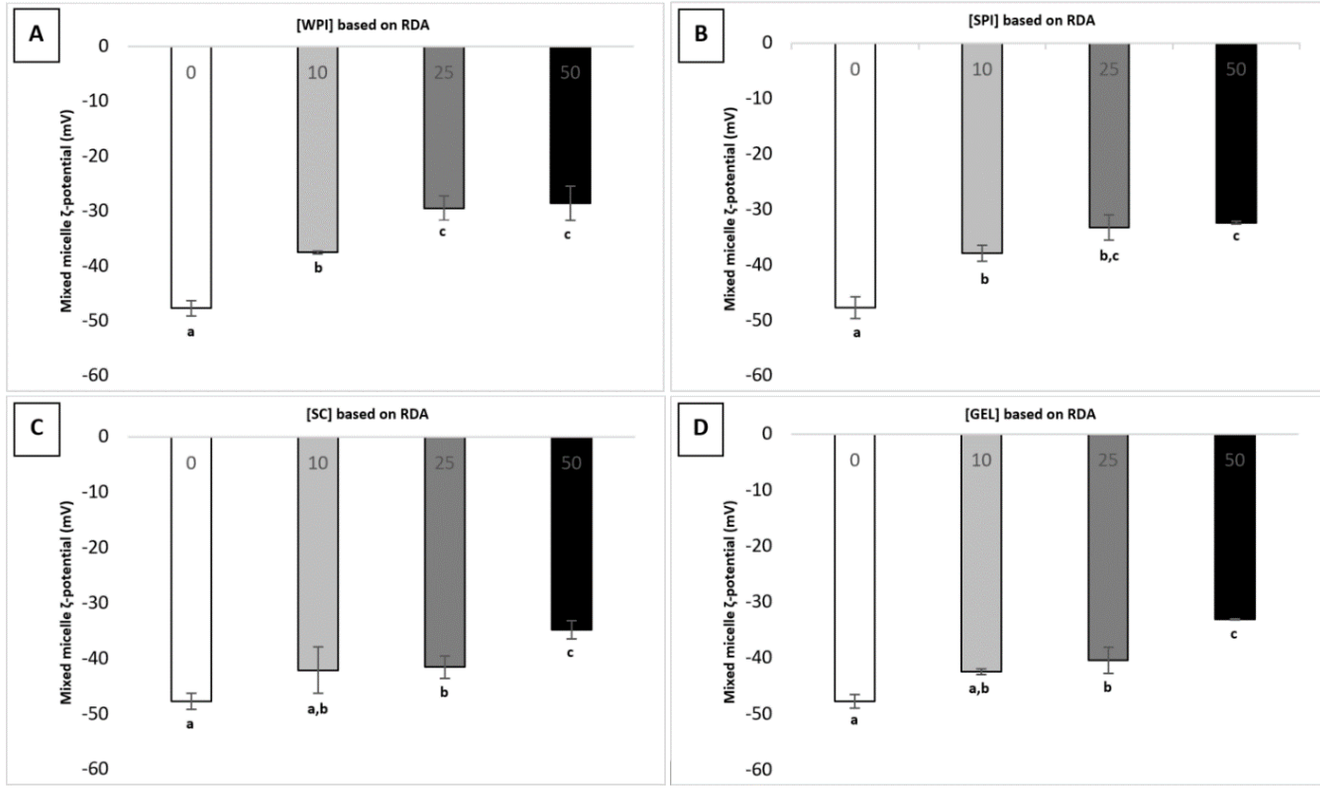


Figure 6.6. Effect of whey protein isolate (panel A), soy protein isolate (panel B), sodium caseinate (panel C) and gelatin (panel D) at various concentrations on the mixed micelle ζ-potential following gastro-intestinal *in vitro* digestions. β-Carotene, lycopene and lutein were examined in a digestion system in the presence of WPI, SPI, SC or GEL at different concentrations (0, 10, 25 and 50% of the recommended dietary allowance (RDA)). Filtered aliquots of the aqueous micellar fraction were used to determine mixed micelle size by using photon correlation spectroscopy (Zetasizer Nano Zs, Malvern Instruments) at room temperature. Values represent means ± SD of n=4. Labeled means without a common superscript (alphabetic letters) differ significantly, P<0.001.

6.4. Discussion

In the present study, we investigated the influence of different proteins (WPI, SPI, SC and GEL) at various concentrations equivalent to 0, 10, 25 and 50% of the recommended dietary allowance (RDA), on the bioaccessibility of pure carotenoids (lycopene, β-carotene and lutein). The presence of proteins during the simulated GI digestion influenced the bioaccessibility of carotenoids, either positively or negatively. These effects depended on the type and concentration of protein, but also on the type of carotenoid.

Proteins are surface-active molecules present within the aqueous phase surrounding the lipid droplets during digestion [38]. Once adsorbed, they form a viscoelastic layer that stabilizes emulsions [39]. They have been proclaimed to aid in emulsifying liposoluble dietary constituents [23] and to enable the incorporation of non-polar components into emulsions during GI digestion [38]. However, it has been reported that high protein concentrations at the lipid surface could affect the stability of emulsions, but also negatively influence lipid degradation, impacting the incorporation of lipophilic constituents into lipid droplets during GI digestion [12, 21, 40]. Indeed, in our previous study, we found that β-carotene bioaccessibility was influenced positively by WPI due to the stabilization of emulsions or negatively, due to hampered enzymatic access to lipid droplets [22].

In the present investigation, the addition of proteins enhanced the bioaccessibility of β-carotene up to 50% compared to the protein-free control, while negatively influencing lutein bioaccessibility. In case of lycopene, adding proteins to the simulated digestion did not

influence its bioaccessibility, except in the presence of WPI, with a slight increase up to 27.4%, compared to the control.

Physicochemical properties of carotenoids, such as polarity, could arbitrate the extent of micellization [41]. Borel et *al.* have shown that carotenoids behave differently in biological emulsions depending on their polarity [42]. Indeed, the more polar zeaxanthin, which has a chemical structure very close to lutein, was preferentially solubilized at the lipid droplet surface, while the apolar β-carotene was solubilized almost exclusively in the core of the lipid droplets. Therefore, the presence of proteins at the interface may result in a stronger negative interaction of proteins and the more polar xanthophylls, though this may happen also before micelle incorporation [12].

Regarding individual proteins, WPI at lower concentrations (10 and 25% RDA) enhanced lycopene bioaccessibility, possibly due to its emulsion forming ability [43], while at 50% RDA it only improved the bioaccessibility of β-carotene. It was reported that β-lactoglobulin was largely resistant to digestion by pepsin during the gastric phase [44], and remained adsorbed at lipid droplet surfaces [40], being in line with our results. Another study demonstrated that β-carotene can be bound by β-lactoglobulin with high affinity in the internal cavity of the β-barrel [45], suggesting that this major WPI fraction could play a transporter role for some carotenoids. Such interactions appear specific to a certain structure, i.e. β-ionone cycle and isoprenoid chain [45]; consequently, carotenoids with only a hydrocarbon chain such as lycopene may show low binding affinity, perhaps explaining the absence of positive effects of higher WPI concentration on its bioaccessibility. In addition, WPI significantly decreased the release of

FFAs. We can only speculate that in these emulsions, large peptides adsorbed to lipid droplet surfaces could form a biological barrier to digestive enzymes, rendering WPI-stabilized emulsions more resistant to lipolysis [40], finally interfering with mixed micelle formation and lycopene micellization.

In case of SC, the bioaccessibility of β-carotene increased in a dose-dependent manner, clearly a different behavior compared to WPI. SDS-PAGE showed that SC was almost completely digested following gastric phase. It is acknowledged that caseins are rapidly hydrolyzed into small peptides, covering the surface of produced emulsions [46, 47]. Furthermore, FFAs release was reduced in the presence of SC, possibly due to a film formed at o/w interface, with high viscoelasticity [48]. Thus, we hypothesize that the presence of these surface-active molecules enhanced the bioaccessibility of β-carotene (and for lycopene, though not significantly, by stabilizing the emulsions and preventing the aggregation of droplets.

Similar mechanisms seemed to occur with GEL, enhancing β-carotene bioaccessibility at 25 and 50% RDA, while only a slight increase was observed for lycopene. GEL was also fully digested at the end for the GI digestion, and its presence did not influence lipid digestion. Recent nanoemulsion applications have demonstrated the ability of GEL to adsorb on the o/w interfaces and produce emulsions that are stable toward coalescence [49], finally promoting the micellization of the encapsulated β-carotene [50].

Low concentrations of SPI enhanced β-carotene bioaccessibility, while reducing it at 50% RDA. The protein was incompletely digested following the gastric phase. SPI in the digesta led to the formation of

insoluble fractions which precipitated and accumulated at the bottom of the digested phases, likely due to the low solubility of SPI [51, 52]. It was earlier shown that SPI influenced the physicochemical behavior of o/w emulsions during *in vitro* digestion; affecting emulsion stability and extensively increasing lipid digestion, mainly dictated by the type of the liberated peptides [44]. This increased lipid digestion is in accordance with our results regarding FFAs release. On one hand, if being partially digested, i.e. at low concentrations (10 and 25% RDA), the resulting short peptides were able to emulsify lipid droplets and prevented their aggregation, enhancing lipolysis as well as β-carotene bioaccessibility. This is similar to hydrolyzed SPI which stabilized emulsions thanks to a thick elastic film formed on lipid droplet surfaces [48]. On the other hand, the accumulation of remaining long peptide chains from SPI digestion may aggregate in the aqueous phase, especially at high concentrations, due to hydrophobic interactions between partially denatured proteins. These may be adsorbed on individual lipid droplets [53], and carotenoids could be trapped in these aggregates, negatively affecting their transfer into mixed micelles.

Despite the ambivalent influence that proteins had on carotenoid bioaccessibility, the viscosity of the digested protein solutions did not depend on the protein concentration and remained in the viscosity range of the control solution. In addition, the analysis of the complete shear rate range revealed that the presence of proteins did not affect shear thinning, except a slight apparent increase in the presence of GEL, which is typical for polymer solutions. Therefore, the drop of viscosity in combination with the lack of shear thinning could be taken

as indication for full protein hydrolysis (except for SPI) due to digestive enzymes.

Surprisingly, adding proteins to the simulated GI digestion increased surface tension, most strongly by SPI. However, the absolute increase compared to water was quite small. A previous study reported similar findings, assuming that the increase in surface tension is reflecting the effects of pepsinolysis on the adsorbed proteins at the interface [54], and the incorporation of intestinal proteases exerted an additional hydrolysis of adsorbed proteins. Consequently, protein digestion reduced the interfacial layer and caused an increment of the surface tension [55]. Furthermore, other studies reported that these changes could be associated with the very complex characteristics of the interface, such as the adsorption of bile salts to the interface, the interaction between bile salts and protein fragments, or the adsorption and activity of pancreatic lipase [56-58].

The investigated physicochemical properties such as micelle size, ζ-potential as well as confocal microscopy visualization, yielded additional insights regarding the stability of emulsions. At 25% RDA, the presence of WPI and SPI during the simulated GI digestion outperformed other proteins (SC and GEL) in reducing mixed micelle size. In addition, the absolute ζ-potential of WPI and SPI was lower, compared to SC and GEL, indicating weaker repulsive forces and probably less stable micelles. However and interestingly, microscopic visualization of the emulsions following GI digestion showed that all investigated proteins reduced lipid droplet size dose-dependently. These results suggest that various processes govern the emulsifying

properties of the investigated proteins, with perhaps ζ-potential not playing a major role.

Previous studies have reported that in emulsions stabilized by hydrolyzed soy or whey proteins, the repulsive forces between the droplets were greatly lessened, and even no longer strong enough to resist the attractive inter-droplet interactions, i.e. van der Waals and hydrophobic interactions [59, 60]. This could lead to the formation of WPI- or SPI-stabilized emulsions, forming a network of bridged lipid droplets, as a result of enhanced hydrophobic interactions between the adsorbed proteins on individual droplets, without changing their individual integrities [61, 62]. Our results indicated that besides the low solubility of SPI, and pepsinolysis-resistance of β-lactoglobulin in case of WPI, these proteins could have higher surface hydrophobicity, a crucial parameter determining their emulsification performance [62]. Thus, the biological barrier at the interface could hinder the transfer and incorporation of carotenoids into mixed micelles, reducing their bioaccessibility. On the other hand, SC and GEL, which were better hydrolyzed even in the early stage of digestion, could be involved in the formation of emulsions with more dispersed oil droplets, as the repulsive forces were slightly stronger, enhancing the bioaccessibility of carotenoids.

Taken together, the low solubility of SPI resulted in a reduction in protein digestion due to the formation of aggregates in the aqueous phase, and accordingly, higher surface tension and a decrease in the ζ-potential were observed, which adversely affected carotenoid bioaccessibility. This is probably due to the trapping of carotenoids in the aggregates, even if it had a positive effect on the digestion of lipids

and the formation of micelles. However, GEL and SC had a better solubility as shown by their effect on the viscosity of the digests. Their digestion was effective even in the early stage of digestion, which resulted in greater repulsive forces between the particles and a better emulsification, as shown by the ζ-potential and the surface tension, respectively, leading to the formation of emulsions with more dispersed lipid droplets. Consequently, GEL and SC had a better effect on carotenoid bioaccessibility compared to PSI, even if their presence slightly reduced lipid digestion and therefore the formation of mixed micelles. The same negative effect was obtained in the presence of WPI, probably due to the pepsinolysis-resistance of β-lactoglobulin, preventing access to the surface of the lipid droplets at the early stage of intestinal digestion, but this effect disappeared with the inclusion of the pancreatic enzymes, resulting in a complete digestion of WPI and enhancing mixed micelle formation. In addition, WPI had a better emulsifying effect, as shown by the surface tension, compared to the other investigated proteins, resulting in the formation of bridged lipid droplets as the repulsive forces were reduced and, consequently, enhanced the bioaccessibility of carotenoids.

6.5. Conclusions

Proteins added to the simulated GI digestions significantly influenced carotenoid bioaccessibility, either positively or negatively. The effects depended on the type and concentration of the protein, but also on the type of carotenoid. Proteins enhanced β-carotene bioaccessibility by up to 189%, while a decrease of up to 50% was

observed for lutein. The influence was somewhat limited regarding lycopene bioaccessibility (increase by up to 135%). On the one hand, differences in polarity of each carotenoid reflect a different rate of micellization [41, 42] and on the other hand, the contribution of proteins, via their emulsifying properties, depended on their type and concentration [21, 63]. This study also investigated the association between the digestion of macronutrients (proteolysis and lipolysis) and their impact on digesta characteristics such as surface tension and macroviscosity, which in turn could influence emulsion stability, repulsive forces, mixed micelle size, all of which could also influence carotenoid bioaccessibility. The results highlight the influence of proteins on carotenoid micellization, constituting an important stage in their bioavailability. However, studying further potential interactions of proteins or/with carotenoid-rich food-matrices would be of great interest. It also remains to be investigated which interactions occur following the interaction of carotenoids from a more mixed diet containing various types of proteins. In addition, investigating the subsequent stages of bioaccessibility, i.e. cellular uptake, will certainly provide additional insights, and of course, final confirmation of these results *in vivo* should be targeted.

6.6. References

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6.7. Supplementary Materials

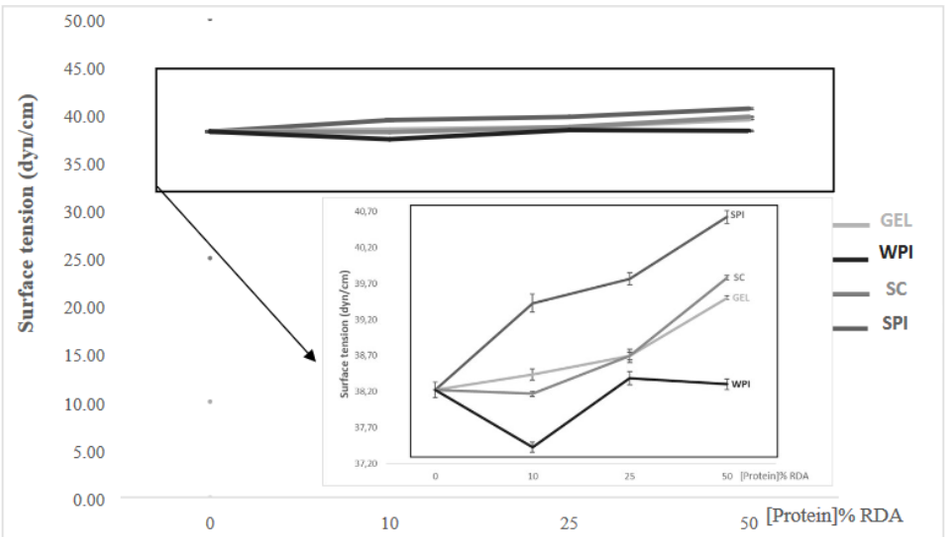

Figure S6.1. Effect of pure proteins at various concentrations on the surface tension of the digesta (value of pure water: 71.99 dyn/cm), following gastro-intestinal digestion. Whey protein isolate (WPI), soy protein isolate (SPI), sodium caseinate (SC) or gelatin (GEL) were subjected to simulated GI digestion at different concentrations (0, 10, 25 and 50% of the recommended dietary allowance (RDA)). The surface tension of digesta, pre-conditioned at 25 ± 0.1 °C, was determined by the weight-drop method. Values represent means ± SD of n = 16 replicates.

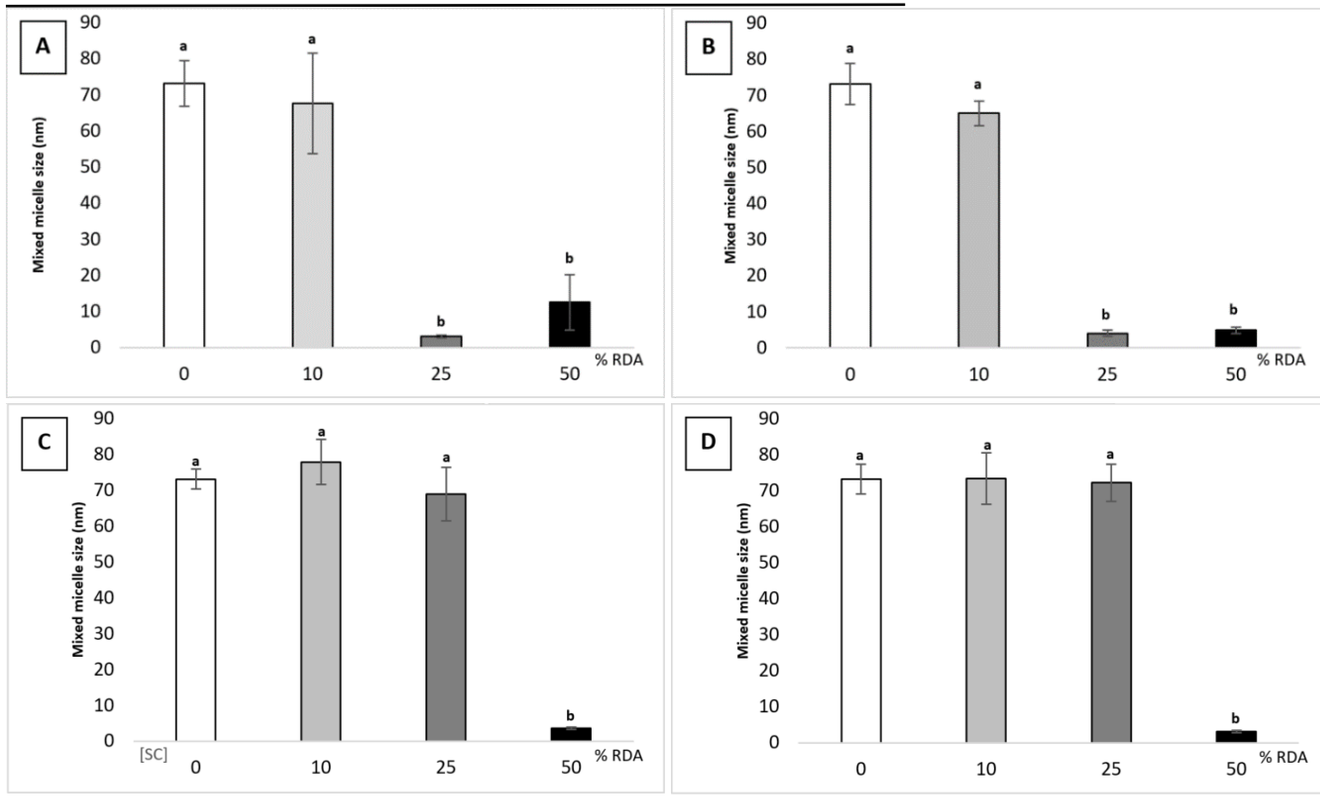

Figure S6.2. Effect of whey protein isolate (WPI, panel A), soy protein isolate (SPI, panel B), sodium caseinate (SC, panel C) and gelatin (Gel, panel D) at various concentrations on the size of the produced mixed micelles following gastro-intestinal *in vitro* digestions. WPI, SPI, SC or GEL were subjected to the simulated digestion at different concentrations (0, 10, 25 and 50% of the recommended dietary allowance (RDA)). Filtered aliquots of the aqueous micellar fraction were used to determine mixed micelle size by photon correlation spectroscopy (Zetasizer Nano Zs, Malvern Instruments) at room temperature. Values represent means ± SD of $n \geq 12$. Labeled means without a common superscript (alphabetic letters) differ significantly, $P<0.05$.

Chapter 7

Impact of protein-enriched plant food items on the bioaccessibility and cellular uptake of carotenoids

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Foreword

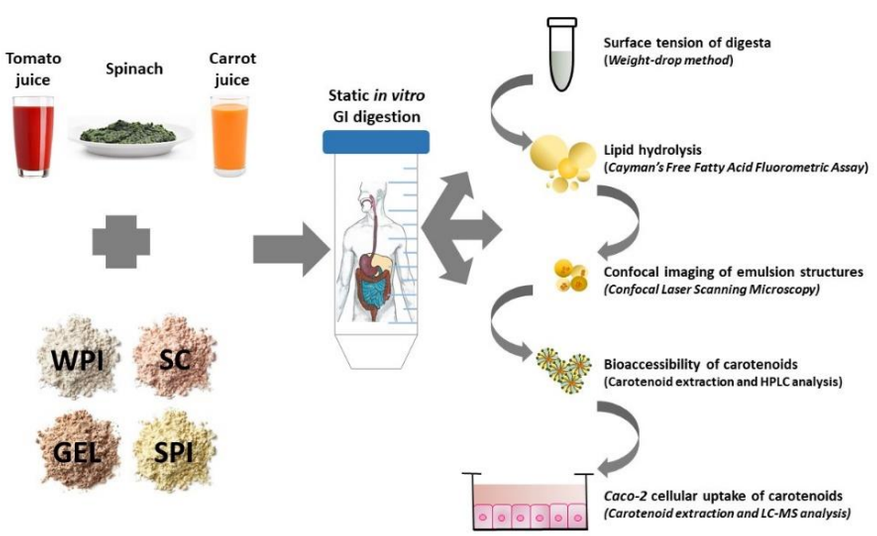
Following on from Chapter 6, in the present chapter we aim to investigate the effect of proteins with different structure, and thus digestibility, on the bioaccessibility and cellular uptake of carotenoids from selected food sources. Toward this end, previously tested pure proteins (WPI, SPI, SC and GEL), and protein-rich food matrices (turkey and cod, both chosen based on the low fat content), at different concentrations (equivalent to the addition of 0/10/25/50% of RDA) will be co-digested with food matrices rich in carotenoids (tomato juice, spinach and carrot juice), using the INFOGEST consensus digestion model with further adaptations as outlined in Chapter 4. Following simulated digestion, we will examine the impact of proteins on the cellular uptake of carotenoids by employing Caco-2 cells as a model for the intestinal epithelium. Additional mechanistic parameters, such as surface tension, free fatty acid release and microscopic visualization of the emulsions, will also be studied.

Abstract

Carotenoids are lipophilic pigments which have been associated with a number of health benefits, partly related to antioxidant effects. However, due to their poor solubility during digestion, carotenoids bioavailability is low and variable. In this study, we investigated the effect of frequently consumed proteins on carotenoid bioaccessibility and cellular uptake. Whey protein isolate (WPI), soy protein isolate (SPI), sodium caseinate (SC), gelatin (GEL), turkey and cod, equivalent to 0/10/25/50% of the recommended dietary allowance (RDA, approx. 60g/d) were co-digested gastro-intestinally with carotenoid-rich food matrices (tomato and carrot juice, spinach), and digesta further studied in Caco-2 cell models. Lipid digestion, surface tension and microscopic visualization were also carried out. Co-digested proteins positively influenced the micellization of carotenes (up to 3-fold, depending on type and concentration), especially in the presence of SPI ($p<0.001$). An increased cellular uptake was observed for xanthophylls/carotenes (up to 12/33%, $p<0.001$), which was stronger for matrices with an initially poor carotenoid micellization (i.e. tomato juice, $p<0.001$), similar as encountered for bioaccessibility. Turkey and cod had less strong an impact. Significant interactions between carotenoids, lipids, and proteins were observed during digestion. Co-digested proteins generally improved lipid digestion in all matrices ($p<0.001$), especially for carrot juice, though slight decreases were observed for GEL. Protein impact on the surface tension was limited. In conclusion, proteins generally improved both carotenoid bioaccessibility and cellular uptake, depending on the matrices and carotenoid-type (i.e.

carotene vs. xanthophylls), which may be relevant under specific circumstances, such as intake of carotenoid-rich food items low in lipids.

Keywords: INFOGEST protocol, tetraterpenoids, micellization, small intestinal uptake, emulsions, lipid droplets, mixed micelles; free fatty acids, bioavailability.



Graphical abstract 7. Impact of protein-enriched plant food items on the bioaccessibility and cellular uptake of carotenoids.

7.1. Introduction

Carotenoids belong to the category of tetraterpenoids. They can be divided into two main classes; carotenes, which are of linear carbon structure, and xanthophylls, carrying at least one oxygen molecule in their structure. These phytochemicals are naturally synthetized by plants, algae, bacteria and fungi [1, 2]. In addition to exhibiting a color spectrum from yellow to red, carotenoids are essential pigments in photosynthetic organisms, as they absorb violet and blue-green light. They also act as a photoprotector against overexposure to light, as well as contributing to attract animals for pollination and dispersal of plant seeds [3, 4].

Humans and other animals do not synthetize carotenoids de novo, i.e. their presence in these organisms is exclusively due to dietary intake. The physiological functions of these secondary plant compounds in the human body appear to be complex and are the topic of much debate. Numerous studies have shown that carotenoids exert protective and/or preventive roles in human health and diseases, which have in part been related to their antioxidant functions, quenching reactive oxygen species such as singlet oxygen or lipid-peroxides [5]. Carotenoid plasma concentrations were associated with reduced risk of chronic diseases such as cardiovascular diseases [6] and type 2 diabetes [7], as well as certain types of cancer [8]. In addition, the presence of certain types of carotenoids in the eye is associated with reduced risk of developing several ocular diseases such as age-related macular degeneration and cataract, caused by photo-damage of the retina, especially among the elderly [9, 10]. Furthermore, certain types of carotenoids, i.e. β-carotene, α-carotene,

and β-cryptoxanthin are cleaved into retinal by β-carotene oxygenase 1 (BCO1) and/or β-carotene oxygenase 2 (BCO2), which could place carotenoids as the main source of vitamin A for vegetarians/vegans and in many countries with low consumption of animal-based food items [11]. Some of these metabolites could also interact with nuclear and transcription factors such as NF-κB and Nrf-2, with anti-inflammatory and anti-oxidant effects [12].

The close relation between carotenoid consumption and clinical outcomes has recently accelerated the pace of carotenoid research directed towards human nutrition, including questions related to bioavailability. In fact, carotenoids go through several stages following their dietary intake, starting by the release from the food matrix to allow their dissolution in lipid droplets in the stomach [13, 14] and their later incorporation, following processing by gastric-and pancreatic lipase activity [15] into mixed micelles in the small intestine [16]. This is largely thought to determine the fraction of carotenoids that are available for absorption, which takes place via either passive diffusion and/or via transporter-dependent processes [17], and is a good approximation of their bioavailability [18], a prerequisite for further physiological functions.

However, the bioavailability of carotenoids is highly variable [19, 20], and is known to be influenced by several intrinsic and extrinsic factors [21], including the food matrix. For instance, several studies have concluded that dietary lipids [22, 23], divalent minerals [24, 25], and dietary fiber [26] could influence carotenoid bioavailability. However, the potential influence of co-consumed dietary proteins on digestion processes of carotenoids has started

more recently to gain attention. It is acknowledged that during gastro-intestinal (GI) digestion, proteins go through structural modifications, which maximizes interactions between hydrophobic and hydrophilic phases, which may aid in emulsifying apolar dietary constituents [27]. These structural modifications have been observed for example in soy protein isolate (SPI), generating short protein chains, resulting in a better amphiphilic property and a decrease in the interfacial tension and the size of the emulsion droplets [28]. Another study reported that protein segments generated by the digestion of sodium caseinate (SC) formed a stable emulsion, carrying lipophilic compounds such as β-carotene, preventing its oxidation and facilitating its transfer into mixed micelles, which increased the bioaccessibility of β-carotene [29]. However, while proteins have received much interest from pharmaceutical and food industries, especially for encapsulation and delivery of lipophilic compounds [30, 31], including carotenoids [32], very little is known about the influence that proteins could have on the bioavailability of carotenoids, under conditions resembling the *in vivo* environment.

Results from our previous investigation demonstrated that whey protein isolate (WPI) was able to modulate β-carotene bioaccessibility during *in vitro* GI digestion, improving its bioaccessibility when the amount of co-digested dietary lipids is limited. However, a negative effect was observed under insufficient digestion parameters (i.e. limited concentrations of digestive enzymes and bile), likely via interactions during the processing of lipid droplets into mixed micelles by limiting the extent of lipolysis, therefore hindering carotenoid transfer [33]. Similarly, a study by Goupy et *al.*

showed that the transfer of pure carotenoids to oil droplets was dramatically impaired, when the emulsions were stabilized by bovine serum albumin [34]. In addition, we have reported in a recent study that various proteins (WPI, SPI, SC, and gelatin (GEL)) had both positive and negative effects on the bioaccessibility of isolated carotenoids (β-carotene, lycopene and lutein) [35]. These influences depended not only on the type of carotenoids, where higher polarity was reflected in a higher degree of micellization, but also on the type and concentration of proteins, where their digestibility and hydrophobicity were related to their emulsifying properties.

In the present study, we aimed to investigate the effect of several, frequently consumed proteins with different structure and thus digestibility, on the bioaccessibility and cellular uptake of carotenoids from selected food sources. Toward this end, pure proteins (WPI, SPI, SC, and GEL) or protein-rich food matrices (turkey and cod), at different concentrations (equivalent to the addition of 0/10/25/50% of the recommended dietary allowance (RDA)) were co-digested with food matrices rich in carotenoids (tomato juice, spinach, and carrot juice), using the INFOGEST consensus digestion model [36]. Following simulated digestion, we examined the impact of proteins on the cellular uptake of carotenoids, by employing Caco-2 cells as a model for the intestinal epithelium. For obtaining additional mechanistic insights, parameters such as surface tension, free fatty acid release, and microscopic visualization of the emulsions were also studied.

7.2. Materials and methods

7.2.1. Enzymes, chemicals and carotenoid standards

Digestive enzymes such as pepsin from porcine gastric mucosa (powder, ≥250 U/mg, measured as trichloroacetic acid-soluble products using hemoglobin as substrate, Art. No. P7000), pancreatin from porcine pancreas (activity equivalent to 4x USP specifications. Art. No. P1750), as well as porcine bile extract (Art. No. B8631) were ordered from Sigma-Aldrich (Overijse, Belgium).

All chemicals were of analytical grade or superior. Potassium chloride, potassium phosphate, sodium bicarbonate, sodium chloride, magnesium chloride hexahydrate, ammonium carbonate, sodium hydroxide solution (1 M), calcium chloride dihydrate, ammonium acetate, butylated hydroxytoluene (BHT), Nile red, fluorescein isothiocyanate isomer I (FITC), and phenolphthalein were acquired from Sigma-Aldrich, while hydrochloric acid solution (1 M), and acetic acid were obtained from VWR (Leuven, Belgium). Acetonitrile, methanol, ammonium acetate, and dichloromethane were purchased from Biosolve (Dieuze, France). Methanol (MeOH), acetonitrile (ACN), and dichloromethane (DCM), and methyl tert-butyl ether (MTBE) were purchased from Carl Roth GmbH+ Co. KG (Rotisol®, Karlsruhe, Germany). The solvents hexane, acetone, and methanol were obtained from VWR, while diethyl ether was from Sigma-Aldrich. 18 MΩ water was used throughout the study, it was either prepared with a purification system from Millipore (Brussels, Belgium) or procured from B. Braun Medical SA (Sempach, Switzerland).

Standards of *all-trans*-β-carotene (β-Car, powder, ≥97%), *all-trans*-lycopene (Lyc, powder, ≥85%), α-carotene (α-Car, powder, ≥97%), neoxanthin[1] (Neo, powder, ≥97%), and *trans*-β-Apo-8′-carotenal (IS, powder, ≥96%) were purchased from Sigma-Aldrich; lutein (Lut, powder, ≥95%), zeaxanthin (Zea, powder, ≥98), and β-cryptoxanthin (β-Cry, powder, ≥97%) were obtained from Extrasynthese (Genay, France); violaxanthin[2] (Vio, powder, ≥95%), (9*Z*)-β-carotene ((*9Z*)-β-Car, powder, ≥95%), phytoene (PTE, oily, ≥95%), and phytofluene (PTF, oily, ≥95%) were acquired from CaroteNature GmbH (Ostermundigen, Switzerland).

The penicillin/streptomycin mixture (Pen/Strep, Lonza), trypsin/EDTA solution (Lonza), Dulbecco's modified Eagle's medium (DMEM + GlutaMAX™, Gibco™), and heat-inactivated fetal bovine serum (FBS, Gibco) were acquired from Fisher Scientific (Illkirch, France). Non-essential amino acids (100x NEAA, Lonza) and Dulbecco's phosphate buffered saline (DPBS, Lonza) were purchased from VWR.

7.2.2. Food matrices

Whey protein isolate (WPI) was acquired from Pure Nutrition USA (95% purity, Oxnard, CA), and soy protein isolate (SPI) was obtained from Self Omninutrition® (≥90% purity, Stockholm, Sweden), while casein sodium salt (SC) from bovine milk (≥85% purity) and gelatin (GEL) from bovine skin (≥70% purity) were both

[1] 9′-*cis*-Neoxanthin
[2] *all-trans*-violaxanthin

purchased from Sigma-Aldrich. All protein solutions were prepared by dissolving 0, 3, 7.5, and 15 g/L of the final digestion volume, corresponding to approx. 0, 10, 25 and 50% of the recommended dietary allowance (RDA, approx. 60 g/d for human adults), respectively [35]. Cod fillet (average nutritional values per 100 g: 0.7 g fat / 0 g carbohydrates: / 17.8 g proteins / 0.1 g salt) and sliced turkey fillet (average nutritional values: 1.2 g fat / <0.5 g carbohydrates: / 25 g proteins / 0.13 g salt) were purchased from a local supermarket (Cora, Bertrange, Luxembourg). Pieces of fillet were cut and chopped as much as possible using a kitchen blender (Moulinette chopper, Moulinex). Aliquots containing 2.19 g of cod fillet or 1.56 g of turkey fillet on average were prepared, representing the equivalent of 50% RDA for total proteins (when digesting this in 25 ml and extrapolating this to 2 L of digestive fluid in the GI tract). Aliquots were placed in thermo-resistant glassware containing boiling water (≥ 75 ° C) until cooked (approximately 15 min.), and after being cooled down, they were flushed with argon and stored at -80°C until *in vitro* GI digestion.

Organic 100% carrot juice (Delhaize, other ingredients: 1% concentrate from lemon juice), 100% tomato juice (Delhaize, other ingredients: 0.5% salt), and frozen spinach (Iglo, other ingredients: 0.04% salt) were purchased from a local supermarket (Delhaize, Strassen, Luxembourg). Using a kitchen blender, the spinach was vigorously grinded. All tested matrices were aliquoted into portions of approx. 35 g in 50 mL Falcon tubes, flushed with argon and stored at -80°C until further analysis.

7.2.3. Simulation of gastro-intestinal digestion of carotenoids

In vitro simulated digestion was carried out according to the harmonized INFOGEST protocol [36], with some modification [33, 35]. As the matrices were either liquid or ground, and were low in complex carbohydrates, the oral phase was omitted. The gastric digestion procedure was carried out following the recommended ratio of matrix (protein solution + food matrix) to simulated gastric fluids of 50:50 (v/v). Each sample consisted of a freshly prepared pure protein solution with the desired concentration equivalent to 0, 10, 25 or 50% of the RDA for proteins, or previously thawed cod and turkey fillet aliquots at a concentration equivalent to 50% of the RDA for proteins, plus 4 g of plant food matrix (tomato juice, spinach, or carrot juice). The *in vitro* gastric passage was performed as reported earlier with a final volume of 13 mL [35]. Similarly, the simulated intestinal digestion was carried out by maintaining the ratio of chyme to simulated intestinal fluids of 50:50 (v/v). Again similar, the remaining steps of the intestinal digestion were performed in the same manner as previously described, with a final GI digestion volume of 26 mL, [35]. At the end of the simulated digestion, aliquots of 12 mL were removed from the digesta and centrifuged at 3300xg for 1h (4°C). Then, 5 mL were collected from the middle aqueous phase and filtered through a 0.2 um nylon membrane syringe filter. The filtered micellar fractions present in the aqueous phase were taken for the extraction of carotenoids [24], while aliquots of complete digesta samples were flushed with argon and stored at -80 °C for further analysis.

7.2.4. Cell culture and uptake experiments

Parental Caco-2 cells, originating from a human colorectal carcinoma [37] were obtained from LGC Standards (Molsheim, France, Art. No. ATCC-HTB-37). Caco-2 cells used in this study were between passage 66 and 75. The cell culture and uptake procedures were adapted from earlier studies [38, 39] and are briefly described below.

7.2.4.1. Cell culture

Caco-2 cells were grown in 75 cm^2 flasks (Nunclon™ Delta Surface, Denmark) at 37°C in an atmosphere of 5% CO_2 (Thermo Electron Corporation, Massachusetts, USA), in DMEM+GlutaMAX™ supplemented with 10% FBS, 1% of NE AA, and 1% of Pen/Strep (10,000 U/mL of each antibiotic). The medium was changed regularly until the flasks reached approximately 80% confluence [38]. Thereafter, cells were seeded in six well-plates (Nunclon™ Delta Surface, Denmark) at a density of $5*10^4$ cells/cm^2 for 15 days to allow a proper cell differentiation [38], similar as described in earlier trials where differentiation was followed visually and based on TEER experiments [40, 41].

7.2.4.2. Cellular uptake experiment

Medium was removed and cells were washed with PBS, and 3 mL of medium diluted complete digesta[3] (obtained at the end of GI

[3] Cytotoxicity tests based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant (LDH cytotoxicity assay, Roche, n° 11 644 793 001).

digestion, centrifuged for 2 min at 1000x*g* to remove larger particles) (digesta/DMEM; 1/8; v/v) were added before incubation for 4 h. The digesta was removed after incubation, and cells were washed with cold PBS and detached with medium as described earlier [39]. Cellular uptake experiments were performed in triplicates, which were pooled in a 15 mL Falcon tube before further extraction of carotenoids.

7.2.5. Extraction of carotenoids

The extraction procedure from plant food matrices was carried out from an aliquot of 4 g of each matrix, using successively hexane/acetone mixture (2/1, v/v), hexane and diethyl ether [42]. For the green leafy matrix, i.e. spinach, the extraction was preceded by a step of saponification with 1 mL of 30% aqueous KOH [42]. Similarly, the filtered micellar fraction was extracted using hexane/acetone mixture (2/1, v/v), and then repeated twice with only pure hexane [35].

Regarding the extraction of carotenoids from Caco-2 cells, the fresh pools of three replicates were centrifuged and the cells were resuspended in ice cold water, followed by a brief vortex and sonication in the dark [38]. Multiple extractions with hexane/acetone mixture (2/1,v/v), hexane, and diethyl ether were performed, all followed by a quick vortex and 2 min. sonication before centrifugation for 2 min. at 4000xg (4°C). An aliquot of all combined extracts was collected and dried under a stream of nitrogen (TurboVap LV from Biotage®, Uppsala, Sweden). The dried extracts were flushed with argon and stored at -80°C until analysis.

7.2.6. Quantification of carotenoids

The percentage of carotenoid micellization was used as a measure of bioaccessibility. Both the bioaccessibility and cellular uptake were expressed as the percentage of carotenoid concentration measured in the dried extracts following *in vitro* digestion and cellular uptake experiments, respectively, compared to the initial concentration measured in the original matrix.

7.2.6.1. Spectrophotometric measurements

As a first evaluation of the impact of the proteins studied (i.e. cod, GEL, SC, SPI, turkey, and WPI, at a concentration of 50% RDA) on carotenoid micellization, the total carotenoid concentration of the bioaccessible fraction was calculated by a method based on the mean absorption coefficients in hexane and mean absorption wavelength, and concentration was calculated as described by Biehler et *al*, 2010 [43].

7.2.6.2. HPLC analysis

For the analysis of carotenoid extracts from the bioaccessible fraction obtained following *in vitro* GI digestion of carotenoid-rich matrices in the absence or presence of selected protein types (GEL, SC, SPI, and WPI), the dried extracts were re-dissolved in a total volume of 500 µL of MTBE:MeOH (30/70, v/v). In order to enhance dissolution of more apolar carotenoids, first, 150 µL of cold MTBE were added to the extracts, and then 350 µL of MeOH, both steps were followed by a brief vortex and sonication. The Internal Standard (IS, *trans*-β-Apo-8′-

carotenal) was added to each sample with a final concentration of 200 ng/mL. The same procedure was applied to the dried extracts from food matrices, which were re-dissolved in a total volume of 6 mL of MTBE:MeOH (30/70, v/v). An aliquot of 80 µL was transferred into an HPLC amber vial (equipped with a glass insert). HPLC separation of carotenoids was carried out using an Agilent 1260 Infinity U-HPLC instrument (Agilent Technologies, Santa Clara, USA), equipped with a C30 reversed phase column (2.6 µm particle size, 100 mm length, 2.1 mm diameter, Thermo Fisher Scientific). The mobile phase consisted of water/MeOH (60/40, v/v) with 30 mM of ammonium acetate as eluent A and ACN:DCM (85/15, v/v) as eluent B. Elution gradient was as follows: 0 min. 42% B; 4 min. 48% B; 5 min. 52% B; 11 min. 52% B; 13 min. 75% B; 18 min. 90% B; 40 min. 90% B; 41 min. 42% B. The flow rate was fixed at 0.8 mL/min., the injection volume was 10 µL, and column temperature was 28^{o}C. Seven point-linear calibration curves were prepared with external standards for each compound, with concentrations ranging from 60 to 500 ng/mL. Peaks were integrated at 286 nm (PTE), 350 nm (PTF), 440 nm (NEO and VIO), 450 nm (ZEA, LUT, α-CAR, β-CAR, and (*9Z*)-β-CAR), 455 nm (β-CRY and IS), and at 470 nm (LYC). Carotenoids were detected with a diode array detector, and identified by comparing each carotenoid's retention time and absorption maxima [44], with those of the available standards.

7.2.6.3. LC-MS analysis

For the analysis of dry carotenoids residues obtained following cellular uptake experiments, the cellular extracts were reconstituted

in 1 mL of MeOH/DCM (60/40, v/v), vortexed thoroughly, and an aliquot of 50 µL was transferred into an amber vial (equipped with a glass insert) containing 5 ng of IS. Each sample of cellular extract was prepared and analyzed in triplicate (n=3). Working solutions with concentration levels ranging from 0.01 µg/L to 1 mg/L were prepared. In order to prepare matrix-matched calibration curves, twelve aliquots of untreated cellular extracts were resuspended in MeOH/DCM mixture (as for the samples). The injection volume was 2 µL, and carotenoid concentrations in the cellular extracts were determined according to external calibration curves. The analysis were performed with a Waters Acquity UPLC H-Class PLUS equipped with a BEH C_{18} column (100 mm, 2.1 mm ID, 1.7 µm particle size, Waters, Milford, MA) combined with a Waters Xevo TQ-S tandem mass spectrometer operating with electrospray ionization (Table S7.4). Similar as carried out by Bukowski et *al*. [45], the mobile phases were: MeOH/water (90/10, v/v) + 20 mM ammonium acetate (mobile phase A) and ACN/DCM/MeOH (70/20/10, v/v/v) + 20 mM ammonium acetate + 0.3% acetic acid (mobile phase B). The column temperature and sample compartment were 28°C and 20°C, respectively. The flow rate applied was set at 0.35 mL/min. The solvent gradient was as following: 1.6 min. of initial composition at 100% A, 1 min. of linear increase to 100% B, composition hold from 2.6 to 9 min. and 7 min. back to initial composition for next injection and equilibration. The parameters set for MS/MS acquisition were the following: capillary voltage at 2 kV, source and desolvation temperatures at 150°C and 650°C, respectively, cone gas flow at 150 L/h, desolvation gas flow at 1200 L/h, collision gas flow at 0.15 mL/min and nebulizer set at 6 bar.

7.2.7. Free fatty acid determination, surface tension and confocal laser scanning microscopy

Complete digesta aliquots stored at -80°C after GI digestion of spinach and carrot juice were used to evaluate lipid hydrolysis by measuring the amount of free fatty acid (FFA) release. This was determined by Cayman's Free Fatty Acid Fluorometric Assay (Cayman Chemical, Art. No. 700310, Ann Arbor, MI) according to the manufacturer's protocol.

Surface tension of digesta samples were determined by the weight-drop method as previously described [46].

Confocal imaging of emulsion structures was carried out after the gastric phase and at the end of the GI digestion, using the confocal laser scanning microscope (Zeiss LSM 880, Airyscam SR, Jena, Germany). Nile red and FITC were mixed into the aliquots of digesta for fat and protein staining, respectively, while carotenoids were visualized by their natural fluorescence. An argon 488 nm laser excited the fluorescent dyes while a 403 nm laser excited the carotenoids (β-carotene used as reference). The emitted light of the samples was collected as following: 450-490 for carotenoids, 500-540nm for proteins, and 590-650 nm for lipids. The resultant images were processed as described previously [35].

7.2.8. Statistical analyses and data treatment

Unless otherwise specified, all data is presented as mean ±SD. Linear mixed models were created to study the effects of added proteins or proteins from food matrices on the bioaccessibility and

cellular uptake of individual carotenoids, and independently the effect of various gastric lipases on carotenoid bioaccessibility. For this purpose, non-normally distributed data (following Q-Q plots and box plots) or data with unequal variances were log-transformed. Linear mixed models thus contained the amount of protein (0, 10, 25 or 50 % RDA), type of protein (WP, SP, GEL, SC, turkey and cod), type of matrix (tomato juice, carrot juice, spinach), free fatty acid release and surface tension as well as the type of carotenoid as fixed factors, while bioaccessibility or cellular uptake were the observed dependent factors. P-values below 0.05 (2-sided) were considered significant. Following Fisher-F-tests, all group wise comparisons were carried out by Fisher-protected LSD tests (3 groups) or Tukey's (>3 groups). Following significant interactions, models were re-run keeping one of the factors in the interaction constant in order to re-do all group-wise comparisons. Therefore, findings for pooled results (several conditions combined) are generally reported first, followed by individual comparisons. All analyses were carried out in SPSS (vs. 25, IBM statistics, Chicago, IL).

7.3. Results

7.3.1. Carotenoid patterns for each food matrices

Spinach contained the highest amount of total quantified carotenoids (15.4 mg/100g), followed by tomato juice (10.9 mg/100g) and carrot juice (7.6 mg/100g, Table 7.1). While carotenes (α-Car, β-Car, and Lyc) were the major carotenoids in tomato and carrot juices, representing 74% and 81% of total carotenoid content, respectively,

xanthophylls (Neo, Vio, Zea, Lut and β-Cry) represented approx. 74% of total quantified carotenoids in spinach. In juice matrices, the contribution of the colorless carotenoids phytoene and phytofluene to the total detected carotenoid content was approx. 26% in tomato juice, and approx. 18% in carrot juice, while their contribution was rather negligible in spinach (< 1% of total carotenoid content) (Table 7.1).

7.3.2. Influence of various proteins on the bioaccessibility of total carotenoids

Under control conditions (no added proteins), the overall bioaccessibility of carotenoids (all carotenoids combined) differed significantly between the food matrices (p < 0.001, Table S7.1). Following *in vitro* GI digestion, overall carotenoid micellization was higher from spinach (19.0 ±0.4%) than from carrot juice (12.1 ±0.3%) and tomato juice (2.3 ±0.3%).

Following linear mixed model analysis, the effect of proteins on the bioaccessibility of total carotenoids was statistically significant (p < 0.001), with highest carotenoid micellization in the presence of SC and GEL, followed by turkey, SPI and cod, and finally WPI.

Table 7.1. Carotenoid content (mg/100g) for each tested plant food matrix[1], as determined by HPLC.

Food matrix	Neo	Vio	Zea+Lut	β-Cry	PTF	PTE	α-Car	β-Car	Lyc	Total
Spinach	2.03 ± 0.1	3.29 ± 0.4	6.00 ± 0.5	0.12 ± 0.0	0.02 ± 0.0	0.07 ± 0.0	0.02 ± 0.0	3.67 ± 0.3	n.d.	15.4 ± 0.3
Tomato juice	n.d.	n.d.	0.04 ± 0.0	n.d.	1.15 ± 0.0	1.63 ± 0.1	0.02 ± 0.0	0.25 ± 0.1	7.79 ± 0.4	10.9 ± 0.2
Carrot juice	n.d.	n.d.	0.09 ± 0.0	n.d.	0.88 ± 0.2	0.49 ± 0.1	1.70 ± 0.1	4.40 ± 0.2	0.04 ± 0.0	7.6 ± 0.1

[1] Each value represents the mean ± SD of $n \geq 3$ replicates.
Neo: neoxanthin; Vio: violaxanthin; Zea+Lut: lutein+zeaxanthin; β-Cry: β-cryptoxanthin; PTF: phytofluene; PTE: phytoene; α-Car: α-carotene; β-Car: β-carotene; Lyc: lycopene. n.d.: not detectable.

Their addition during digestion at various concentrations had a significant influence compared to the control condition ($p < 0.001$, except at 10% RDA concentration). When investigating all proteins together, a higher bioaccessibility was found at a concentration of 25% RDA, while a slight but significant decrease of total carotenoid micellization was obtained following the addition of a concentration of 50% RDA. Regarding the matrices, the proteins increased carotenoid micellization in tomato juice (from 2.3 to 3.4%), while a slight decrease was observed in carrot juice (from 12.1 to 9.3%), and in spinach (from 19.0 to 16.9%).

More specifically, only co-digested turkey proteins improved in general the bioaccessibility of carotenoids from spinach compared to the control ($p < 0.05$), while other protein types significantly decreased it ($p < 0.001$, Figure 7.1). The opposite effect was found for digested tomato juice, i.e. turkey proteins had a negative effect on the bioaccessibility, and except for the neutral effect of cod proteins, all other types of proteins significantly improved overall carotenoid bioaccessibility compared to the control ($p < 0.001$, Figure 7.1). In carrot juice, a significant decrease was found in the presence of co-digested cod proteins, WPI, SPI ($p < 0.001$), and SC ($p < 0.05$, Figure 7.1), while turkey proteins and GEL had no effect.

Thus, the proteins seemed to improve the bioaccessibility of those matrices with an initially poor carotenoid micellization, such as tomato juice. In contrast, matrices with an originally rather higher carotenoid bioaccessibility, such as spinach and carrot juice, showed a more pronounced decrease in bioaccessibility when co-digested with proteins.

7.3.3. Influence of proteins on the bioaccessibility of individual carotenoids

7.3.3.1. General effects on different carotenoids

In the absence of proteins, the bioaccessibility of individual carotenoids in spinach followed the order: lutein+zeaxanthin > neoxanthin > β-carotene > violaxanthin, while their contributions to total bioaccessible carotenoids followed a similar order (Table 7.2).

For carotenoid species present in tomato juice, their bioaccessibility followed the order: lutein+zeaxanthin > β-carotene > phytoene > phytofluene > lycopene (Table 7.2). However, the contribution of each individual carotenoid in the micellar fraction differed compared to their micellization. In carrot juice, the bioaccessibility of individual carotenoids followed the order: lutein+zeaxanthin > phytoene > phytofluene > α-carotene > β-carotene (Table 7.2).

The effect of carotenoid species was statistically significant ($p < 0.001$), similar to that of the matrix ($p < 0.001$). In addition, a significant interaction between matrix type and carotenoid type was encountered ($p < 0.001$), thus the effects were further studied at individual carotenoids and food items.

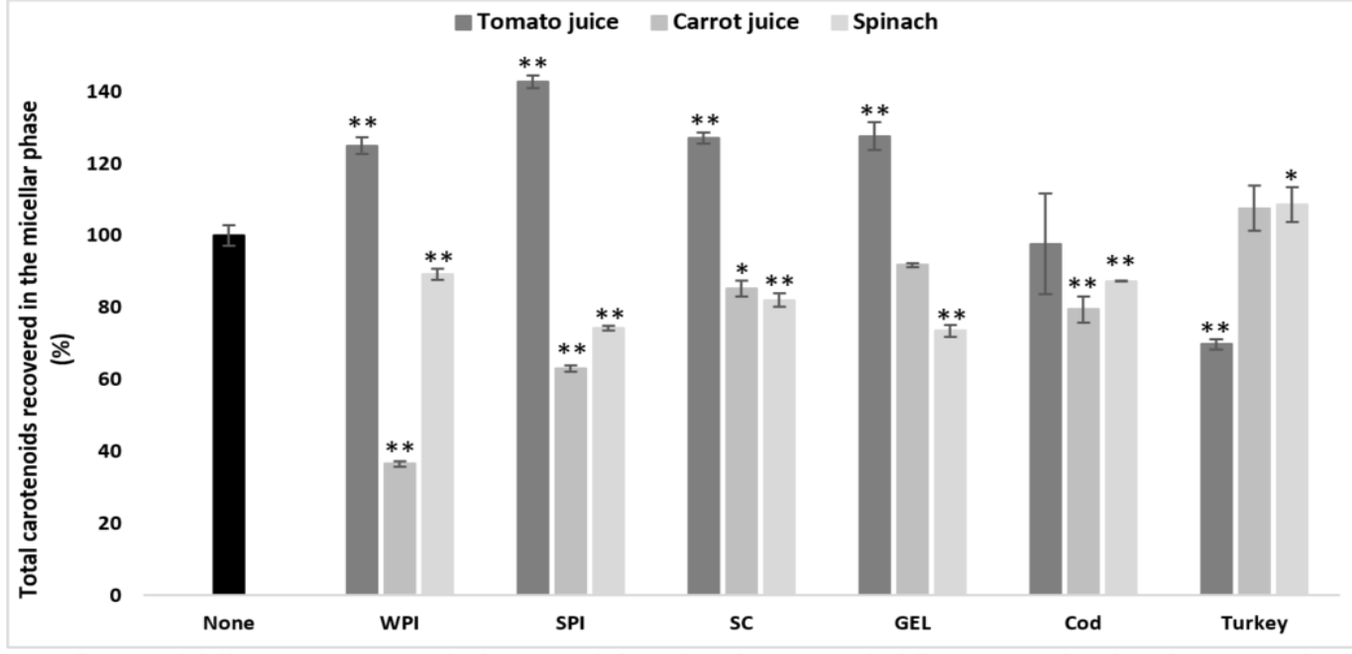


Figure 7.1. Influence of different proteins on the bioaccessibility of total carotenoids following simulated GI digestions of tomato juice, spinach, and carrot juice. Carotenoid rich-food matrices were digested in the absence or presence of different protein rich food items/supplements (Cod, turkey, WPI, SPI, SC, and GEL) at a concentration of 50% of protein recommended dietary allowance (RDA, approx. 60 g/d for human adults [35]). Bioaccessibility is expressed as the percentage of carotenoids recovered from the aqueous micellar fraction at the end of the GI digestion, compared to the amount present in each matrix at the beginning of digestion. Values represent means ± SD of $n \geq 4$. Bars represent means ± SD of $n \geq 4$ and N≥2 (sets repeated at different days). Columns labelled with either *($P < 0.05$) or **($P < 0.001$) were significantly different from the control condition (no added protein).

Table 7.2. Absolute amount[1] of carotenoids, bioaccessibility[2] as well as their relative contribution to the bioaccessible fraction[3] after simulated *in vitro* gastrointestinal digestion of each tested plant food matrices, as determined by HPLC. Values represent means of $n \geq 4$ replicates and N≥2 (sets repeated at different days).

Carotenoid	Spinach			Tomato juice			Carrot juice		
	Amount mg/100g	Bioacc. %	Contrib. %	Amount mg/100g	Bioacc. %	Contrib. %	Amount mg/100g	Bioacc. %	Contrib. %
Neo	0.4 (±0.0)	22.1 (±0.5)	15.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Vio	0.1 (±0.0)	2.8 (±0.1)	3.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Zea+Lut	2.2 (±0.1)	37.5 (±0.9)	77.3	0.03 (±0.0)	66.5 (±1.4)	10.6	0.1 (±0.0)	61.9 (±1.2)	6.1
PTF	n.d.	n.d.	n.d.	0.1 (±0.0)	5.1 (±0.1)	23.0	0.3 (±0.0)	35.6 (±0.8)	34.0
PTE	n.d.	n.d.	n.d.	0.1 (±0.0)	7.6 (±0.1)	48.6	0.3 (±0.0)	53.4 (±1.4)	28.5
α-Car	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1 (±0.0)	4.8 (±0.1)	8.9
β-Car	0.1 (±0.0)	3.3 (±0.1)	4.1	0.02 (±0.0)	8.0 (±0.2)	8.0	0.2 (±0.0)	4.7 (±0.1)	22.5
Lyc	n.d.	n.d.	n.d.	0.02 (±0.0)	0.3 (±0.0)	9.7	n.d.	n.d.	n.d.
Total car.	2.9 (±0.5)	19.0 (±0.4)	100	0.3 (±0.0)	2.3 (±0.3)	100	0.9 (±0.0)	12.1 (±0.3)	100

[1] Absolute amount of carotenoids after simulated *in vitro* gastrointestinal digestion of each tested plant food matrices in the obtained bioaccessible fractions.

[2] Bioaccessibility expressed as the percentage of carotenoids recovered from the aqueous micellar fraction at the end of the *in vitro* GI digestion, compared to the initial amount present in the undigested test meal.

[3] Values represent the contribution (%) of each individual carotenoid, relative to the total amount of carotenoids recovered from the aqueous micellar fraction after the *in vitro* GI digestion of each matrices.

Neo: neoxanthin; Vio: violaxanthin; Zea+Lut: lutein+zeaxanthin; PTF: phytofluene; PTE: phytoene; α-Car: α-carotene; β-Car: β-carotene; Lyc: lycopene. n.d.: not detectable.

7.3.3.2. Comparison across matrices- overall effects

To better compare the effects of proteins on carotenoid bioaccessibility across all tested matrices, β-carotene as well as lutein+zeaxanthin were chosen, as they were the only carotenoids present in all of the investigated matrices (Table 7.2). Likewise, further focus was placed on pure proteins regarding studying their influence on the bioaccessibility of β-carotene and lutein + zeaxanthin, as they showed a stronger impact compared to cod and turkey proteins on the bioaccessibility of total carotenoids.

In addition to the significant interaction between the food matrix and carotenoid species, the effects of protein type and concentration were also found significant ($p < 0.001$), and the interaction between all these effects was statistically significant ($p < 0.001$). Thus the effects on the bioaccessibility of lutein+zeaxanthin and β-carotene were further studied per protein and matrix. Of note, when studying all matrices combined, the micellization of lutein+zeaxanthin in the presence of proteins was higher than that of β-carotene ($p < 0.001$).

7.3.3.3. Comparison across matrices – lutein + zeaxanthin

The bioaccessibility of lutein+zeaxanthin differed significantly between the plant food matrices ($p < 0.001$), and was found higher from tomato juice than from carrot juice and from spinach (lowest micellization, Table S7.2). The presence of proteins had a negative effect on the micellization of lutein+zeaxanthin in all investigated

matrices (Figure 7.2). In the presence of SC (considering all protein concentrations combined), the bioaccessibility of lutein+zeaxanthin was higher compared to WPI ($p < 0.001$), with no significant effect with GEL, while lowest micellization of lutein+zeaxanthin was observed in the presence of SPI ($p<0.001$). Additionally, the co-digested proteins negatively influenced the bioaccessibility in a concentration-dependent manner ($p < 0.001$).

The co-digestion of WPI together with either spinach or tomato juice had no influence on lutein+zeaxanthin bioaccessibility, except for a slight decrease in spinach at 50% RDA ($p < 0.05$). However, its presence during the digestion of carrot juice reduced, in a concentration-dependent manner, the micellization of lutein+zeaxanthin ($p < 0.05$, Figure 7.2). Regarding the effect of SPI, a drastic decrease of lutein+zeaxanthin bioaccessibility in a concentration-dependent manner in all tested matrices was found ($p < 0.001$). The co-digestion of SC to the digestion of spinach resulted in a slight significant decrease of lutein+zeaxanthin micellization, while it had no influence when added to carrot juice or tomato juice (except for a slight increase at 50% RDA for tomato juice, $p < 0.05$). Regarding GEL, its presence had no influence on the bioaccessibility of lutein+zeaxanthin from tomato juice, while a significant decrease was found at higher concentrations (25 and 50% RDA) in spinach and carrot juice ($p < 0.001$).

7.3.3.4. Comparison across matrices – β-carotene

β-Carotene bioaccessibility also differed significantly between the investigated matrices ($p < 0.001$). Similar as for lutein+zeaxanthin, the bioaccessibility of β-carotene was higher from tomato juice compared to carrot juice, while being lowest for digested spinach (Table S7.2).

The effect of protein type significantly influenced β-carotene recovery in the micellar fraction ($p < 0.001$). The presence of SPI (considering all concentrations combined) resulted in highest β-carotene bioaccessibility, followed by SC, WPI, and finally GEL. While at 10% RDA proteins had no influence on β-carotene bioaccessibility, the addition of proteins at higher concentrations (25 and 50% RDA) resulted in an increased β-carotene micellization, compared to the control condition ($p < 0.001$). Regarding the different, matrices, the presence of proteins strongly improved β-carotene micellization in tomato juice, as well as in spinach, while the micellization of β-carotene from carrot juice was most negatively affected (Figure 7.2)

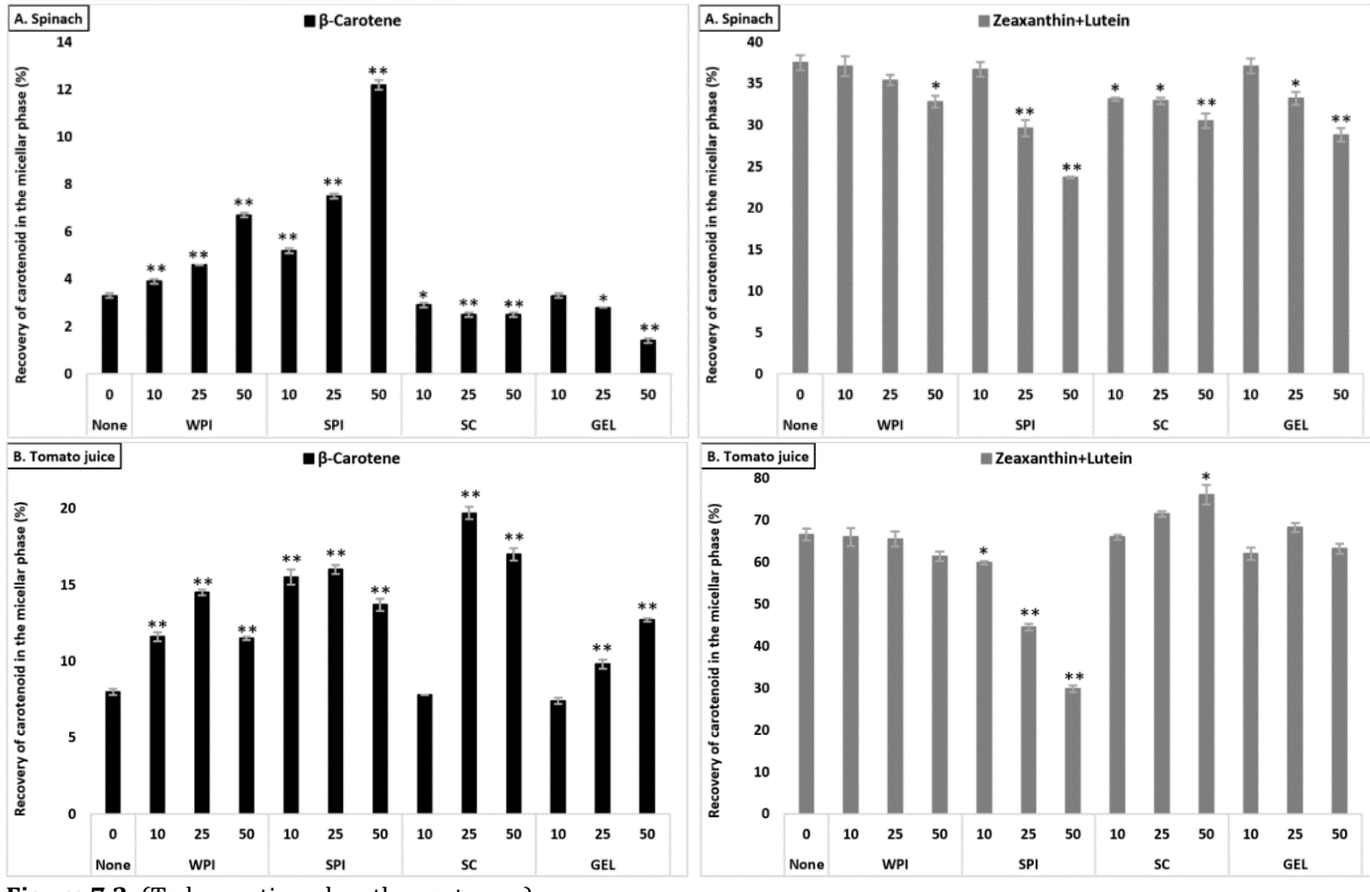


Figure 7.2. (To be continued on the next page).

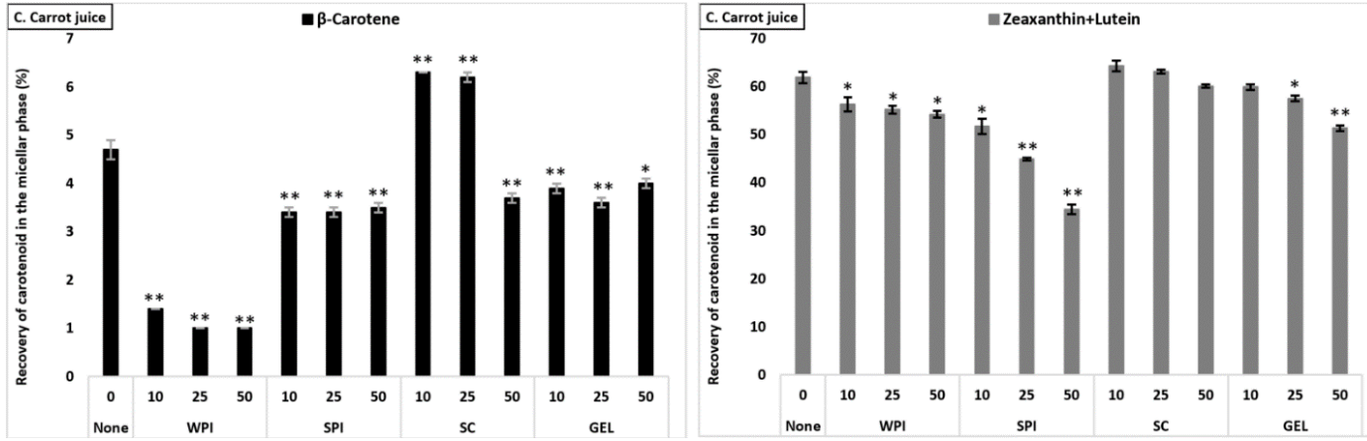


Figure 7.2. (Continued). Average bioaccessibility (%) of carotenoids following simulated GI digestions of spinach (A), tomato juice (B), and carrot juice (C). Each bar represents the bioaccessibility of either β-carotene or lutein+zeaxanthin recovered from the aqueous micellar fraction at the end of the *in vitro* GI digestion. Bioaccessibility is expressed as explained in Figure 7.1 heading. Values represent means ± SD of $n \geq 4$. Bars represent means ± SD of $n \geq 4$ and N≥2 (sets repeated at different days). Columns labelled with either *($P < 0.05$) or **($P < 0.001$) were significantly different from the control condition (no added protein).

The co-digestion of WPI with either spinach or tomato juice significantly increased β-carotene bioaccessibility ($p < 0.001$), while in carrot juice, the micellization significantly dropped ($p < 0.05$, Figure 7.2). Similarly, the presence of SPI during digestion positively affected the bioaccessibility of β-carotene from spinach and tomato juice ($p < 0.001$), while a slight decrease was found for carrot juice ($p < 0.001$). Adding SC significantly deceased β-carotene micellization in spinach, while a positive effect was found in tomato and carrot juice ($p < 0.001$), except at the highest concentration, where a slight decrease was found for carrot juice ($p < 0.001$). Regarding GEL, a negative effect on the bioaccessibility of β-carotene for spinach and carrot juice was observed. However, a concentration-depend increase was found when co-digested with tomato juice ($p < 0.001$, Figure 7.2).

Overall, changes regarding bioaccessibility were somewhat limited, especially for the xanthophylls (small relative decreases of ca. 1/10th), while up to almost a 3-fold increase was seen under certain conditions for carotenes.

7.3.4. Influence of various proteins on cellular uptake of total carotenoids

Under control conditions, fractional cellular uptake of total carotenoids (the sum of the individual carotenoids, compared to the original amount present in the matrix) differed significantly between the investigated matrices ($p < 0.001$, Table S7.3). Overall, carotenoid uptake was higher from spinach (1.3% ±0.08) than from tomato juice (0.7% ±0.03) and carrot juice (0.2% ±0.01).

The effect of proteins on the cellular uptake of total carotenoids (considering different concentrations combined) was statistically significant ($p < 0.001$), with no significant difference between WPI, SPI and GEL, though SC resulted in higher total carotenoid uptake compared to other protein types ($p < 0.001$). The addition of various proteins and their concentrations resulted in an increased total carotenoid uptake, compared to the control condition (no added protein, $p < 0.001$), following the order 10>50>25% RDA. Similarly, cellular uptake of total carotenoids was significantly different between the investigated matrices in the presence of proteins ($p < 0.001$). Co-digested proteins appeared to influence more strongly tomato juice, with an increase from 0.7 to 1%, while a slight decrease of total carotenoid uptake from carrot juice and spinach was found (Table S7.3).

In spinach (considering all protein concentrations combined), co-digestion with GEL resulted in the highest cellular uptake of total carotenoids, followed by WPI and SC and SPI ($p < 0.001$). Similarly, in the digested carrot juice, the presence of GEL and SC resulted in a higher overall carotenoid uptake than SPI ($p < 0.05$), while WPI significantly reduced carotenoid uptake compared to other tested proteins ($p < 0.05$). In tomato juice, a significant difference was found between the tested proteins ($p < 0.001$), with higher uptake of total carotenoids in the presence of WPI, followed by SPI, GEL and SC.

Though cellular uptake was low overall, and effects of protein additions apparently limited, relative increases of up to almost 2-fold were seen under certain conditions, though also decreases to about half the original levels. However, the effect of protein on the uptake of

total carotenoids was comparable to the bioaccessibility, with an overall positive effect on carotenoids in tomato juice, while a negative influence was observed for especially carrot juice, and also for spinach.

7.3.5. Influence of proteins on cellular uptake of individual carotenoids

7.3.5.1. General effects on different carotenoids

Under control conditions, fractional cellular uptake of individual carotenoids in spinach followed the order: lutein+zeaxanthin > β-carotene > neoxanthin+violaxanthin > β-cryptoxanthin, while their contributions to total amounts taken up differed (Table 7.3). For carotenoids present in tomato juice, their fractional cellular uptake followed the order: lutein+zeaxanthin > β-carotene > α-carotene > phytoene > phytofluene > lycopene. (Table 7.3). In carrot juice, the cellular uptake (%) of individual carotenoids followed the order: lutein+zeaxanthin > phytofluene > β-carotene > α-carotene > lycopene (Table 7.3).

The effect of matrix as well as carotenoid species were statistically significant ($p < 0.001$), thus the effects were further studied at individual carotenoid species and food items.

7.3.5.2. Comparison across matrices- overall effects

Again, lutein+zeaxanthin as well as β-carotene were selected to better compare the effects of proteins at various concentrations on carotenoid cellular uptake across all tested matrices (Table 7.3).

The interaction between protein type, protein concentration, carotenoid species, and matrix type, was statistically significant ($p < 0.001$), similar as reported for the bioaccessibility, thus the effects on the uptake of lutein+zeaxanthin and β-carotene were further studied for individual matrices and proteins. The cellular uptake of lutein+zeaxanthin in the presence of proteins was higher than that of β-carotene ($p < 0.001$, all matrices and protein concentrations considered).

7.3.5.3. Comparison across matrices – lutein + zeaxanthin

Under control conditions, a higher uptake of lutein+zeaxanthin was obtained from tomato juice, followed by carrot juice, and spinach, with a significant difference between all matrices ($p < 0.001$, Table 7.3). A slight difference was found between protein types regarding their influence on the cellular uptake of lutein+zeaxanthin, which followed the order: GEL > WPI > SPI > SC. In addition, 10 and 25% RDA protein addition significantly increased the uptake of lutein+zeaxanthin, up to 10.4%, compared to the control (7.7%, $p < 0.001$), while 50% RDA slightly decreased lutein+zeaxanthin uptake ($p < 0.05$).

Contrary to what was observed for bioaccessibility, the presence of proteins had a positive effect on the uptake of lutein+zeaxanthin in all investigated matrices, with the strongest increase in tomato juice (up to 22.0% cellular uptake), compared to the control (17.1%, Figure 7.3). Positive effects were more limited for carrot juice and lowest for spinach, in line with bioaccessibility showing least negative effects for tomato juice and strongest reductions for spinach.

Table 7.3. Absolute amount[1] of carotenoids, percentage of their cellular uptake[2], as well as their relative contribution to cellular uptake[3] to total carotenoids in the fractional cellular uptake phase of carotenoids into Caco-2 cells, as determined by LC-MS-MS. Values represent means of *n*=3 measurements.

Carotenoid	**Spinach**			**Tomato juice**			**Carrot juice**		
	Amount ng/3wells	CellUp. %	Contrib. %	Amount ng/3wells	CellUp. %	Contrib. %	Amount ng/3wells	CellUp. %	Contrib. %
Neo+Vio	14.1 (±0.9)	0.5 (±0.0)	12.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Zea+Lut	87.3 (±5.6)	2.5 (±0.2)	77.3	4.0 (±0.2)	17.1 (±0.8)	9.8	1.9 (±0.0)	3.6 (±0.1)	28.1
β-Cry	0.1 (±0.0)	0.05 (±0.0)	0.05	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PTF	n.d.	n.d.	n.d.	8.1 (±0.5)	1.2 (±0.1)	19.7	1.0 (±0.0)	0.2 (±0.0)	14.5
PTE	n.d.	n.d.	n.d.	13.4 (±0.8)	1.4 (±0.1)	32.6	n.d.	n.d.	n.d.
α-Car	0.1 (±0.0)	0.02 (±0.0)	n.d.	0.2 (±0.0)	1.8 (±0.1)	0.5	0.5 (±0.0)	0.05 (±0.0)	8.0
β-Car	11.5 (±0.7)	0.5 (±0.0)	10.2	11.2 (±0.6)	7.6 (±0.3)	27.3	3.3 (±0.1)	0.13 (±0.0)	49.2
Lyc	n.d.	n.d.	n.d.	4.2 (±0.2)	0.09 (±0.0)	10.2	0.01 (±0.0)	0.05 (±0.0)	0.2
Total car.	112.9 (±15.3)	1.3 (±0.1)	100	41.2 (±3.5)	0.7 (±0.0)	100	6.6 (±0.5)	0.2 (±0.0)	100

[1] Absolute amount of carotenoid cellular uptake from Caco-2 cells following simulated *in vitro* gastrointestinal digestion of each tested plant food matrices.

[2] Cellular uptake represents the fraction of carotenoid recovered in the Caco-2 cell fraction compared to the original matrix content, considering all dilution factors.

[3] Values represent the contribution (%) of each individual carotenoid, relative to the total amount of carotenoids taken up by the cells.

Neo+Vio: neoxanthin+violaxanthin; Zea+Lut: lutein+zeaxanthin; PTF: phytofluene; PTE: phytoene; α-Car: α-carotene; β-Car: β-carotene; Lyc: lycopene. n.d.: not detectable.

7.3.5.4. Comparison across matrices – β-carotene

Cellular uptake of β-carotene differed between the tested matrices ($p < 0.001$), with highest uptake from tomato juice, followed by spinach and carrot juice (Table 7.3). The investigated proteins influenced differently cellular uptake of β-carotene ($p < 0.001$, except in the presence of WPI and GEL), with a higher uptake observed in the presence of SPI, and followed by GEL, WPI and SC, when considering all protein concentrations combined. Their presence at various concentrations enhanced β-carotene uptake in a concentration-dependent manner, with an increase up to 6.0%, compared to the control (2.7%). The influence of proteins on the cellular uptake appeared to mimic the observed impact on the bioaccessibility of β-carotene, i.e. being positive in all investigated matrices, with the strongest increase in tomato juice (from 7.6 to ca. 12.5%, Figure 7.3).

Overall, co-digested proteins had a positive influence on cellular uptake, with a relative increase of up to 12% for xanthophylls, and up to 33% for carotenes (all matrices, proteins and concentrations considered).

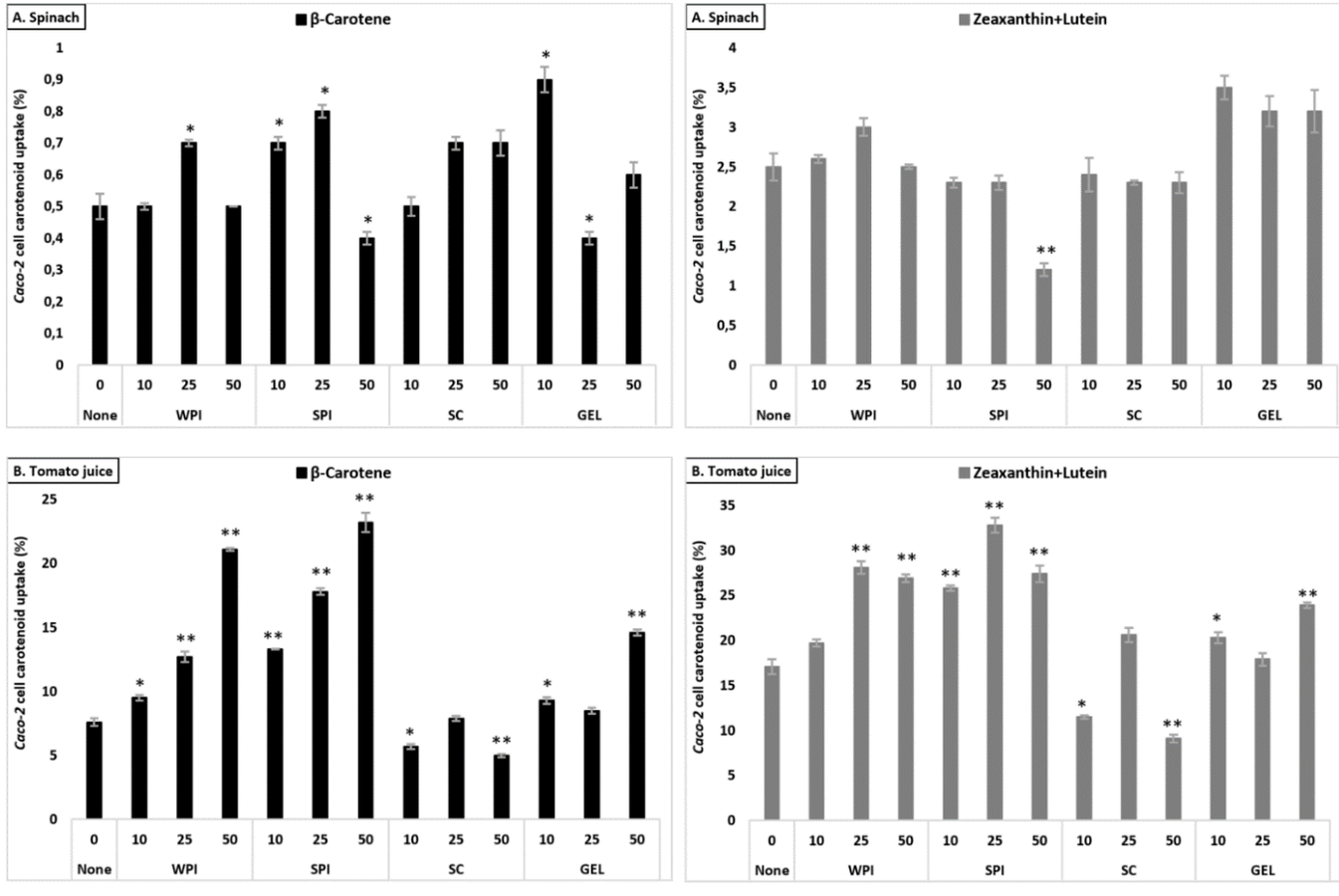


Figure 7.3. (To be continued on the next page).

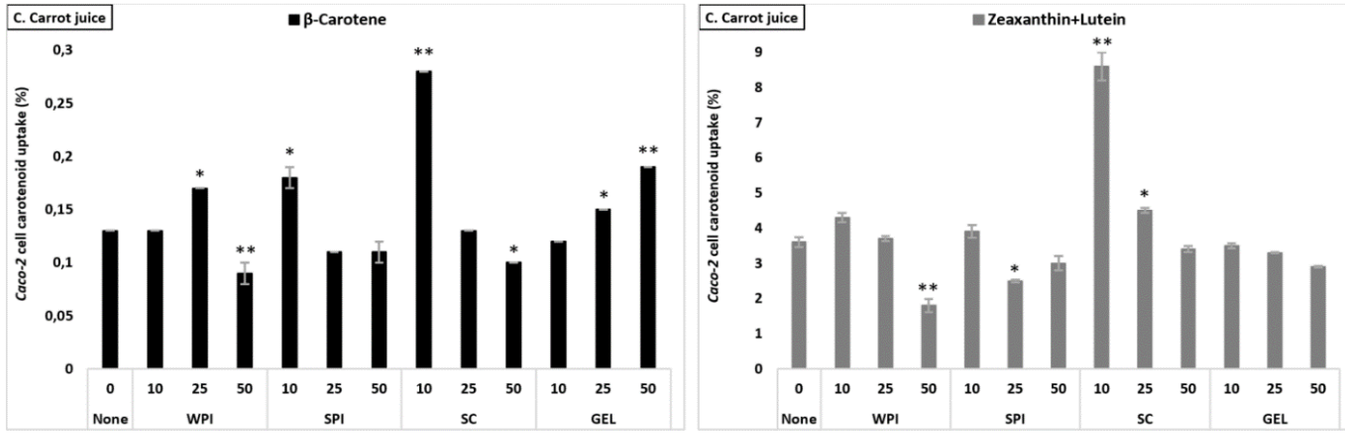


Figure 7.3. (Continued). Average cellular uptake (%) of carotenoids from spinach (A), tomato juice (B), and carrot juice (C). The investigated matrices were studied following *in vitro* GI digestion. Each bar represents the cellular uptake of either β-carotene or lutein+zeaxanthin recovered in the cell fraction compared to the original, matrix content (considering all dilutions). Bars represent means ± SD of n=3. Columns labelled with either *($P < 0.05$) or **($P < 0.001$) were significantly different from the control condition (no added protein).

7.3.6. Lipid analysis

Under control conditions, the release of FFAs differed depending on the type of the matrix ($p < 0.001$), and was higher in spinach (0.019 μM), compared to carrot juice (0.013 μM).

Adding proteins at various concentration to digestion differentially influenced FFA release ($p < 0.001$, Figure 7.4). Co-digestion of proteins with the investigated food matrices influenced differently lipid digestibility compared to controls ($p < 0.001$), with higher FFA release in the presence of SC, followed by WPI and SPI, and finally GEL. Additionally, their presence during the digestion influenced FFA release in a concentration-depended manner ($p < 0.001$), with a relative increase of up to 86% compared to the control condition. Similarly, the presence of proteins had a positive effect on lipid digestibility in all investigated matrices, with an average increase of up to 46%.

In spinach, a better lipid digestibility was obtained in the presence of SC, WPI and SPI, compared to GEL which had a negative influence on FFA release ($p < 0.001$). Globally, increasing protein concentrations slightly improved FFA release, with a significant effect at 50% RDA, compared to the control ($p < 0.001$). Co-digestion of WPI with spinach improved significantly lipid digestibility at all tested concentrations, while SC and SPI improved FFA release only at 50 % RDA ($p < 0.001$, Figure 7.4). However, GEL at 10 and 25% RDA significantly lowered the digestion of lipids ($p < 0.05$, Figure 7.4A).

In carrot juice however, a higher FFA release was obtained in the presence of SC, as well as by WPI and SPI, while GEL had a negative

impact ($P < 0.001$). Similar as found for spinach, increasing the concentration of proteins enhanced the release of FFAs during the digestion of carrot juice, being significant at 25 and 50% RDA ($p < 0.001$). The presence of SC in the digesta enhanced lipid digestibility in a concentration-depended manner ($p < 0.001$), with an almost 3-fold increase. For SPI and WPI, only higher concentrations significantly improved the release of FFAs ($p < 0.001$).

With less impact, the addition of GEL to the carrot juice digesta lowered the release of FFAs at a concentration of 10% RDA, while there was no significant influence at higher concentrations (Figure 7.4B).

Thus, proteins appeared to influence more strongly carrot juice, although slight decreases were observed in the presence of GEL.

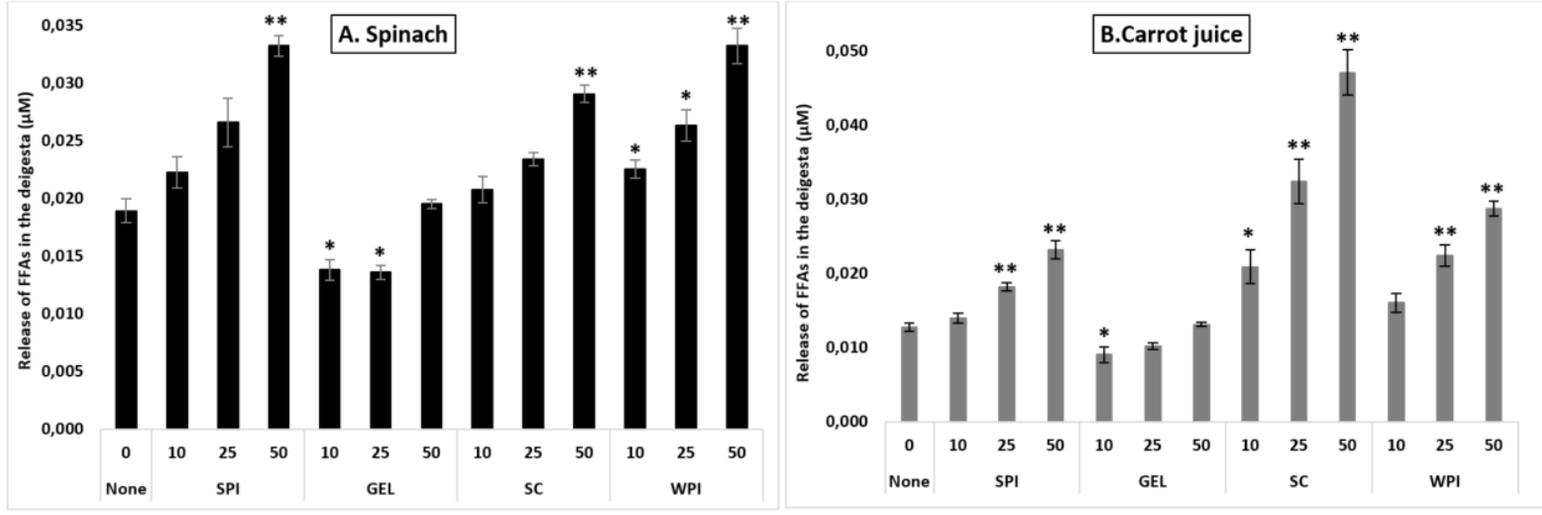


Figure 7.4. Influence of proteins on the release of free fatty acids (FFAs) during simulated GI digestion of spinach (panel A) and carrot juice (panel B). Carotenoid-rich matrices were co-digested in the presence of whey protein isolate, soy protein isolate, sodium caseinate or gelatin at various concentrations (0, 10, 25 and 50% of the recommended dietary allowance (RDA, approx. 60 g/d for human adults [35])), and the release of FFAs was evaluated at the end of GI digestion (Cayman's Free Fatty Acid Fluorometric Assay). Values represent means ± SD of n=4. Columns labelled with either *($P < 0.05$) or **($P < 0.001$) were significantly different from the control condition (no added protein).

7.3.7. Physicochemical characteristics of the digesta

7.3.7.1. Microscopic visualization

Microscopic visualization also revealed the extent of lipid digestibility through the confocal imaging of emulsion structures taken after gastric and GI digestion (Figure 7.5). The presence of proteins in the simulated digestion resulted in different emulsion structures, though all proteins remarkably appeared to reduce the size of the emulsified particles after complete GI digestion, compared to emulsions obtained in digestas collected after the gastric phase.

At concentrations of 25 and 50 % RDA, WPI and SPI showed a higher degree of aggregation compared to SC and GEL. This difference was even accentuated at the end of the gastric stage. A co-localization of the labelled compounds was observed in all digesta, indicating an interaction between carotenoids, lipids, and proteins during the *in vitro* GI digestion (Figure 7.5).

7.3.7.2. Surface tension

Although the overall effect of co-digested proteins was low and limited (an average decrease from 46.9 to 45.0 dyn/cm, Figure 7.6), the effect of added proteins apparently had more impact on the surface tension of digested spinach than liquid matrices (up to 8% relative decrease).

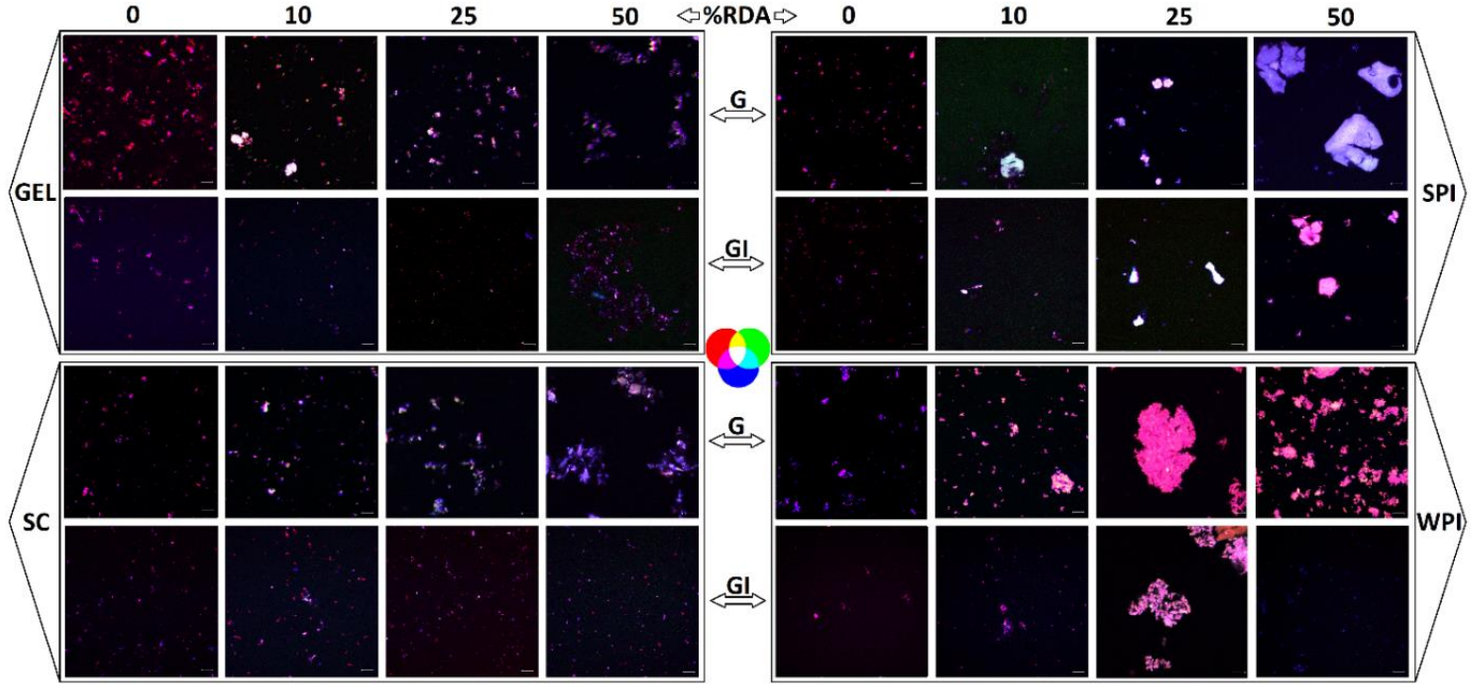


Figure 7.5. Confocal microscopy images taken after gastric (G) and gastrointestinal (GI) digestion with whey protein isolate (WPI), soy protein isolate (SPI), sodium caseinate (SC), and bovine gelatin (GEL). The grey bar is equal to 10 µm. Confocal imaging of emulsion structures was carried out at room temperature with a confocal laser scanning microscope (Zeiss LSM 880, Airyscam SR, Jena, Germany), using a 63x objective. The fluorescent dyes were excited by an Argon laser (488 nm) and the emitted light was collected at 522 nm for protein and 635nm for the fat phase. Lipids were labelled with Nile red (red colour) and proteins were labelled with fluorescent isothiocyanate (FITC, green colour), while carotenoids are naturally fluorescent (blue colour).

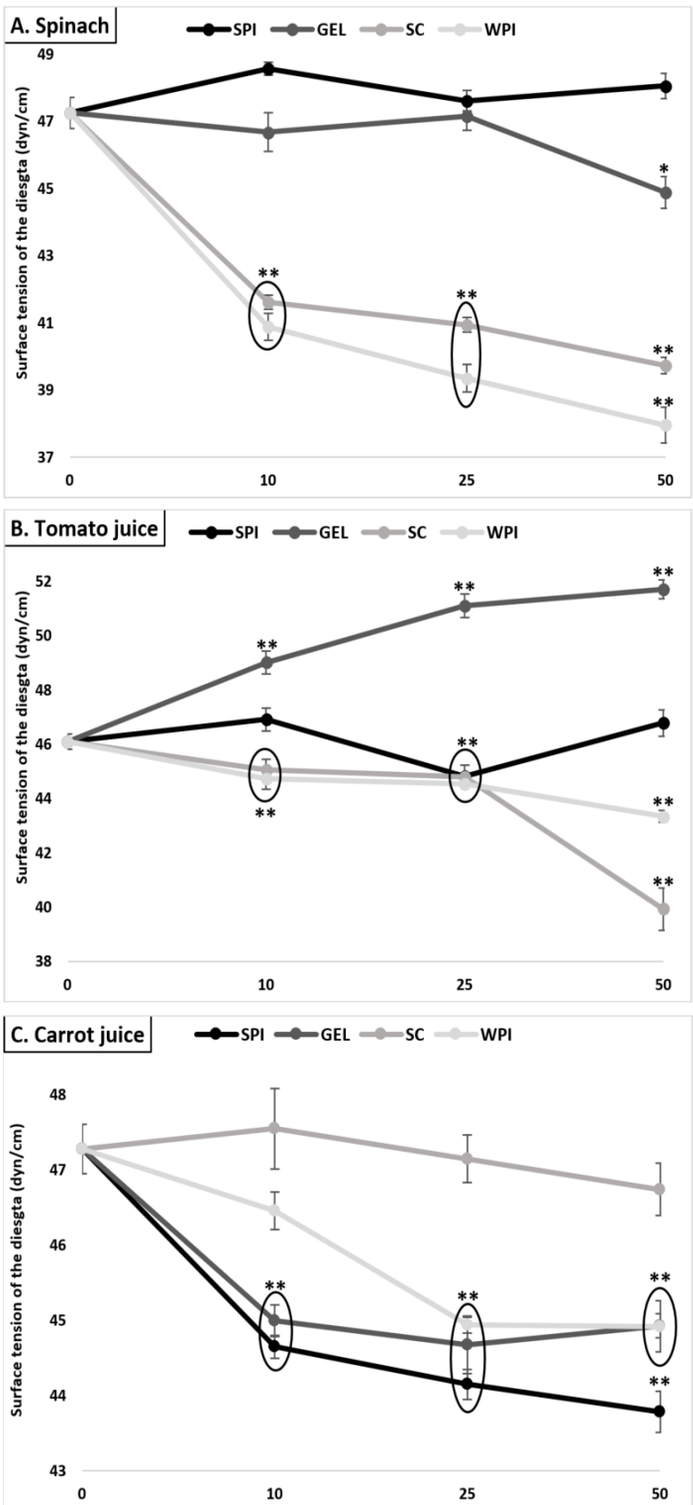


Figure 7.6. Effect of proteins on the surface tension of the digesta (value of pure water: 71.99 dyn/cm), following GI digestion of spinach (panel A), tomato juice (panel B), and carrot juice (panel C). Carotenoid-rich matrices were digested in the presence of whey protein isolate, soy protein isolate, sodium caseinate or gelatin at various concentrations (0, 10, 25 and 50% of the recommended dietary allowance (RDA, approx. 60 g/d for human adults [35])). The surface tension of digesta samples, pre-conditioned at 25 ± 0.1 °C, was determined by the weight-drop method. Values represent means ± SD of *n* = 4 replicates.

7.4. Discussion

In this study, we tested whether various frequently consumed types of proteins could interact with carotenoid bioaccessibility or cellular uptake. Indeed, especially discriminative interactions between xanthophylls and carotenes were found, with up to 3-fold improvement in carotene bioaccessibility but likewise a reduction of 1/10th in xanthophyll bioaccessibility. Such potential changes of bioavailability could be important toward assuring optimal intake of carotenoids, especially in sight of their disease-related aspects such as acting as direct or indirect antioxidants.

WPI, SPI, SC and GEL were co-digested with carotenoid-rich food matrices (tomato juice, carrot juice and spinach), following the INFOGEST model for *in vitro* GI digestion [36, 47]. When upscaled to a daily food intake, amounts were approx. equivalent to 0, 10, 25 and 50% of the recommended dietary allowance (RDA) of protein intake of adults. The food matrices represent regularly consumed fruits and vegetables, providing a wide range of carotenoids. It was found that the bioaccessibility of total carotenoids was highest from spinach, while being lowest for tomato juice. The investigated matrices differed in carotenoid content, i.e. carotenes represented the major quantified carotenoids in tomato and carrot juice (74 and 81%, respectively), while xanthophylls represented approx. 74% of total carotenoids in spinach. As carotenoid bioaccessibility has been inversely correlated with their hydrophobicity [48], the findings of highest bioaccessibility of polar xanthophylls (mostly lutein+zeaxanthin) from spinach vs. the

nonpolar carotenes in carrot juice (mostly β-carotene) and tomato juice (mostly lycopene) are not too surprising.

However, in addition to an effect on carotenoids, matrix effects were also registered, as lutein+zeaxanthin and β-carotene from liquid matrices represented better bioaccessibility compared to their homologues in spinach, following the sequence tomato juice> carrot juice > spinach. The physical state of the matrix in which carotenoids are incorporated likely affects aspect of release during digestion and hence their bioaccessibility [49]. Fiber content in spinach is known to limit carotenoid micellization [26], and carotenoids in spinach have been reported to be mostly present within chloroplasts [50], while carotenoids occur in a solid crystalline deposition form in both tomato and carrot chromoplasts [51]. Though the original carotenoid location has likely changed due to the more drastic heat treatment, some cells may remain intact even after juice processing [52]. It has been shown that carrots have small cell size paired with thicker, very fibrous and denser cell walls, compared to the very large cells and thin cell walls of tomato [53], which could be one of the reasons why tomato juice exhibited a higher total bioaccessibility than carrot juice, similar as observed earlier [54].

Regarding the influence of proteins, it has been acknowledged that proteins are predominantly adsorbed at the oil/water (o/w) interfaces via their hydrophobic segments, which are dissolved in the oil phase [55]. Surrounding the lipid droplets, proteins aid in emulsifying liposoluble dietary constituents [56] and enable the incorporation of non-polar components into emulsions during digestion [57]. In the present study, the presence of proteins improved

total carotenoid bioaccessibility of tomato juice, while decreasing that of spinach and carrot juice. We have previously reported these two diverging effects of proteins on the bioaccessibility of pure carotenoids during *in vitro* GI digestion [35]. While co-digested proteins enhanced the bioaccessibility of β-carotene by up to 50% compared to the protein-free control, it hampered lutein bioaccessibility. As the digested matrices differed greatly in their content and profile of carotenoids, this likely arbitrated their distribution in the lipid droplets, and thus the extent of micellization [58]. Borel et *al*. have reported that polar xanthophylls such as zeaxanthin were preferentially solubilized at the lipid droplet surface, while the apolar β-carotene was solubilized almost exclusively in the core of the lipid droplets [13]. This may lead to a negative interaction of the adsorbed proteins and xanthophylls at the o/w interface, especially in view of proteins often rearranging their molecular conformation to facilitate adsorption of hydrophobic groups at the interface in order to obtain an energetically favorably conformation [59]. Furthermore, only a limited amount of carotenoids can be solubilized within mixed micelles, due to the limited loading capacity [60]. This may explain, at least in part, why the presence of proteins during digestion could interfere with the trend of bioaccessibility, being inversely correlated with their aqueous solubility [48]. Therefore, it is proposed that on one hand, the high original bioaccessibility of xanthophylls was hampered by the presence of adsorbed proteins, limiting their incorporation into mixed micelles, while on the other hand proteins, via their emulsifying properties, enhanced the initially poorly micellized carotenes by increasing their solubilization and transition into the mixed micelles.

The co-digested proteins influenced differentially the bioaccessibility of carotenoids. All investigated proteins showed their ability to enhance lipid digestibility, while their emulsifying capability was somehow limited as illustrated by small changes in surface tension. Though all proteins appeared to reduce the size of the emulsified particles after complete GI digestion, the presence of proteins in the simulated digestion resulted in different emulsion structures. WPI and SPI showed a higher degree of aggregation compared to SC and GEL. In our previous work, we reported that the major protein fraction of WPI (i.e. β-lactoglobulin) remained intact only at the early stage of the intestinal digestion, while SPI digestibility was incomplete due to low-size polypeptide fragments remaining even following complete GI digestion [35]. The ensuing undigested peptides could have resulted in binding of carotenoids.

Indeed, the hydrophobicity of the investigated proteins may play a decisive role in the extent of carotenoid micellization. It has been reported that the solubility of SPI is low [52], leading to high surface hydrophobicity [61]. The reason has been attributed to the denaturation induced by heating, drying, and storage conditions [62, 63]. A recent study showed that β-carotene bioaccessibility was highly related to the type of proteins used to stabilize the emulsions [64]. SPI-based emulsions possessed the highest bioaccessibility compared to compared WPI and SC, which were insufficient to stabilize the emulsions, leading to coalescence. Similarly, SPI was successfully used as a nano-carrier to enhance the stability and bioaccessibility of β-carotene in a concentration-dependent manner, through hydrophobic intermolecular interactions (i.e. van der Waals forces and hydrogen

bonds) occurring mainly in the hydrophobic sites of SPI [65, 66]. The high surface hydrophobic nature and aggregated state were suggested to impart SPI a key role to perform as nano-carriers for lipophilic compounds, including β-carotene. Once denatured by proteases during digestion, the structure of SPI will unfold and the hydrophobic clusters initially buried within the structure of proteins will be exposed, which may foster β-carotene-protein interactions. These findings are in line with our results, as highest β-carotene bioaccessibility was obtained when co-digested with SPI (up to 50% RDA), compared to other proteins, improving micellization by almost 3-fold (e.g. in spinach).

Regarding the bioaccessibility of lutein+zeaxanthin, in addition to their negative interactions that may occur at the interface with the adsorbed proteins, the more polar xanthophylls may also show low binding affinity to the hydrophobic sites of proteins, perhaps explaining the strong negative influence of proteins on lutein+zeaxanthin bioaccessibility, with co-digested proteins decreasing their micellization in a concentration-dependent manner ($p < 0.001$). Lowest micellization was observed in the presence of SPI. Conversely, the higher solubility of SC and WPI (also considering the complete hydrolysis of other protein fractions such as α-lactalbumin) may produce more peptides that have amphiphilic structure, and these may be more beneficial for the more polar carotenoids, facilitating their interactions with the micelles [67] and enhancing their bioaccessibility, in line with results from the present study.

Despite the standardized protein amounts, the effect of protein-rich food matrices (i.e. turkey and cod) on the bioaccessibility of total

carotenoids were smaller than pure proteins. A slightly higher bioaccessibility was obtained in the presence of turkey, compared to cod. It has been reported that isolated proteins have weaker network structures and more cleavage sites, and are hydrolyzed faster and to a greater extent than protein-rich foods [68]. Also, co-digested plant food matrices may have an additional impact on the protein network structure of the digested animal matrices, possibly by preventing enzymes from accessing proteins, and therefore reducing protein digestibility [68]. Additionally, a higher protein digestibility was found in turkey-rich meals (60% turkey and 40% poultry products) compared to fish meal (sardine), using casein as reference protein [69]. Thus, we can only speculate that the limited effect of rich-protein matrices on the bioaccessibility of carotenoids is probably due to the less complete or slowed-down protein digestion.

In line with bioaccessibility findings, fractional cellular uptake of total carotenoids from spinach was higher than from carrot and tomato juices. Also in this case, xanthophylls were taken up better compared to carotenes, reflecting bioaccessibility. A recent study evaluating the bioaccessibility and cellular uptake by Caco-2 cells of carotenoids from orange peels reported the same findings [70], i.e. the bioaccessibility and cellular uptake of xanthophylls being significantly higher than that of carotenes. Sy et *al*, indicated that this may be due to the fact that xanthophylls reside at the surface of the mixed micelles, making them potentially more available for cellular uptake [48]. However, when comparing lutein+zeaxanthin and β-carotene fractional cellular uptake across matrices, it was highest from tomato juice and lowest for spinach (except for β-carotene where cellular

uptake was lowest from carrot juice). As reported earlier, we hypothesize that these differences are due to differences in the respective carotenoid content between the matrices, and that higher absolute amounts are taken up at a lower percentage, as realized in the present study with the main carotenoid in spinach (β-carotene, cellular uptake 0.13% as opposed from 7.6% from tomato juice), and lutein/zeaxanthin from spinach (cellular uptake 2.5% as opposed to 17.1% in tomato juice), perhaps pointing out to saturation processes including involved transporters for carotenoids, i.e. NPC1L1 for lutein/zeaxanthin and SR-B1 and CD36 for β-carotene[17]. Such transporters could also explain why fractional cellular uptake differed less drastically between lutein+zeaxanthin vs. β-carotene than the bioaccessibility results, a finding that was earlier reported by Kaulmann et *al*. [38]. In their study, carotenes such as β-carotene showed indeed a better cellular uptake alone compared to xanthophylls such lutein+zeaxanthin.

Interestingly, somewhat contrarily to bioaccessibility, the presence of proteins had a positive effect on the Caco-2 cellular uptake of lutein+zeaxanthin (up to 12%), with the strongest increase for tomato juice, while a limited influence for carrot juice followed by spinach was found. The cellular uptake of carotenes appeared to well reflect the impact of proteins on the bioaccessibility of β-carotene, i.e. being positive in all investigated matrices (up to 33%), with the strongest increase in tomato juice, especially in the presence of SPI (50% RDA), with up to a 3-fold increase. Thus, despite the negative effect of co-digested proteins on the bioaccessibility of lutein+zeaxanthin, they were better taken up in the presence of

proteins. A recent study on astaxanthin-loaded emulsions using WPI as emulsifier indicated that protein-based emulsions had a higher potential for improving the cellular uptake of astaxanthin than free astaxanthin [71]. Similarly, for carotenes, Lu et *al.* investigated the bioaccessibility and cellular uptake of β-carotene loaded emulsions, and found that emulsifiers such as WPI and SC improved the cellular uptake by facilitating the interaction of micelles with Caco-2 cells [67].

Regarding the influence of different protein types on the cellular uptake of carotenoids, results are also aligned with bioaccessibility findings, apparently fostering β-carotene-protein interactions and therefore improving its cellular uptake. A higher cellular uptake of β-carotene was obtained in the presence of co-digested SPI, followed by WPI and GEL and finally SC. The high solubility of SC [35] was apparently playing a lesser role than the potentially more hydrophobic SPI digestion products. Contrarily, xanthophylls were better taken up in the presence of more soluble proteins, such as WPI, while the presence of SPI strongly reduced their cellular uptake.

7.5. Conclusions

The addition of proteins to the simulated GI digestion resulted in an improved fractional bioaccessibility in matrices containing higher amounts of nonpolar carotenes, compared to matrices rich in more polar xanthophylls. Proteins with a high surface hydrophobic nature such as SPI may foster β-carotene-protein interactions, and improved micellization close to 3-fold, while negative interactions

with xanthophylls at O/W interface may occur, limiting their incorporation into the mixed micelles. Similarly, co-digested proteins fostered the cellular uptake of carotenes (β-carotene, to up to 33% uptake), and counteracted the somewhat negative effect of proteins on xanthophyll bioaccessibility by improving zeaxanthin+lutein cellular uptake (up to 12%), especially in the presence of soluble WPI and SC, which may produce peptides that have more amphiphilic structure that can be more beneficial for the more polar carotenoids, facilitating the interactions at the surface of the mixed micelles, making them potentially more available for cellular uptake. Regarding proteins from regular food matrices, their effects were more limited regarding altering carotenoid bioaccessibility compared to matrix-free proteins. Additional work on protein-carotenoid interactions is warranted in sight of the potential health-associated benefits of this group of dietary antioxidants.

7.6. References

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7.7. Supplementary Materials

Table S7.1. The bioaccessibility of total carotenoids[1] from plant food matrices in the absence or presence of various proteins following *in vitro* gastrointestinal digestion. Tomato juice, spinach, and carrot juice were digested in the absence or presence of different protein-rich food items or supplements (Cod, turkey, WPI, SPI, SC, and GEL) at a concentration of 50% of protein recommended dietary allowance (RDA[2]). Values represent means ± SD of $n \geq 4$ replicates and N≥2 (sets repeated at different days). Values in a row represented without a common alphabetic letters differ significantly, $P < 0.05$.

	None (%)	Cod (%)	Turkey (%)	WPI (%)	SPI (%)	SC (%)	GEL (%)	P value[3]
Spinach	19,0 ±0.4^{A}	16.6 ±0.0^{B}	20.6 ±0.9^{C}	16.9 ±0.3^{B}	14.1 ±0.1^{D}	15.5 ±0.4^{B}	13.9 ±0.3^{D}	P<0.001
Tomato juice	2.3 ±0.3^{A}	2.3 ±0.3	1.6 ±0.0^{C}	2.9 ±0.1^{D}	3.3 ±0.0^{D}	3.0 ±0.0^{D}	3.0 ±0.1^{D}	P<0.001
Carrot juice	12.1 ±0.3^{A,B}	9.6 ±0.4^{C}	13.0 ±0.8^{B}	4.4 ±0.1^{D}	7.6 ±0.1^{E}	10.3 ±0.3^{C}	11.1 ±0.1^{A,C}	P<0.001

[1]Bioaccessibility is expressed as the percentage of total carotenoids recovered from the aqueous micellar fraction at the end of the *in vitro* GI digestion, compared to the amount present in each matrix at the beginning of digestion.
[2]RDA: corresponding to approx. 60 g/d for human adults [34].
[3]Fisher F-test followed by Bonferroni post-hoc tests.

Table S7.2. Average bioaccessibility[1] (± SD) of carotenoids from plant food matrices co-digested with different proteins at varying concentrations[2]. Values represent means of $n \geq 4$ replicates and N≥2 (sets repeated at different days). Values labelled with either *($P < 0.05$) or **($P < 0.001$) were significantly different from the control condition (no added protein).

			Carotenoid bioaccessibility (%)								
Matrix	Prot. type	Prot. conc. %[3]	Neo	Vio	Zea+Lut	PTF	PTE	α-Car	β-Car	Lyc	Tot. car.
Spinach	None	0	22,1 ±0.5	2,8 ±0.1	37,5 ±0.9	n.d.	n.d.	n.d.	3,3 ±0.1	n.d.	19,0 ±0.4
	WPI	10	18,8 ±0.4**	2.4 ±0.1*	37.1 ±1.2	n.d.	n.d.	n.d.	3.9 ±0.1**	n.d.	18.5 ±0.5
		25	18,0 ±0.4**	2,5 ±0.0*	35.4 ±0.6	n.d.	n.d.	n.d.	4.6 ±0.0**	n.d.	17,9 ±0.3
		50	15,1 ±0.4**	2,2 ±0.1**	32.8 ±0.7*	n.d.	n.d.	n.d.	6.7 ±0.1**	n.d.	16.9 ±0.3*
	SPI	10	24,0 ±0.5	3,2 ±0.1*	36,7 ±0.9	n.d.	n.d.	n.d.	5.2 ±0.1**	n.d.	19,5 ±0.5
		25	17,2 ±0.2**	2,4 ±0.0*	29,6 ±1.0**	n.d.	n.d.	n.d.	7,5 ±0.1**	n.d.	16,2 ±0.4**
		50	11,6 ±0.1**	1.8 ±0.1**	23.7 ±0.1**	n.d.	n.d.	n.d.	12,2 ±0.2**	n.d.	14.1 ±0.1**
	SC	10	23,5 ±0.5	3,0 ±0.1	33,1 ±0.2*	n.d.	n.d.	n.d.	2.9 ±0.1*	n.d.	17,5 ±0.1
		25	22,2 ±0.1	2,6 ±0.0	32,9 ±0.4*	n.d.	n.d.	n.d.	2,5 ±0.1**	n.d.	17,0 ±0.2*
		50	18,6 ±0.3**	2.6 ±0.1	30,5 ±0.9**	n.d.	n.d.	n.d.	2,5 ±0.1**	n.d.	15.5 ±0.4**
	GEL	10	22,1 ±0.6	2,8 ±0.1	37,1 ±0.9	n.d.	n.d.	n.d.	3.3 ±0.1	n.d.	18,9 ±0.4
		25	16,3 ±0.3**	2.7 ±0.0	33,2 ±0.8*	n.d.	n.d.	n.d.	2.8 ±0.0*	n.d.	16,4 ±0.3*
		50	13.2 ±0.2**	3,0 ±0.1	28,8 ±0.8**	n.d.	n.d.	n.d.	1.4 ±0.1**	n.d.	13.9 ±0.3**

Table S7.2. Average bioaccessibility[1] (± SD) of carotenoids from plant food matrices co-digested with different proteins at varying concentrations[2] (continued).

Matrix	Prot. type	Prot. conc. %[3]	Carotenoid bioaccessibility (%)								
			Neo	Vio	Zea+Lut	PTF	PTE	α-Car	β-Car	Lyc	Tot. car.
Tomato juice	None	0	n.d.	n.d.	66,5 ±1.4	5,1 ±0.1	7,6 ±0.1	n.d.	8,0 ±0.2	0,3 ±0.0	2.3 ±0.3
	WPI	10	n.d.	n.d.	65.9 ±2.1	8,6 ±0.2**	15,3 ±0.3**	n.d.	11,6 ±0.3**	0.5 ±0.0**	4.1 ±0.1**
		25	n.d.	n.d.	65,4 ±1.8	10,6 ±0.4**	18,6 ±0.4**	n.d.	14.5 ±0.2**	0.4 ±0.0**	4.8 ±0.1**
		50	n.d.	n.d.	61,3 ±1.1	6,1 ±0.2*	11,9 ±0.4**	n.d.	11.5 ±0.1**	n.d.	2.9 ±0.1**
	SPI	10	n.d.	n.d.	59.8 ±0.4*	11.0 ±0.3**	14.4 ±0.2**	n.d.	15.5 ±0.5**	0.6 ±0.0**	4.3 ±0.1**
		25	n.d.	n.d.	44.5 ±0.8**	11.9 ±0.1**	13.1 ±0.3**	n.d.	16.0 ±0.3**	0.5 ±0.0**	4.1 ±0.0**
		50	n.d.	n.d.	29.8 ±0.8**	8.8 ±0.1**	11.1 ±0.3**	n.d.	13.7 ±0.4**	0.4 ±0.0**	3.3 ±0.0**
	SC	10	n.d.	n.d.	65.9 ±0.6	5.0 ±0.1	7.4 ±0.1	n.d.	7.8 ±0.0	0.3 ±0.0	2.3 ±0.0
		25	n.d.	n.d.	71.4 ±0.7	11,0 ±0.3**	12.9 ±0.3**	n.d.	19,7 ±0.4**	0.5 ±0.0**	4.2 ±0.1**
		50	n.d.	n.d.	76,0 ±2.3*	6.9 ±0.2**	8,4 ±0.2*	n.d.	17,0 ±0.4**	0.4 ±0.0**	3.0 ±0.0**
	GEL	10	n.d.	n.d.	61.9 ±1.5	4.7 ±0.1	7.9 ±0.2	n.d.	7.4 ±0.2	0.2 ±0.0*	2.2 ±0.1
		25	n.d.	n.d.	68.2 ±1.1	6.0 ±0.2*	9.7 ±0.2*	n.d.	9.8 ±0.3**	0.3 0.0	2,8 ±0.0*
		50	n.d.	n.d.	63.1 ±1.2	6.2 ±0.1**	10.3 ±1.6**	n.d.	12,7 ±0.1**	0.3 ±0.0	3.0 ±0.1**

Table S7.2. Average bioaccessibility[1] (± SD) of carotenoids from plant food matrices co-digested with different proteins at varying concentrations[2] (continued).

Matrix	Prot. type	Prot. conc. %[3]	Carotenoid bioaccessibility (%)								
			Neo	Vio	Zea+Lut	PTF	PTE	α-Car	β-Car	Lyc	Tot. car.
Carrot juice	None	0	n.d.	n.d.	61,9 ±1.2	35,6 ±1.6	53,4 ±1.4	4,8 ±0.1	4,7 ±0.2	n.d.	12.1 ±0.3
	WPI	10	n.d.	n.d.	56,3 ±1.5*	19,4 ±0.6**	31,5 ±0.8**	1,8 ±0.1**	1,4 ±0.0**	n.d.	6,1 ±0.1**
		25	n.d.	n.d.	55,2 ±0.8*	16,2 ±0.4**	26,4 ±0.7**	1,5 ±0.0**	1,0 ±0.0**	n.d.	5,1 ±0.1**
		50	n.d.	n.d.	54,2 ±0.7*	12,1 ±0.2**	23,8 ±1.6**	1,2 ±0.0**	1,0 ±0.0**	n.d.	4.4 ±0.1**
	SPI	10	n.d.	n.d.	51,7 ±1.6*	18,8 ±0.4**	37,7 ±1.1**	2,6 ±0.1**	3,4 ±0.1**	n.d.	7,8 ±0.2**
		25	n.d.	n.d.	44,9 ±0.3**	18,7 ±0.3**	39,0 ±0.4**	2,6 ±0.1**	3,4 ±0.1**	n.d.	7,7 ±0.1**
		50	n.d.	n.d.	34,4 ±1.0**	18,3 ±0.1**	38,4 ±0.9**	2,6 ±0.0**	3,5 ±0.1**	n.d.	7.6 ±0.1**
	SC	10	n.d.	n.d.	64,3 ±1.1	45,3 ±1.1**	69,8 ±1.8**	6,1 ±0.0**	6,3 ±0.0**	n.d.	15,6 ±0.2**
		25	n.d.	n.d.	63,1 ±0.4	40,6 ±0.9*	68,6 ±2.1**	5,9 ±0.1**	6,2 ±0.1**	n.d.	14,8 ±0.2**
		50	n.d.	n.d.	60,1 ±0.4	29,6 ±1.0**	51,6 ±1.8	3,1 ±0.1**	3,7 ±0.1**	n.d.	10.3 ±0.3**
	GEL	10	n.d.	n.d.	59,9 ±0.6	27,4 ±0.8**	49,4 ±0.7	4,1 ±0.3*	3,9 ±0.1**	n.d.	10,2 ±0.2**
		25	n.d.	n.d.	57,5 ±0.6*	29,6 ±0.8**	48,9 ±1.3	4,2 ±0.3*	3,6 ±0.1**	n.d.	10,3 ±0.1**
		50	n.d.	n.d.	51,3 ±0.6**	34,2 ±0.5	51,0 ±0.4	4,1 ±0.1*	4,0 ±0.1*	n.d.	11.1 ±0.1*

[1]Bioaccessibility expressed as the percentage of carotenoids recovered from the aqueous micellar fraction at the end of the *in vitro* GI digestion, compared to the initial amount present in the undigested test meal.
[2]Concentrations given in final digestion volume are 0, 10, 25, and 50% based-RDA (approx. 60 g/d for human adults [34]).
[3]RDA: recommended dietary allowance; WPI: whey protein isolate; SPI: soy protein isolate; SC: sodium caseinate; GEL: gelatin; Neo: neoxanthin; Vio: violaxanthin; Zea+Lut: lutein+zeaxanthin; PTF: phytofluene; PTE: phytoene; α-Car: α-carotene; β-Car: β-carotene; Lyc: lycopene. n.d.: not detectable.

Table S7.3. Average cellular uptake[1] (%, ±SD) of carotenoids from **spinach** co-digested with different proteins at varying concentrations[2]. Values represent means of *n*=3 replicates. Values labelled with *(*P* < 0.05) were significantly different from the control condition (no added protein).

Prot. type	Prot. conc.%[3]	Neo+Vio	Zea+Lut	β-Cry	PT F	PT E	α-Car	β-Car	Lyc	Tot. car.
None	0	0.5 ±0.03	2.5 ±0.17	0.05 ±0.0	n.d.	n.d.	0.02 ±0.0	0.5 ±0.04	n.d.	1,3 ±0,08
WPI	10	0.4 ±0.0	2.6 ±0.05	0.05 ±0.0	n.d.	n.d.	0.02 ±0.0	0.5 ±0.01	n.d.	1,3 ±0,02
	25	0.5 ±0.0	3.0 ±0.11	0.06 ±0.0	n.d.	n.d.	0.03 ±0.0	0.7 ±0.01	n.d.	1,5 ±0,04
	50	0.3 ±0.0*	2.5 ±0.03	0.05 ±0.0	n.d.	n.d.	0.02 ±0.0	0.5 ±0.0	n.d.	1,2 ±0,01
SPI	10	0.4 ±0.0	2.3 ±0.06	0.05 ±0.0	n.d.	n.d.	0.02 ±0.0	0.7 ±0.02*	n.d.	1,2 ±0,03
	25	0.3 ±0.0**	2.3 ±0.09	0.05 ±0.0	n.d.	n.d.	0.03 ±0.0	0.8 ±0.02*	n.d.	1,2 ±0,04
	50	0.1 ±0.0**	1.2 ±0.08**	0.04 ±0.0	n.d.	n.d.	0.02 ±0.0	0.4 ±0.02*	n.d.	0,6 ±0,04**
SC	10	0.5 ±0.03	2.4 ±0.21	0.05 ±0.0	n.d.	n.d.	0.02 ±0.0	0.5 ±0.03	n.d.	1,2 ±0,1
	25	0.3 ±0.01*	2.3 ±0.03	0.06 ±0.0	n.d.	n.d.	0.03 ±0.0	0.7 ±0.02	n.d.	1,2 ±0,02
	50	0.3 ±0.01*	2.3 ±0.13	0.06 ±0.0	n.d.	n.d.	0.03 ±0.0	0.7 ±0.04	n.d.	1,2 ±0,06
GEL	10	0.7 ±0.03*	3.5 ±0.15*	0.04 ±0.0	n.d.	n.d.	0.03 ±0.0	0.9 ±0.04*	n.d.	1,8 ±0,07*
	25	0.4 ±0.01	3.2 ±0.19	0.03 ±0.0*	n.d.	n.d.	0.02 ±0.0	0.4 ±0.02	n.d.	1,5 ±0,07
	50	0.3 ±0.02**	3.2 ±0.27	0.03 ±0.0*	n.d.	n.d.	0.02 ±0.0	0.6 ±0.04	n.d.	1,5 ±0,12

Table S7.3. Average cellular uptake[1] (%, ±SD) of carotenoids from **tomato juice** co-digested with different proteins at varying concentrations[2] (continued).

Prot. type	Prot. conc.%[3]	Neo+ Vio	Zea+Lut	β-Cry	PTF	PTE	α-Car	β-Car	Lyc	Tot. car.
None	0	n.d.	17.1 ±0.83	n.d.	1.2 ±0.06	1.4 ±0.09	1.8 ±0.08	7.6 ±0.3	0.09 ±0.01	0,7 ±0,03
WPI	10	n.d.	19.7 ±0.4	n.d.	1.5 ±0.05	1.7 ±0.09	2.3 ±0.05**	9.5 ±0.2*	0.3 ±0.0**	0,9 ±0,0*
	25	n.d.	28.1 ±0.7**	n.d.	2.1 ±0.07**	2.1 ±0.14	2.7 ±0.04**	12.7 ±0.4**	0.3 ±0.01**	1,1 ±0,04**
	50	n.d.	26.9 ±0.4**	n.d.	2.2 ±0.04**	3.3 ±0.11**	2.3 ±0.04**	21.1 ±0.1**	0.5 ±0.01**	1,5 ±0,04**
SPI	10	n.d.	25.8 ±0.3**	n.d.	2.3 ±0.03**	2.6 ±0.08**	2.0 ±0.03	13.3 ±0.03**	0.16 ±0.01**	1,2 ±0,02**
	25	n.d.	32.8 ±0.8**	n.d.	2.8 ±0.09**	3.3 ±0.04**	2.6 ±0.04**	17.8 ±0.25**	0.12 ±0.0**	1,4 ±0,02**
	50	n.d.	27.4 ±0.9**	n.d.	4.1 ±0.14**	4.1 ±0.31**	3.2 ±0.1**	23.2 ±0.75**	0.33 ±0.01**	1,9 ±0,08**
SC	10	n.d.	11.5 ±0.2*	n.d.	0.8 ±0.03*	1.0 ±0.07	1.6 ±0.07	5.7 ±0.2*	0.06 ±0.0*	0,5 ±0,02*
	25	n.d.	20.6 ±0.8	n.d.	1.7 ±0.13	1.5 ±0.18	1.6 ±0.08	7.9 ±0.2	0.08 ±0.0	0,7 ±0,05
	50	n.d.	9.1 ±0.4**	n.d.	1.0 ±0.06	1.0 ±0.1	1.6 ±0.08	5.0 ±0.13**	0.12 ±0.0	0,5 ±0,02
GEL	10	n.d.	20.3 ±0.6*	n.d.	1.3 ±0.03	1.4 ±0.17	2.3 ±0.06**	9.3 ±0.26*	0.06 ±0.0**	0,7 ±0,03
	25	n.d.	17.9 ±0.7	n.d.	1.1 ±0.03	1.3 ±0.08	2.1 ±0.03*	8.5 ±0.25	0.08 ±0.0	0,6 ±0,02
	50	n.d.	23.9 ±0.3**	n.d.	2.7 ±0.09**	3.3 ±0.28**	3.1 ±0.05**	14.6 ±0.24**	0.15 ±0.0**	1,3 ±0,04**

Table S7.3. Average cellular uptake[1] (%, ±SD) of carotenoids from **carrot juice** co-digested with different proteins at varying concentrations[2] (continued).

Prot. type	Prot. conc.%[3]	Neo +Vio	Zea+Lut	β-Cry	PTF	PTE	α-Car	β-Car	Lyc	Tot. car.
None	0	n.d.	3.6 ±0.14	n.d.	0.2 ±0.05	n.d.	0.05 ±0.0	0.13 ±0.0	0.05 ±0.01	0,2 ±0,0
WPI	10	n.d.	4.3 ±0.13	n.d.	0.2 ±0.01	n.d.	0.05 ±0.0	0.13 ±0.0	0.03 ±0.0	0,2 ±0,0
	25	n.d.	3.7 ±0.07	n.d.	0.2 ±0.01	n.d.	0.08 ±0.0	0.17 ±0.0*	0.03 ±0.0	0,2 ±0,0
	50	n.d.	1.8 ±0.19**	n.d.	0.01 ±0.01**	n.d.	0.05 ±0.01	0.09 ±0.01**	0.16 ±0.13	0,09 ±0,01*
SPI	10	n.d.	3.9 ±0.18	n.d.	0.3 ±0.02*	n.d.	0.08 ±0.01*	0.18 ±0.01*	0.06 ±0.01	0,2 ±0,01
	25	n.d.	2.5 ±0.04*	n.d.	0.2 ±0.01	n.d.	0.05 ±0.0	0.11 ±0.0	0.06 ±0.01	0,12 ±0,0
	50	n.d.	3.0 ±0.2	n.d.	0.1 ±0.01	n.d.	0.05 ±0.0	0.11 ±0.01	0.07 ±0.01	0,13 ±0,01
SC	10	n.d.	8.6 ±0.4**	n.d.	0.7 ±0.04**	n.d.	0.11 ±0.01**	0.28 ±0.0**	0.09 ±0.01	0,4 ±0,02**
	25	n.d.	4.5 ±0.07*	n.d.	0.3 ±0.0**	n.d.	0.04 ±0.0*	0.13 ±0.0	0.07 ±0.01	0,2 ±0,0
	50	n.d.	3.4 ±0.09	n.d.	0.2 ±0.01	n.d.	0.04 ±0.0*	0.10 ±0.0*	0.14 ±0.08	0,14 ±0,0
GEL	10	n.d.	3.5 ±0.07	n.d.	0.2 ±0.01	n.d.	0.06 ±0.0	0.12 ±0.0	0.05 ±0.01	0,15 ±0,0
	25	n.d.	3.3 ±0.02	n.d.	0.2 ±0.02	n.d.	0.06 ±0.0	0.15 ±0.0*	0.05 ±0.0	0,16 ±0,0
	50	n.d.	2.9 ±0.02**	n.d.	0.2 ±0.01	n.d.	0.10 ±0.0**	0.19 ±0.0**	0.05 ±0.0	0,20 ±0,0**

[1]Cellular uptake represents the fraction of carotenoid recovered in the Caco-2 cell fraction compared to the original matrix content, considering all dilutions.

[2]Concentrations given in final digestion volume are 0, 10, 25, and 50% based-RDA (approx. 60 g/d for human adults [34]).

[3]RDA: recommended dietary allowance; WPI: whey protein isolate; SPI: soy protein isolate; SC: sodium caseinate; GEL: gelatin; Neo: neoxanthin; Vio: violaxanthin; Zea+Lut: lutein+zeaxanthin; PTF: phytofluene; PTE: phytoene; α-Car: α-carotene; β-Car: β-carotene; Lyc: lycopene. n.d.: not detectable.

Table S7.4. The MRM transitions scanned for detection of compounds of interest.

Compound	Retention time (min)	Parent ion	Quantification transition (Da)	CE (V)	Confirmation transition (Da)	CE (V)	Confirmation transition (Da)	CE (V)	Confirmation transition (Da)	CE (V)
Vio	2.26	$[M+H]^+$	601.6 > 221.1	19	601.6 > 583.2	9	601.6 > 491.3	17		
Neo	2.31	$[M+H]^+$	601.6 > 167.2	15	601.6 > 583.2	9	601.6 > 221.1	19		
Zea	3.88	$[M]^{+\bullet}$	568.5 > 476.2	12	568.5 > 550.4	11	568.5 > 338.4	15		
Lut	3.91	$[M]^{+\bullet}$	568.5 > 338.4	15	568.5> 476.2	12	568.5 > 430.3	15		
β-apo-Car	4.50	$[M]^{+\bullet}$	416.4 > 324.3							
β-Cry	4.90	$[M]^{+\bullet}$	552.4 > 460.2	13	552.4 > 537.1	17				
Lyc	5.41	$[M]^{+\bullet}$	536.5 > 444.2	9	536.5 > 467.3	11	536.5 > 375	13		
α-Car	6.22	$[M]^{+\bullet}$	536.5 > 444.2	13	536.5 > 321.1	15	536.5 > 413.3	13		
β-Car	6.34	$[M]^{+\bullet}$	536.5 > 444.2	13	536.5 > 346.3	13	536.5 > 399.3	14	536.5 > 281	18
(9Z)-β-Car	6.38	$[M]^{+\bullet}$	536.5 > 444.2	13	536.5 > 346.3	13	536.5 > 399.3	14	536.5 > 281	18
PTF	6.43	$[M]^{+\bullet}$	542.5 > 337.2	13	542.5 > 404.9	11				
PTE	6.54	$[M]^{+\bullet}$	544.5 > 81	35	544.5 > 450.3	8	544.5 > 339.2	12		

Chapter 8

Whey- and soy protein isolates added to a carrot-tomato juice alter carotenoid bioavailability in healthy adults

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Foreword

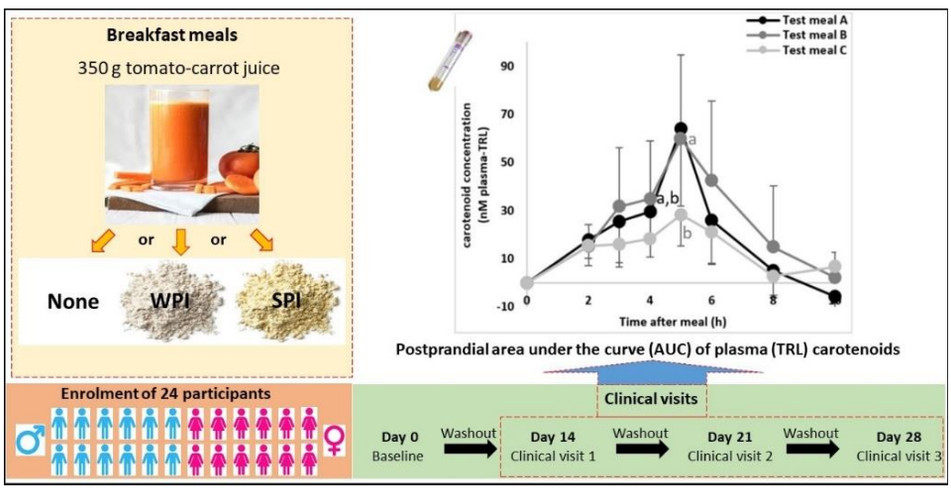
In the present crossover randomized human study, we aim to prove or disprove the hypothesis that proteins, and specifically WPI and SPI, can impair postprandial carotenoid bioavailability in healthy human adults. For this purpose, a carotenoid-rich test meal, i.e. a mixture of tomato and carrot juice, will be served in the absence or presence of proteins at a concentration equivalent to 50% of the RDA (≈ 60 g/d). Absorption kinetics of carotenoids will be evaluated by measuring the triacylglycerol-rich lipoprotein (TRL) fraction response, representing the newly absorbed carotenoids, at timed intervals up to 10 h after test meal intake.

Abstract

Recent findings suggested that proteins can differentially affect carotenoid bioaccessibility during gastro-intestinal digestion. In this crossover, randomized human trial, we aimed to confirm that proteins, specifically whey- and soy-protein isolates (WPI/SPI) impact postprandial carotenoid bioavailability. Healthy adults (n = 12 males, n = 12 females) were recruited. After 2-week washout periods, 350 g of a tomato-carrot juice mixture was served in the absence/presence of WPI or SPI (50% of the recommended dietary allowance, RDA ≈ 60 g/d). Absorption kinetics of carotenoids and triacylglycerols (TAGs) were evaluated via the triacylglycerol-rich lipoprotein (TRL) fraction response, at timed intervals up to 10 h after test meal intake, on three occasions separated by 1 week. Maximum TRL-carotenoid concentration (C_{max}) and corresponding time (T_{max}) were also determined. Considering both genders and carotenoids/TAGs combined, the estimated area under the curve (AUC) for WPI increased by 45% vs. the control (p = 0.018), to 92.0 ± 1.7 nmol × h/L and by 57% vs. SPI (p = 0.006). Test meal effect was significant in males (p = 0.036), but not in females (p = 0.189). In males, significant differences were found for phytoene (p = 0.026), phytofluene (p = 0.004), α-carotene (p = 0.034), and β-carotene (p = 0.031). C_{max} for total carotenoids (nmol/L ± SD) was positively influenced by WPI (135.4 ± 38.0), while significantly lowered by SPI (89.6 ± 17.3 nmol/L) vs. the control (119.6 ± 30.9, p < 0.001). T_{max} did not change. The results suggest that a well-digestible protein could enhance carotenoid bioavailability, whereas the less digestible SPI results in negative effects. This is, to our knowledge, the

first study finding effects of proteins on carotenoid absorption in humans.

Keywords: carotenoids; enzymes; micellization; plant vs. animal proteins; emulsification; xanthophylls.



Graphical abstract 8. Whey- and soy protein isolates added to a carrot-tomato juice alter carotenoid bioavailability in healthy adults.

8.1. Introduction

Carotenoids are lipid-soluble pigments produced by plants, bacteria, and certain fungi. These secondary plant compounds cannot be produced by the human body, but are present in the blood and tissues as they are taken up from a large variety of fruits and vegetables. These pigments have been associated in epidemiological studies with the reduced incidence of several chronic diseases [1], such as lowered risk of coronary heart diseases and type 2 diabetes [2], certain types of cancer [3], as well as age-related macular degeneration [4]. The possible role of carotenoids in disease prevention is far from fully understood. However, this may be attributed to their anti-inflammatory properties, but also to their antioxidant functions, where carotenoids are mostly involved in the scavenging of reactive oxygen species (ROS) such as singlet oxygen or peroxyl radicals, thanks to the number of conjugated double bonds present in the molecule, which determines to a large extent their antioxidant properties [5-7]. Carotenoids have also been shown to exert an influence on the cellular level, by acting on gene transcription [8]. Beyond these properties, certain carotenoids act as precursors of vitamin A and constitute a dietary source for many subjects not consuming sufficient animal-based foods, notably vegetarians/vegans, but also for many living in developing countries [9, 10].

These aspects stimulated interest in carotenoid absorption and metabolism, though the bioavailability of carotenoids is low and

variable due to the multiple factors that affect their release, absorption, transport, metabolism, and storage [11, 12]. The type of carotenoid species [13, 14], dietary aspects such as matrix, as well as host-related factors [15, 16] are known to interfere with these above-mentioned metabolic steps. The type of carotenoid species may play a role as they differ in their polarity, which could arbitrate the extent of micellization, also known as bioaccessibility. It has also been acknowledged that the food matrix from which they are absorbed plays a role. For instance, lipids can enhance carotenoid absorption efficiency, by improving their micellization and fostering chylomicron sequestration [17, 18], while dietary fibers could negatively affect their bioavailability, by hampering the transition of lipid droplets into mixed micelles and/or affecting the activity of digestion enzymes [19, 20]. Additionally, it has been hypothesized that co-consumed dietary proteins, being highly surface-active, adsorb to the lipid droplets and may aid in emulsifying apolar dietary constituents by modulating their transfer into mixed micelles [21]. During digestion, proteins have also been reported to have the potential to act as antioxidants. This may be increased by exposing their amino acids by disrupting their tertiary structures, which can inhibit lipid oxidation through multiple pathways, including the inactivation of ROS, scavenging free radicals, chelation of pro-oxidative transition metals, reduction of hydroperoxides, and alterations of the physical properties of food systems [22]. In our previous *in vitro* studies, we have shown that various proteins (whey protein isolate (WPI), soy protein isolate (SPI), sodium caseinate (SC), and gelatin (GEL)) had positive and negative effects on the bioaccessibility of carotenoids originating from different matrices such as carrot juice, tomato juice, and spinach, depending on

the type of carotenoids [23], but also on the type and concentration of proteins [14]. In fact, higher carotenoid polarity was associated with a stronger negative influence of proteins on their bioaccessibility, while hydrophobic carotenoids appeared in part to benefit from the presence of proteins regarding their micellization. As carotenoid micellization is assumed to be a key step for carotenoid bioavailability, we have suggested that if a similar interaction occurs *in vivo*, the presence of proteins may also influence carotenoid absorption efficiency.

In the present crossover randomized human study, we aimed to prove or disprove the hypothesis that proteins, and specifically WPI and SPI, can impair postprandial carotenoid bioavailability in healthy humans. For this purpose, a carotenoid-rich test meal, i.e., a mixture of tomato and carrot juice, was served in the absence or presence of proteins at a concentration equivalent to 50% of the recommended dietary allowance (RDA ≈ 60 g/d). Absorption kinetics of carotenoids were evaluated by measuring the triacylglycerol-rich lipoprotein (TRL) fraction response, representing the newly absorbed carotenoids, at timed intervals up to 10 h after test meal intake.

8.2. Materials and methods

8.2.1. Chemicals, carotenoid standards and food matrices

All chemicals were of analytical grade or superior. Methanol (MeOH), acetonitrile (ACN), dichloromethane (DCM), diethyl ether (DEE), hexane (HEX) were purchased from Carl Roth GmbH + Co. KG (Rotisol®, Karlsruhe, Germany), while methyl tert-butyl ether (MTBE)

and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and ammonium acetate purchased from Scientest Biokemix GmbH (Leese, Germany), ethanol, sodium chloride and anhydrous sodium sulfate from VWR (Leuven, Belgium). Ultrapure water (18 MΩ) was used throughout the study and was prepared with a purification system from Millipore (Brussels, Belgium).

Standards of all-trans-β-carotene (β-Car, powder, ≥97%, Art. No. 22040), all-trans-lycopene (Lyc, powder, ≥85%, Art. No. 75051), α-carotene (α-Car, powder, ≥97%, Art. No. 50887), neoxanthin (Neo, powder, ≥97%, Art. No. 72994), and trans-β-apo-8′-carotenal (internal standard (IS), powder, ≥96%, Art. No. 10810) were purchased from Sigma-Aldrich (St. Louis, MO, USA); lutein (Lut, powder, ≥95%, Art. No. 0306S), zeaxanthin (Zea, powder, ≥98%, Art. No. 0307S), and β-cryptoxanthin (β-Cry, powder, ≥97%, Art. No. 0317S) were obtained from Extrasynthese (Genay, France); violaxanthin (Vio, powder, ≥95%, Art. No. 0259), (9Z)-β-carotene ((9Z)-β-Car, powder, ≥95%, Art. No. 0003.1), phytoene (Pte, oily, ≥95%, Art. No. 0044), and phytofluene (Ptf, oily, ≥95%, Art. No. 0042) were acquired from Carotenature GmbH (Münsingen, Switzerland).

Tomato juice (Delhaize Le Lion, Belgium), organic carrot juice (Delhaize Le Lion, Belgium), Delhaize peanut oil (free of native carotenoids [24] and own blank examinations), butter (Carlsbourg, Luxembourg province, Belgium), cream cheese (La Vache qui rit, Suresnes, France), bread (Harrys, Boulogne-Billancourt, France), minced turkey fillet (Delhaize Le Lion, Belgium), Greek yogurt (Mevgal, Thessaloniki, Greece), apples (Golden, Delhaize Le Lion, Belgium), and

water (SPA Monopole, Spa, Belgium) were purchased from a local supermarket (Delhaize, Strassen, Luxembourg).

Soy protein isolate (SPI) was obtained from Self Omninutrition® (≥90% purity, Stockholm, Sweden), and whey protein isolate (WPI) was acquired from Pure Nutrition USA (≥90% purity, Oxnard, California, USA).

8.2.2. Participants

Twenty-four individuals (twelve men and twelve women) from or living in the region of the Grand Duchy of Luxembourg were recruited for this study[1]. Subjects were 20 to 50 years old, non-smokers (abstinent for at least 2 years), and body-mass index (BMI) < 30 kg/m^2 (Table 8.1).

Participants had no history of metabolic disease, malabsorption disorders, hyperlipidemia, hyper-glycaemia, food allergies/intolerances/special diets that are not compatible with test meals or washout periods, and were not (1) consuming regularly dietary supplements during the study, (2) eating more than 5 portions of fruits and vegetables per day, (3) taking any medication for chronic conditions or recent illness (e.g., antibiotics), (4) practicing daily intense physical activity (≥120 min). Vegan/vegetarian subjects were likewise excluded. The number of participants was based on a randomized block design, consisting of six blocks of four subjects each,

[1] Based on statistical sample size calculation, assuming a power of 80% and alpha level of 5%, considering an effect size of one (i.e. similar effect size than in intra-individual variability, e.g. 20%), and the overall design of the study (considering three test meals served within six sequences).

with each being served different sequences (A/B/C; A/C/B; B/A/C; B/C/A; C/A/B and C/B/A) of test meals (A = control; B = WPI; C = SPI) in order to cancel out any potential effect of sequence of meals (Figure 8.1).

The National Research Ethics Committee of Luxembourg (CNER) approved the present study (n ° 201710/04). Procedures followed were in accordance with the Declaration of Helsinki, and according to the ICH-GCP (international Conference on Harmonization-Good Clinical Practice) and with European General Data Protection Regulations (GDPR). The study has been registered under the reference number NCT04078646 on the website of clinicaltrials.gov.

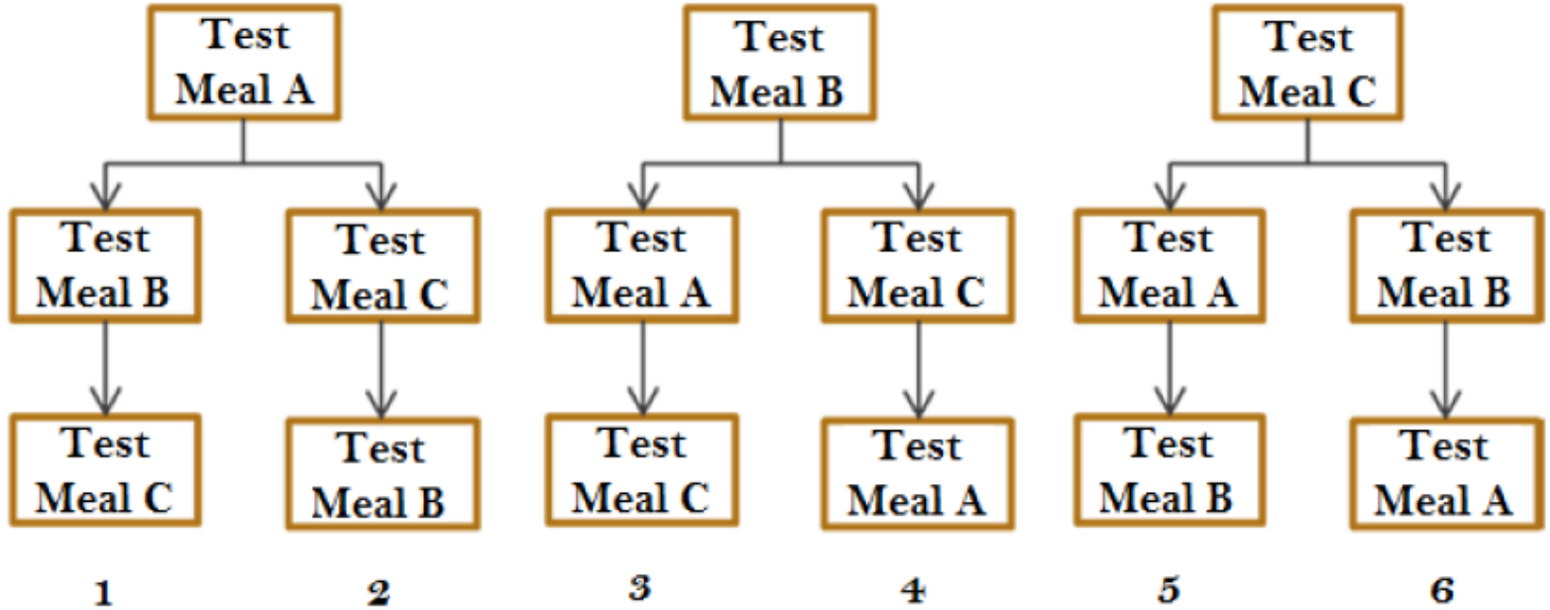


Figure 8.1. Scheme of breakfast meal sequences for each treatment pattern during the clinical trial phase. Meal A: control, no added protein; Test meal B: added WPI, Test meal C: added SPI. Six final test meal sequences were possible.

Table 8.1. Anthropometric characteristics and fasting blood biochemistry from the twenty-four participants, at the time of recruitment (mean values and standard deviations).

	Male (*n* = 12)			**Female (*n* = 12)**			**All participants (*n* = 24)**		
	Mean (SD)	**Max**	**Min**	**Mean (SD)**	**Max**	**Min**	**Mean (SD)**	**Max**	**Min**
				Anthropometric measures[1]					
Age (years)	30.9 (6.4)	46.0	22.0	29.7 (6.3)	42.0	22.0	30.3 (6.3)	46.0	22.0
BMI (kg/m^2)	25.1 (2.3)	28.7	19.7	22.8 (1.8)	25.7	18.9	23.9 (2.4)	28.7	18.9
Body fat (%)	18.4 (5.7)	27.5	8.9	31.5 (5.0)	38.4	22.9	24.9 (8.5)	38.4	8.9
Waist/hip ratio	0.9 (0.1)	1.0	0.8	0.8 (0.1)	0.9	0.5	0.9 (0.1)	1.0	0.5

[1] Measured using impedance method.
[2] Fasting blood levels.
[3] Calculated with the Friedewald equation.

Table 8.1. Anthropometric characteristics and fasting blood biochemistry from the twenty-four participants, at the time of recruitment (mean values and standard deviations) (continued).

	Male (*n* = 12)			**Female (*n* = 12)**			**All participants (*n* = 24)**		
	Mean (SD)	**Max**	**Min**	**Mean (SD)**	**Max**	**Min**	**Mean (SD)**	**Max**	**Min**
Blood biochemistry profile[2]									
Glucose (mg/dL)	96.6 (8.0)	108.0	82.0	87.9 (7.3)	106.0	79.0	92.3 (8.7)	108.0	79.0
Total cholesterol (mg/dL)	152.3 (31.2)	193.0	94.0	176.4 (36.3)	230.0	99.0	164.3 (35.3)	230.0	94.0
HDL-cholesterol (mg/dL)	48.5 (15.4)	88.0	32.0	67.3 (14.9)	95.0	48.0	57.9 (17.7)	95.0	32.0
Non-HDL-cholesterol (mg/dL)	103.6 (33.7)	161.0	43.0	109.1 (31.6)	152.0	37.0	106.3 (32.1)	161.0	37.0
LDL-cholesterol (mg/dL)[3]	95.1 (29.3)	144.0	43.0	106.5 (29.7)	143.0	32.0	100.8 (29.4)	144.0	32.0
TAG (mg/dL)	88.7 (44.7)	192.0	44.0	75.4 (29.9)	129.0	39.0	82.0 (37.8)	192.0	39.0

[1] Measured using impedance method.
[2] Fasting blood levels.
[3] Calculated with the Friedewald equation.

8.2.3. Study procedure

The main steps of the study included recruitment, an information session, a first screening visit, and the experimental parts.

8.2.3.1. Recruitment

The study was carried out at the Clinical and Epidemiological Investigation Center (CIEC) of the Luxembourg Institute of Health (LIH). Candidates were informed about the study by resorting to flyers, local radio and newspapers, e-mails, social media, "word of mouth" communication, and advertisement at the LIH website.

8.2.3.2. Information session

This appointment was meant to inform the participants about the trial, answer all questions that participants might have, and have participants sign the Informed Consent Form (ICF) and fill out our health and lifestyle questionnaire, which was used to determine the person's eligibility for this study.

8.2.3.3. Screening visit

Spot urine and blood samples were collected from the participants, and were meant to check the subjects' anemic status and analyze glucose levels as well as serum lipids such as triacylglycerols (TAGs) and cholesterol fractions.

8.2.3.4. Experimental design

Final eligible participants commenced the four-week trial phase, which included three washout periods, one short enrolment visit, and three full day clinical visits.

The enrolment visit (also termed baseline visit), took place at the beginning of the trial phase. A blood sample was collected to determine the baseline levels of TAGs and plasma carotenoids at the beginning of the trial, prior to the first washout phase.

The first washout period started on day 1 after the baseline visit and had a duration of two weeks during which the participants were asked to stay on a low carotenoid diet (i.e., to avoid the intake of colored fruits and vegetables), to reduce the basal levels of plasma carotenoids, before reporting to the CIEC for the 1st clinical visit (Figure 8.2). Depending on the test day, proteins (either SPI or a WPI) were or were not added to the breakfast meal. This first clinical full-day visit (detailed in the following paragraph) was followed by a third week of washout, during which the participant continued on a low carotenoid diet. At the end of the third week of washout, a second appointment at the CIEC took place. This was followed by the 4th week of washout period and at the end, the participant had the third and last appointment at the CIEC.

A list of foods to be avoided during the washout period, together with a list of alternative food items were given to each participant. The volunteers were asked to fill in a provided food journal, on a daily basis, where they wrote down the portions of what they had eaten

during the day. This was used to check compliance with the washout period and to better interpret later findings.

8.2.3.5. Clinical visit and postprandial experiments

Recruitment procedures resulted in the final inclusion of 12 men and 12 women. On clinical visit days, participants were asked to report to the CIEC's facilities, starting from 7:30 am, and a baseline blood sample (20 mL) was drawn ("0 h" time point). A carotenoid-rich test meal was then immediately served after the baseline blood draw (Table 8.2), and was composed of carrot and tomato juice mixture (1:1, *v/v*) as a rich source of carotenoids (Table 8.3), to which peanut oil was added. Depending on the test day, proteins (either SPI or a WPI) were or were not added to the carotenoid-rich meal. A toasted bread (white wheat) spread with butter and cream cheese, as well as water low in minerals were also served (Table 8.2). The entire test meal was consumed within 30 min, under supervision.

Post-prandial blood samples (20 mL each) were collected at timed intervals (before breakfast meal intake, i.e., hours 0, 2, 3, 4, 5, 6, 8, and 10 after breakfast meal intake) by a trained nurse from the forearm into EDTA-K2 and SSTTM II Advance tubes (BD Vacutainer®, Fisher Scientific, Illkirch, France), for carotenoid and TAGs analysis, respectively. Participants received a standardized lunch 4 h after breakfast meal intake, consisting of a toasted sandwich (white wheat bread), with turkey, some butter to spread on the bread, Greek yogurt, and a small apple. The participants had a courtesy meal at the end of the visits for dinner, i.e., 10 h after breakfast meal intake. No other foods or beverages except water (ad libitum) were allowed during the

day (including during breakfast and lunch if desired). The three breakfast meals were given to the participants in six different permutations (Figure 8.1).

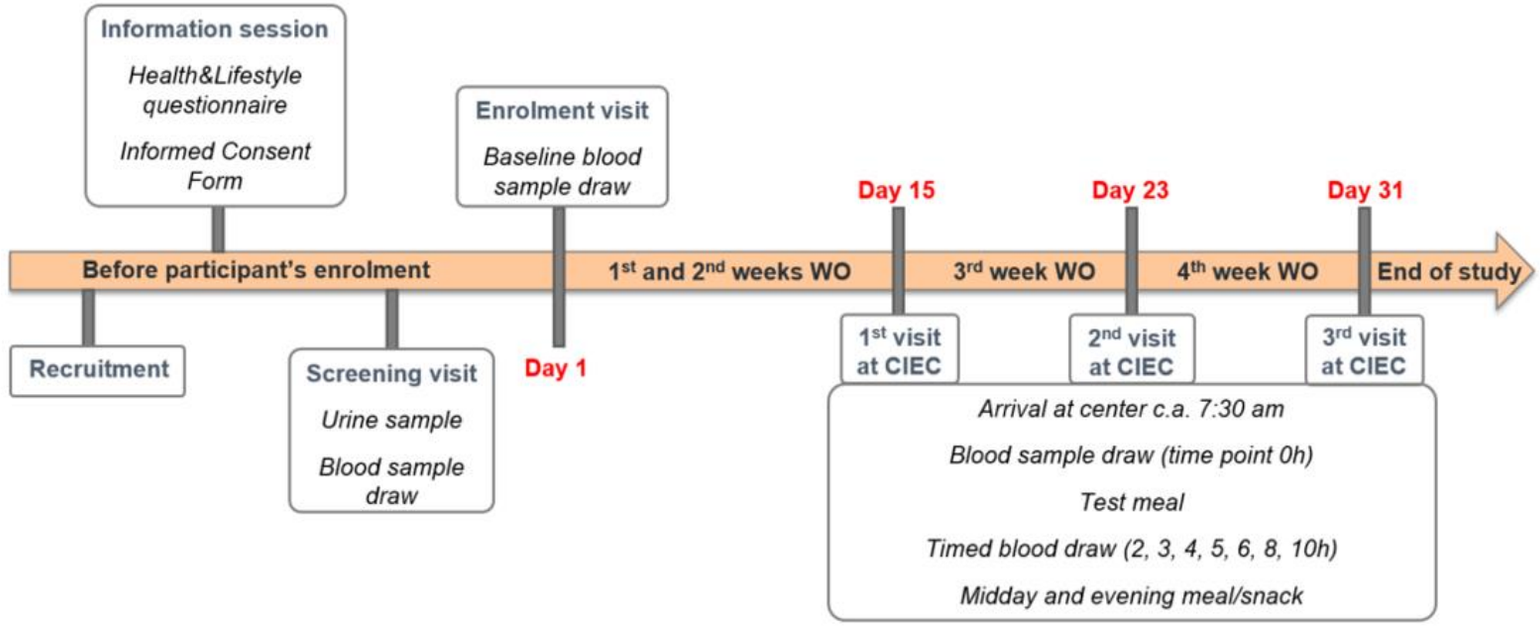


Figure 8.2. Representative scheme of the participants' schedule during the trial phase. WO: Washout.

Table 8.2. Composition of test meals.

Amounts		Ingredients	Nutritional values per serving
Breakfast meal (mornings)			
Mixture carrot juice/ tomato juice (1:1, *v/v*)	350 mL	99% carrot juice, 1% concentrate from lemon 99.5% tomato juice, 0.5% salt	Energy: 282 kJ / 67 kcal / Fat: 0.9 g, of which saturated 0.2 g / Carbohydrates: 13.5 g, of which sugars 13.5 g / Fibers: 1.6 g / Proteins: 2.5 g / Salt: 1.6 g
Peanut oil[1]	5 mL	Refined peanut oil 100%	Energy: 185 kJ / 45 kcal / Fat: 45 g, of which saturated: 0.85 g, mono-unsaturated: 3.3 g, polyunsaturated: 0.85 g
Proteins[1] Control (A)	0 g	n.a.[3]	n.a.[3]
WPI (B)	30 g	Whey protein isolate, emulsifier (soy lecithin)	Energy: 503 kJ / 119 kcal / Fat: 0.3 g, of which saturated 0.2 g / Carbohydrates: 0.5 g, of which sugars 0.5 g / Fibers: 1 g / Proteins: 28.0 g / Salt: 0.2 g
SPI (C)	30 g	Soy protein isolate, emulsifier (E322-sunflower)	Energy: 472 kJ / 113 kcal / Fat: 0.3 g, of which saturated 0.06 g / Carbohydrates: 0.3 g, of which sugars 0.03 g / Fibers: 0.3 g / Proteins: 27.2 g / Salt: 0.09 g
Toasted bread	40 g	Wheat flour (65%), water, sugar, colza oil, salt, vinegar, yeast, bean flour, wheat gluten, aroma (contains alcohol), acerola extract	Energy: 457 kJ / 108 kcal / Fat: 1.6 g, of which saturated 0.2 g / Carbohydrates: 19.6 g, of which sugars 3.0 g / Fibers: 1.6 g / Proteins: 3.0 g / Salt: 0.4 g
Butter[2]	12.5 g	Fat 82%	Energy: 385 kJ / 94 kcal / Fat: 10.3 g, of which saturated 7.1 g / Carbohydrates: 0.1 g, of which sugars 0.1 g / Proteins: 0.1 g / Salt: <0.1 g

Table 8.2. Composition of test meals (continued).

Amounts		Ingredients	Nutritional values per serving
Breakfast meal (mornings)			
Cream cheese[2]	19 g	Rehydrated skimmed milk, cheese, butter, milk mineral concentrate.	Energy: 172 kJ / 41 kcal / Fat: 3.3 g, of which saturated 2.2 g / Carbohydrates: 0.9 g, of which sugars 0.9 g /Proteins: 2.0 g /Salt: 0.32 g
Water	300 mL	Analysis (mg/L): Ca:5; Mg:2; Na:3; K:0.5; Cl:5; SO_4:4; NO_3:1.5; HCO_3:17; SiO_2:7	n.a.[3]
Total energy			
Control meal			**1281 kJ / 355 kcal**
WPI meal			**1759 kJ / 467 kcal**
SPI meal			**1753 kJ / 468 kcal**
Lunch (4 h after breakfast meal)			
Toasted bread	60 g		Energy: 686 kJ/163 kcal / Fat: 2.5 g, of which saturated 0.3 g / Carbohydrates: 29.3 g, of which sugars 4.6 g / Fibers: 2.3 g / Proteins: 4.6 g/ Salt: 0.7 g
Butter[2]	12.5 g	Fat 82%	Energy: 385 kJ/94 kcal / Fat: 10.3 g, of which saturated 7.1 g / Carbohydrates: 0.1 g, of which sugars 0.1 g / Proteins: 0.1 g / Salt: <0.1 g

Table 8.2. Composition of test meals (continued).

Amounts		Ingredients	Nutritional values per serving
Lunch (4 h after breakfast meal)			
Turkey[2]	60 g	Turkey fillet 90%, salt, dextrose, spices, acidity regulator (sodium lactate), stabilizers (sodium citrate, carrageenans), flavor, antioxidant (sodium ascorbate), preservative (sodium nitrite)	Energy: 247 kJ/58 kcal / Fat: 0.9 g, of which saturated 0.3 g / Carbohydrates: 0.5 g, of which sugars 0.4 g / Proteins: 12.0 g /Salt: 1.2 g
Greek yogurt	150 g	Pasteurized cow milk, milk cream, milk protein, yogurt culture	Energy: 804 kJ/194 kcal / Fat: 15 g, of which saturated 11.3 g / Carbohydrates: 5.3 g, of which sugars 5.3 g / Fibers: <0.5 g / Proteins: 8.3 g / Salt: 0.2 g
Apple	150 g	n.a.[3]	Energy: 321 kJ/77 kcal /Fat: >0.5 g, of which saturated >0.1g / Carbohydrates: 16.5 g, of which sugars 15.0 g /Fibers: 3.8 g / Proteins: <0.5 g / Salt: <0.01 g.
Water	300 mL	Analysis (mg/L): Ca:5; Mg:2; Na:3; K:0.5; Cl:5; SO_4:4; NO_3:1.5; HCO_3:17; SiO_2:7	n.a.[3]
Total energy			**2443kJ/586kcal**

[1] Dissolved/added to carrot and tomato mixture. [2] Spread on the toasted bread. [3] Not applicable.

Table 8.3. Carotenoid contents (mg/100 g) of the tomato and carrot juices[1], as determined by HPLC.

breakfast meal	Lut+Zea	Ptf	Pte	α-Car	β-Car	Lyc	Total
Tomato juice	0.04	1.15	1.63	0.02	0.25	7.79	10.9
Carrot juice	0.09	0.88	0.49	1.70	4.40	0.04	7.6
Juice mix 100 g	0.06	1.01	1.06	0.86	2.32	3.91	9.25
Juice mix 350 g	0.2	3.5	3.7	3.0	8.1	13.7	32.4

[1] Each value represents the mean of $n \geq 3$ replicates. Lut+Zea: lutein/zeaxanthin; β-Cry: β-cryptoxanthin; Ptf: phytofluene; Pte: phytoene; α-Car: α-carotene; β-Car: β-carotene; Lyc: lycopene.

8.2.4. Sample processing

The collected blood samples for carotenoid analysis were immediately centrifuged at 2000× *g* for 8 min, at 4 °C (Sigma 2-16KC centrifuge, Thermo Fisher Scientific Inc., Waltham, MA, USA). The obtained blood plasma was used for the separation of the TRL fraction rich in chylomicrons, as described earlier [25, 26]. Briefly, 2.5 mL of plasma was over-layered at room temperature with 2.2 mL of a 1.006 g/l NaCl solution in a 5 mL ultra-clear open-top tube (Beckman Coulter), and TRL fractions were separated at 180,000× *g* for 1 h at 24 °C in an Optima MAX-XP Ultracentrifuge (Beckman Coulter), using the high-capacity swinging-bucket MLS-50 rotor. Following the centrifugation, the bottom of the tubes was punctured with a heated needle to remove the lower plasma phase, and the upper TRL fraction (1–1.2 mL) was collected into 1.5 mL cryovials. The volume was brought up to 1.5 mL using 1.006 g/l NaCl by rinsing the ultra-clear tubes before storage at −80 °C. Likewise, plasma aliquots were stored

at −80 °C for carotenoid analyses in order to measure the effect of the washout periods.

Fasting blood glucose, lipids, and hematology as well as serum-TAG analyses were performed by a local external commercial laboratory (Laboratoires Réunis). The samples were centrifuged for 5 min at 3775x g, at 4 °C, and kept on site at 4 °C until pickup and analysis on the same day.

8.2.5. Extraction of carotenoids

The extraction of carotenoids from tomato and carrot juice samples was performed as described earlier [14, 27]. Blood plasma and plasma TRL fraction extraction protocols were adapted from Unlu et al. [26]. In short, 1.5 mL of TRL fraction or 1.0 mL of plasma were thawed and totally transferred into 15 mL Falcon tubes. A total 3.25 mL of ethanol containing 0.1% of butylated hydroxytoluene (BHT) were added to the samples, which were vortexed and spun down at 4 °C for 2 min, at 600× *g*, in order to collect the supernatant into a new tube. The precipitates were re-extracted with 3 mL of diethyl ether–hexane (1:2, *v/v*) containing 0.02% BHT, and centrifuged at 1250× *g* for 2 min, at 4 °C, and the organic upper phases were combined. A total 2 mL of saturated NaCl as well as 4 mL of hexane (plus 0.02% BHT) were added to the combined supernatants before their spinning down at 1250× *g* for 2 min, at 4 °C. Another extraction step was performed on the combined extracts with 3 mL of diethyl ether–hexane (1:2, *v/v* + 0.02% BHT).

To ensure that the combined organic phases did not contain any traces of water, a small amount of sodium sulfate (water free, ≈ 500 mg) was added to each tube, and the organic phase was transferred into a new 15 mL tube and dried under a stream of nitrogen for ≈ 40 min at 30 °C (TurboVap LV from Biotage, Uppsala, Sweden). Tubes were then flushed with argon and stored at −80 °C until analysis.

8.2.6. Carotenoid analysis

The analysis of carotenoids was carried out as described previously [14]. In brief, the dried extracts were re-dissolved with fridge-cold MTBE/MeOH (30/70, *v/v*) in a total volume of 150 µL (TRL fraction), 600 µL (plasma samples) or 6 mL (tomato and carrot juice extracts).

The internal standard (IS, *trans*-β-apo-8′-carotenal) was added to each sample to reach a final concentration of 0.2 µg/mL. HPLC separation of carotenoids was carried out using an Agilent 1260 Infinity HPLC instrument (Agilent Technologies, Santa Clara, USA), equipped with an Accucore C30 reversed phase column (2.6 µm particle size, 100 mm length, 2.1 mm diameter, Thermo Fisher Scientific). The mobile phase consisted of water/MeOH (60/40, *v/v*) with 30 mM of ammonium acetate as eluent A and ACN:DCM (85/15, *v/v*) as eluent B. Elution gradient was as follows: 0 min 42% B; 4 min 48% B; 5 min 52% B; 11 min 52% B; 13 min 75% B; 18 min 90% B; 40 min 90% B; 41 min 42% B. The flow rate was fixed at 0.5 mL/min, the injection volume was 10 µL, and column temperature was 28 °C. Seven point-linear calibration curves were prepared with external standards for each compound, with concentrations ranging from 60 to 500

ng/mL. Peaks were integrated at 286 nm (Pte), 350 nm (Ptf), 440 nm (Neo and Vio), 450 nm (Zea, Lut, α-Car, β-Car, and (*9Z*)-β-Car), 455 nm (β-Cry and IS), and at 470 nm (Lyc). Carotenoids were detected with a diode array detector, and identified by comparing each carotenoid's retention time and absorption maxima [28], with those of the available standards. Concentrations of carotenoids were determined using the internal standard method.

8.2.7. AUC, Cmax, Tmax

The postprandial AUC of time vs. concentration of the respective carotenoids extracted from TRL fractions was determined on the basis of seven postprandial time points plus the baseline (fasting state, before breakfast meal intake). The AUC was then determined from baseline-subtracted (each concentration was subtracted from its baseline value) values using the trapezoidal method. In case values below baseline were observed, these were included into the AUC calculations (as negative values) in order to maintain a maximum amount of information. Individual C_{max} values reflected the highest carotenoid concentrations measured in the TRL fraction of one participant on one clinical day, irrespective of the time point, and these were used to calculate the average C_{max}. Individual T_{max} values of one person, at each individual visit, were also noted down.

8.2.8. Statistical analyses

Normality of distribution of obtained AUC values as well as equality of variances were tested by Q-Q plots and box-plots, respectively. When needed, non-normally distributed data was log-transformed for further statistical analyses. A linear mixed model was created, with each subject acting as their own control, with AUC or C_{max} as the dependent, observed factor (main outcomes). Fixed factors were type of carotenoid or TAG (with levels of Lyc, β-Car, α-Car, Pte, Ptf, TAG), breakfast meal (A, B, or C), breakfast meal sequence, i.e., random block number (levels from 1-6), as well as the individual (ID number) nested within sequence and gender. Following significant Fisher-F tests for breakfast meal, Fisher protected LSD tests were used to compare the three treatments against one another, while Tukey's post-hoc test was employed for studying all group-wise comparisons if number of groups was >3. Following significant interactions (e.g., between test meal and type of carotenoid), one factor was kept constant to re-investigate statistical findings. *p*-values below 0.05 (2-sided) were considered significant. Unless otherwise reported, all values are means ± SD. SPSS (vs. 25, IBM, Chicago, IL) was used for all analyses.

8.3. Results

8.3.1. Description of subjects

Thirty healthy and free-living participants were enrolled in the study. Among these, six dropped out of the study for personal reasons, and twenty-four participants completed the trial, i.e., twelve males and

twelve females consumed all three breakfast meals, from which data were analyzed. Anthropometric measures and blood chemistry profile of the subjects at baseline are shown in Table 8.1. The baseline characteristics of the participants were in line with the inclusion criteria, but it is worth mentioning that one male participant had borderline TAG values (≈ 190 mg/dl), while two female participants had elevated total cholesterol concentrations (≈ 230 mg/dl). However, in no case both TAG and cholesterol were elevated, and the participants were advised to follow-up their blood lipids. No adverse reactions were observed during the three clinical days or in between.

8.3.2. Breakfast meal and carotenoid composition

Apart from the breakfast meal A (control, no additional proteins (1281 kJ / 355 kcal)), test meals B (added WPI) and C (added SPI) were isocaloric (1759 kJ / 467 kcal and 1753 kJ / 468 kcal, respectively) (Table 8.2). For all test meals, a total of 350 g of the juice were administered to the subjects for breakfast, being a 1/1 mix (*v/v*) of carrot and tomato juices. Carotenoid compositions and concentrations (mg/100 g) are reported in Table 8.3.

8.3.3. Plasma levels and effect of washout

The participants' fasting carotenoid plasma concentrations at baseline are reported in Table 8.4. Women had higher carotenoid plasma levels than men (by approx. 45%). The concentration of total plasma carotenoids was generally reduced by the washout period, from 455 (D0) to 305 (V1) and then to 285 nmol/l (V2 and V3) (Table

8.4). The decrease from D0 to V1 was significant ($p < 0.001$), while V1, V2, and V3 carotenoid plasma values did not differ significantly. This was also observed for each individual plasma carotenoid measured (except Lut+Zea and β-Cry for male). Although the effect of the two weeks of initial washout was significant in both genders, a stronger decrease was found in males compared to females (a 45 vs. 30% reduction, on average).

Table 8.4. Blood plasma carotenoid concentrations (nM)* of the participants at the baseline (D_0), first clinical visit (V_1), second clinical visit (V_2), and third clinical visit (V_3).

Gender	Visits	Lut+Zea	β-Car	α-Car	Lyc	Pte	Ptf	β-Cry	Total carot.†
Female (*n* = 12)	D_0	282.4 (±237.6)[a]	85.3 (±76.0)[a]	12.9 (±8.3)[a]	53.3 (±39.5)[a]	31.7 (±14.2)[a]	60.0 (±46.1)[a]	56.0 (±35.9)[a]	581.3 (±361.8)[a]
	V_1	214.4 (±202.8)[b]	55.4 (±61.6)[b]	10.0 (±5.6)[a]	42.4 (±52.0)[b]	22.6 (±35.6)[b]	44.9 (±45.5)[b]	39.5 (±23.1)[b]	429.3 (±386.1)[b]
	V_2	173.6 (±134.9)[b]	55.2 (±50.7)[b]	10.8 (±4.8)[a]	40.2 (±46.1)[b]	17.8 (±11.9)[b]	44.4 (±39.8)[b]	34.8 (±20.7)[b]	376.9 (±271.6)[b]
	V_3	175.0 (±120.4)[b]	58.0 (±57.7)[b]	11.2 (±5.0)[a]	35.3 (±35.3)[b]	15.8 (±9.0)[b]	46.5 (±46.8)[b]	35.4 (±22.8)[b]	377.3 (±262.1)[b]
Male (*n* = 12)	D_0	100.9 (±59.8)[a]	63.5 (±59.6)[a]	22.5 (±24.3)[a]	40.8 (±26.9)[a]	38.7 (±30.4)[a]	29.4 (±21.0)[a]	32.9 (±16.7)[a]	328.8 (±139.3)[a]
	V_1	61.6 (±37.9)[a]	38.0 (±34.0)[b]	13.5 (±13.9)[b]	23.1 (±15.4)[b]	11.6 (±6.0)[b]	14.2 (±7.4) [b]	19.6 (±10.0)[a]	181.6 (±74.0)[b]
	V_2	79.8 (±61.3)[a]	37.0 (±30.1)[b]	13.0 (±11.9)[b]	19.3 (±11.8)[b]	10.8 (±6.7)[b]	13.8 (±7.0)[b]	19.9 (±9.5)[a]	193.7 (±71.4)[b]
	V_3	79.0 (±62.8)[a]	35.5 (±29.2)[b]	13.2 (±11.7)[b]	18.0 (±10.9)[b]	12.2 (±6.5)[b]	14.5 (±9.7)[b]	21.1 (±13.3)[a]	193.7 (±76.9)[b]
Total Participants (*n* = 24)	D_0	191.7 (±193.1)[a]	74.4 (±67.7)[a]	17.7 (±18.4)[a]	47.0 (±33.6)[a]	35.2 (±23.5)[a]	44.5 (±38.3)[a]	44.7 (±29.8)[a]	455.0 (±297.5)[a]
	V_1	138.0 (±162.6)[b]	46.7 (±49.5)[b]	11.7 (±10.5)[b]	32.7 (±38.8)[b]	17.1 (±25.6)[b]	29.5 (±35.5)[b]	29.6 (±20.2)[b]	305.4 (±299.9)[b]
	V_2	126.7 (±113.1)[b]	46.1 (±41.9)[b]	11.9 (±8.9)[b]	29.8 (±34.6)[b]	14.3 (±10.1)[b]	29.1 (±32.0)[b]	27.4 (±17.5)[b]	285.3 (±215.6)[b]
	V_3	127.0 (±105.9)[b]	46.8 (±46.2)[b]	12.2 (±8.9)[b]	26.7 (±30.3)[b]	14.0 (±8.0)[b]	30.5 (±36.9)[b]	28.3 (±28.3)[b]	285.5 (±210.9)[b]

[1] Each value represents the mean of $n \geq 3$ replicates. Lut/Zea: lutein/zeaxanthin; β-Cry: β-cryptoxanthin; Ptf: phytofluene; Pte: phytoene; α-Car: α-carotene; β-Car: β-carotene; Lyc: lycopene. Values in the same column of the same group with a different superscript letter differ significantly. †sum of all listed carotenoids.

8.3.4. Effect of proteins on carotenoid AUC of plasma-TRL fraction over time

8.3.4.1. Total population (n = 24)

Following linear mixed models, it was tested whether there was any significant effect of the given meals on the AUC of serum TAGs/plasma-TRL fraction of carotenoids. The effect of the employed meals on AUC (all carotenoids investigated considered plus TAGs, i.e., statistically pooled) was significant (p = 0.011), as was the effect of gender and type of carotenoid (both $p < 0.001$). Considering all three meals and all types of carotenoids combined, AUC for carotenoids (nmol × h/l ± SEM) and TAGs (mg × h/dl ± SEM) were higher in males, compared to females (99.0 ± 2.0 vs. 44.7 ± 1.9, $p < 0.001$, and 339.9 ± 7.8 vs. 196.7 ± 7.6, p = 0.01, respectively) (Table 8.5).

Considering both genders and all carotenoids/TAGs investigated (statistically pooled) combined, the overall AUC (nmol × h/l) for test meal B (WPI) increased by 45% compared to the meal A (control, p = 0.018), and by 57% compared to test meal C (SPI, p = 0.006), while the latter did not differ significantly, as compared to test meal A (p = 0.685).

AUC for total carotenoids (nmol × h/l ± SEM) was 186 ± 7.0, 249 ± 7.0 and 124 ± 7.0 for control, WPI and SPI meals, respectively, being significantly different between the WPI meal and SPI meal (p = 0.014, Figure 8.3). Similarly, significant differences were found between SPI and WPI for individual carotenoids, i.e., Pte (p = 0.002), Ptf (p = 0.001), and α-Car (p = 0.002), although the difference between WPI vs. control meal was also significant for Ptf (Table 8.5). AUC values for TAGs (mg

× h/dl ± SEM) were significantly higher for the WPI meal (332.5 ± 9.6), than for the control meal (196.3 ± 9.6) (p = 0.044, Figure 8.3).

8.3.4.2. Males (n = 12)

The effects of the employed meal and carotenoid were significant (p = 0.036 and p < 0.001). Again, the AUC (nmol × h/l) for WPI increased by 46% compared to the control meal (p = 0.045), and by 62% compared to SPI (p = 0.016) (all carotenoids/TAGs considered, i.e., statistically pooled).

AUC for total carotenoids (nmol × h/l ± SEM) was 279.2 ± 17.4 for the control meal, 364.5 ± 17.4 for WPI and 171 ± 17.4 for SPI, with a significant difference between the tested proteins (WPI vs. SPI, p = 0.034). Similarly, significant differences were found between WPI- and SPI-supplemented meals for individual carotenoids including Pte (p = 0.026), Ptf (p = 0.004), α-Car (p = 0.034), and β-Car (p = 0.031) (Table 8.5). However, the AUC for TAGs did not significantly differ between the three meals (Figure 8.3).

Table 8.5. Effect of the test meals on the postprandial plasma AUC of TAG-rich lipoprotein (TRL) concentration of carotenoids and TAGs in serum.

CAR†/ TAGs	Breakfast meal	Female	Male	Total Participants
Lut + Zea	Control	−16.4 ± 4.5^{a}	15.0 ± 2.4^{a}	−0.6 ± 1.8^{a}
	WPI	−7.1 ± 4.5^{a}	17.5 ± 2.4^{a}	5.2 ± 1.8^{a}
	SPI	0.2 ± 4.5^{a}	28.7 ± 2.4^{a}	14.4 ± 1.8^{a}
β-Car	Control	1.7 ± 3.1^{a}	24.9 ± 3.2^{a,b}	13.3 ± 1.4^{a,b}
	WPI	1.7 ± 3.1^{a}	43.4 ± 3.2^{b}	22.6 ± 1.4^{b}
	SPI	−0.2 ± 3.1^{a}	6.6 ± 3.2^{a}	3.2 ± 1.4^{a}
α-Car	Control	4.2 ± 0.4^{a,b}	10.7 ± 1.6^{a,b}	7.4 ± 0.6^{a,b}
	WPI	5.9 ± 0.4^{b}	22.2 ± 1.6^{b}	14.1 ± 0.6^{b}
	SPI	2.0 ± 0.4^{a}	4.2 ± 1.6^{a}	3.1 ± 0.6^{a}
Lyc	Control	−8.8 ± 1.7^{a}	1.7 ± 1.2^{a}	−3.6 ± 0.7^{a}
	WPI	−13.5 ± 1.7^{a}	6.4 ± 1.2^{a}	−3.6 ± 0.7^{a}
	SPI	−8.3 ± 1.7^{a}	4.2 ± 1.2^{a}	−2.0 ± 0.7^{a}
Pte	Control	94.5 ± 2.3^{b}	176.9 ± 9.1^{a,b}	135.7 ± 3.3^{b}
	WPI	112.1 ± 2.3^{b}	207.6 ± 9.1^{b}	159.9 ± 3.3^{b}
	SPI	63.3 ± 2.3^{a}	100.5 ± 9.1^{a}	81.9 ± 3.3^{a}
Ptf	Control	28.9 ± 1.7^{a}	49.2 ± 2.1^{a}	39.0 ± 0.9^{a}
	WPI	40.1 ± 1.7^{a}	69.2 ± 2.1^{b}	54.7 ± 0.9^{b}
	SPI	26.3 ± 1.7^{a}	36.2 ± 2.1^{a}	31.2 ± 0.9^{a}
β-Cry	Control	−10.2 ± 0.7^{a}	0.7 ± 0.8^{a}	−4.7 ±0.7^{a}
	WPI	−6.2 ± 0.7^{a}	−1.5 ± 0.8^{a}	−3.8 ± 0.3^{a}
	SPI	−6.5 ± 0.7^{a}	2.0 ± 0.8^{a}	−2.3 ± 0.7^{a}
Total car‡	Control	93.8 ± 9.7^{a}	279.2 ± 17.4^{a,b}	186.5 ± 7.0^{a,b}
	WPI	133.1 ± 9.7^{a}	364.5 ± 17.4^{b}	248.8 ± 7.0^{b}
	SPI	76.8 ± 9.7^{a}	171.5 ± 17.4^{a}	124.1 ± 7.0^{a}
TAGs	Control	165.3 ± 6.3^{a}	232.1 ± 25.8^{a}	196.3 ± 9.6^{a}
	WPI	238.2 ± 6.3^{b}	426.7 ± 25.8^{a}	332.4 ± 9.3^{b}
	SPI	191.5 ± 6.3^{a,b}	360.8 ± 25.8^{a}	276.2 ± 9.3^{a,b}

Values represent mean ± SEM. Means without a common superscript letter differ significantly, $p < 0.05$. Lut+Zea: lutein/zeaxanthin; β-Cry: β-cryptoxanthin; Ptf: phytofluene; Pte: phytoene; α-Car: α-carotene; β-Car: β-carotene; Lyc: lycopene. Total carot.: total carotenoids. †AUC results for carotenoids in the plasma TAG-rich lipoprotein (TRL, nmol × h/l) fractions, and for serum TAGs (mg/dl × h/l), following the consumption of a test meal without added protein (control meal), or with either whey protein isolate (WPI-supplemented meal) or soy protein isolate (SPI-supplemented meal) in male (n = 12), female (n = 12) or in total participants (n = 24). ‡Sum of all individual carotenoids.

8.3.4.3. Females (n =12)

The effect of carotenoid was significant ($p < 0.001$), while it was not significant for the test meal effect ($p = 0.189$). Regarding the carotenoid effect, AUC for total carotenoids did not differ significantly between the test meals, while for individual carotenoids, significant differences were found only for Pte, with significantly lower AUC values (nmol × h/l ± SEM) for the SPI meal (63.3 ± 11.0), as compared to the control meal (94.5 ± 14.9, $p = 0.013$), and the WPI meal (112.2 ± 21.7, $p <0.001$) (Table 8.5). The AUC values for TAGs (mg × h/dl ± SEM) were significantly lower for the control meal vs. the WPI meal (165.3 ± 6.3 vs. 238.2 ±5.9, $p = 0.024$) (Figure 8.3).

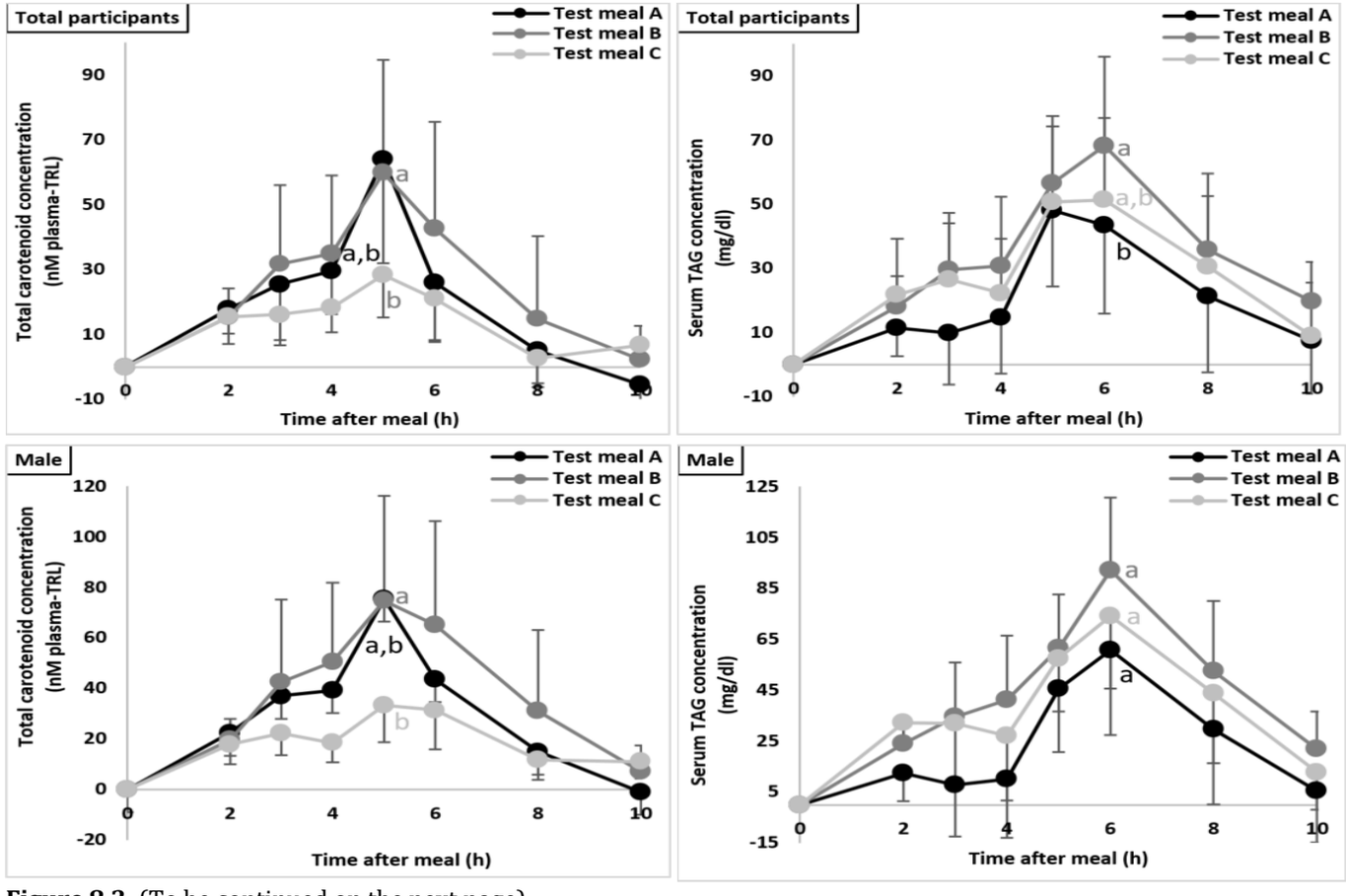


Figure 8.3. (To be continued on the next page).

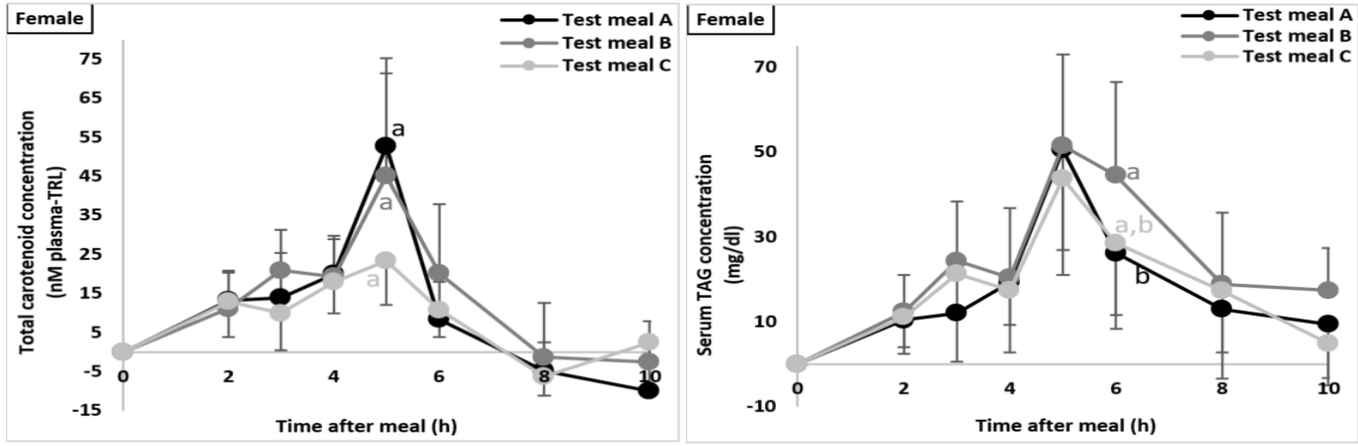


Figure 8.3. (Continued). Effect of different protein types on the postprandial serum TAG concentrations and plasma TAG-rich lipoprotein (TRL) concentration of carotenoids. Changes in plasma TRL-carotenoids (left panel) and serum TAGs (right panel) were assessed over a 10 h period after the intake of a test meal without (control, test meal A) or including one of the two investigated proteins, namely whey protein isolate (WPI, test meal B) or soy protein isolate (SPI, test meal C). Plasma TRL-carotenoid concentrations (nM) and serum TAG concentrations (mg/dl) are expressed as mean values (total participants = *n*24, male/female = *n*12) with their standard errors. AUC were compared for statistically significant differences as outlined in the "Statistical Analyses" section. Solid lines not sharing the same alphabetical letter (a, b, c) are considered significantly different.

8.3.5. Effect of proteins on maximum plasma TRL-carotenoid concentration (Cmax)

8.3.5.1. Total population (n = 24)

The effects of tested meal and carotenoid were significant (both $p < 0.001$) regarding C_{max}, while the effect of gender was not ($p = 0.116$).

C_{max} for total carotenoids (nmol/l ± SD) was 119.6 ± 30.9 vs. 135.4 ± 38.0 vs. 89.6 ± 17.3 nmol/l for test meals A (control, no protein), B (WPI), and C (SPI), respectively, being significantly different between control meal and SPI, as well as between WPI and SPI (both $p < 0.001$, Figure 8.4B). Similarly, the C_{max} of several individual carotenoids such as Lut+Zea, Lyc, Pte, α-, and β-Car were significantly lower in the presence of SPI, as compared to the WPI and control meals ($p < 0.05$, Figure 8.4.A3). However, the C_{max} values for TAGs (mg/dl ± SD) were only significantly different between the control meal and WPI (143.3 ± 41.4 vs. 162.4 ± 49.2 mg/dl, $p = 0.044$, Figure 8.4C).

8.3.5.2. Males (n = 12)

The effects of test meal and carotenoid type on C_{max} were significant (both $p < 0.001$). C_{max} for total carotenoids (nmol/l ± SD) was significantly lower in the presence of SPI, compared to control meal and WPI-supplemented test meal, by approx. 33 and 36%, respectively (88.0 ± 21.1 vs. 131.6 ± 41.9 and 138.6 ± 50.0, respectively; both $p < 0.001$) (Figure 8.4B). Significant differences

were found for C_{max} of several individual carotenoids, such as Pte, Ptf, α-Car, and β-Car, between the SPI-supplemented meal and the two other meals ($p < 0.05$, Figure 8.4.A1), while the C_{max} values for TAGs (mg/dl ± SD) did not significantly differ between the three meals (Figure 8.4C).

8.3.5.3. Females (n = 12)

The effects of tested meal and type of carotenoid were also both significant ($p < 0.001$). C_{max} for total carotenoids (nmol/l ± SD) was significantly different between the control meal (107,7 ± 12.8) and WPI (132.1 ± 23.0, $p = 0.003$), and between control meal and SPI (91.1 ± 13.3, $p = 0.023$), as well as between WPI meal and SPI meal ($p < 0.001$, Figure 8.4B). The C_{max} of several individual carotenoids such as Lut+Zea, Lyc, Pte, Ptf, α-Car, and β-Car were significantly lower ($p < 0.05$) in the presence of SPI, compared to WPI and control meal, except for Lyc and Ptf, where a significant difference was found only between WPI vs. SPI and control vs. SPI, respectively (Figure 8.4.A2). Similar as for males, the C_{max} values for TAGs (mg/dl ± SD) did not significantly differ between the three test meals (Figure 8.4C).

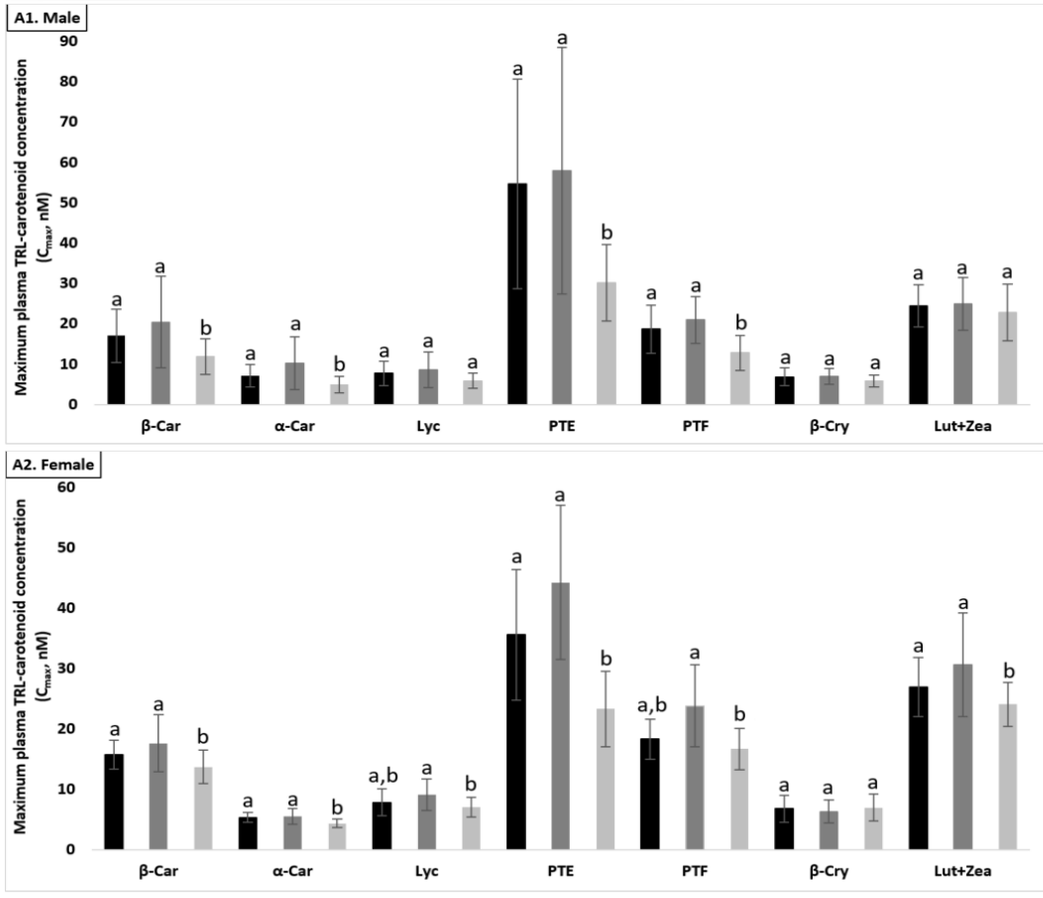


Figure 8.4. (To be continued on the next page).

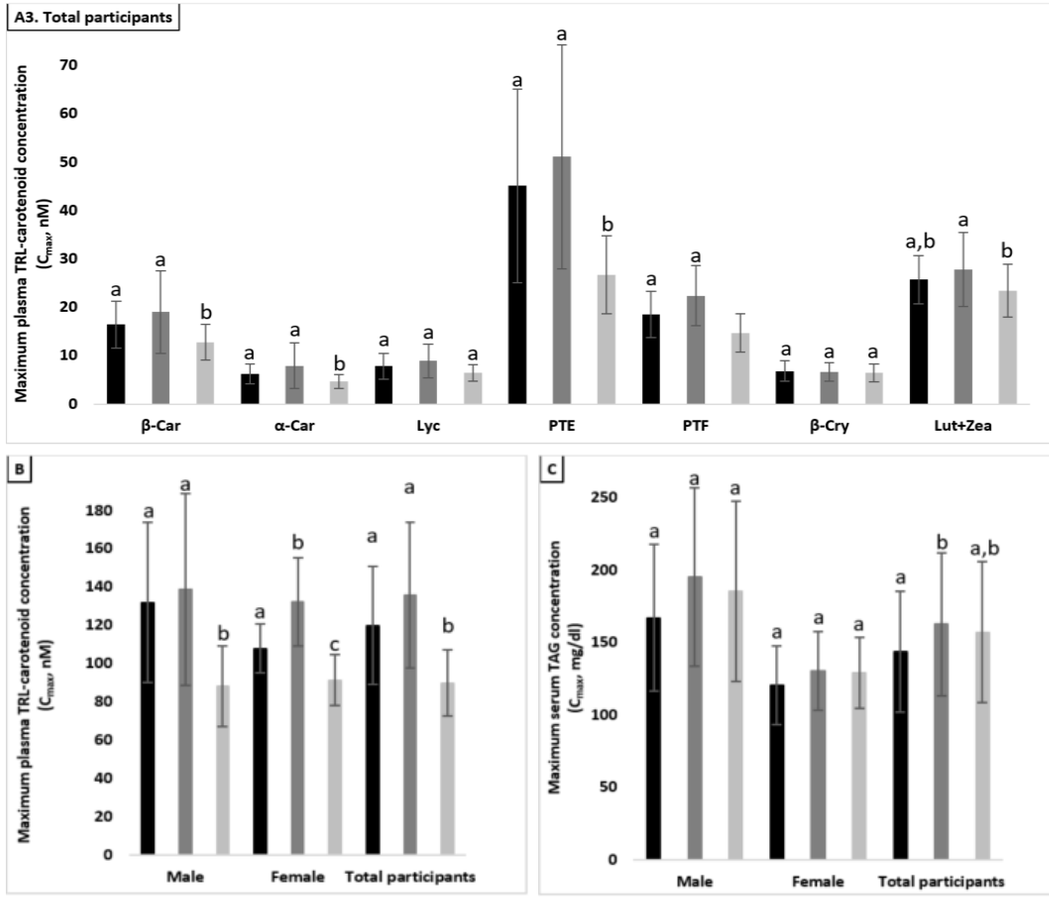


Figure 8.4. (Continued). Effect of the tested meals on the maximum concentrations (Cmax) of plasma carotenoids (**A–B**) and serum TAGs (**C**) in the postprandial plasma TAG-rich lipoprotein (TRL) fraction. Changes in Cmax of the plasma TRL-carotenoids and serum TAGs that reflect the highest concentrations measured, irrespective of the time point, following the consumption of the ■ control meal, ■ whey protein isolate (WPI-supplemented meal), or ■ soy protein isolate (SPI-supplemented meal). A. individual carotenoid concentration (nM) in the plasma TRL fraction; B. total carotenoid concentration (nM) in the plasma TRL fraction; C. serum TAG concentrations (mg/dl). Concentrations are expressed as mean values (total participants = 24, male/female = 12) with their standard errors. AUC were compared for statistically significant differences as outlined in the "Statistical Analyses" section. Columns labelled without a common superscript (alphabetic letters) differ significantly, $p < 0.05$.

8.3.6. Influence of proteins on the time to peak plasma TRL-carotenoid concentration (Tmax)

The observed time (T_{max}) to reach the maximum plasma carotenoid concentration did not change and was found generally at t = 5 h, following the lunch meal. A somewhat bi-phasic absorption pattern was found, with a first peak/plateau at t = 2–3 h and a second one at t = 5 h, which was similar to patterns reported earlier [25]. Of note, at certain times, carotenoid concentrations dropped below baseline, which was attributed to a decreased plasma volume prior to food and fluid intake and an increased circulatory volume after test meal intake.

8.4. Discussion

This study followed our recent *in vitro* investigation, reporting that proteins may interfere with the bioaccessibility and cellular uptake of carotenoids [14]. The main objective of this clinical trial was to verify these results *in vivo*. For this purpose, a carotenoid-rich meal was supplemented or not with proteins at a concentration of 50% of the RDA (60 g), and was served to healthy adults on three occasions interspaced by one week, following a randomized cross-over design. The results confirm the previous *in vitro* observations, as they showed that the two proteins, WPI and SPI, had diverging effects on carotenoid absorption as determined by their AUC of the plasma-TRL fraction. The bioavailability of total carotenoid/TAGs was enhanced by 45% in the presence of WPI, and lowered insignificantly by 8% in the presence of SPI, compared to control meal (no additional proteins).

The tested meals, i.e., a tomato/carrot juice mixture, provided a wide range of carotenoids (Table 8.3) with concentrations similar to those reported previously [29, 30]. At baseline, the participants' fasting carotenoid plasma concentrations were within the range previously reported for healthy subjects [31]. Following the two-week washout period, the concentrations of total plasma carotenoids were significantly reduced, by approx. 35% at the first clinical visit, with no further significant difference between the baseline concentrations prior to the three clinical visits, suggesting that the washout periods were successful and sufficient to cause a steady reduction of the plasma levels.

The effects of the tested meal, gender, and carotenoid were significant in the applied linear mixed model. The presence of WPI in the tested meal resulted in improved AUC of carotenoids/TAGs over the 10 h course, by 45% compared to the control meal without added proteins, and by 57% compared to SPI-supplemented meal. This is, to our knowledge, the first study finding an effect of proteins on carotenoid absorption in humans.

In our previous *in vitro* studies, we have reported these two diverging effects of various proteins on the bioaccessibility of carotenoids [14, 23, 27, 32], which strongly depended on the type of protein and carotenoid. In fact, certain proteins with a high surface hydrophobic nature such as SPI, when partially digested, were suspected to aggregate in the aqueous phase and/or adsorb in the form of long polypeptides at the O/W interface [23], thus trapping carotenoids and producing negative interactions, limiting their processing from lipid droplets to mixed micelles [14]. In addition, this

would be fostered by the general lower digestibility of SPI vs. WPI [23]. On the other hand, more digestible proteins, such as WPI [23], appeared to foster bioaccessibility and the cellular uptake of carotenoids, via producing peptides of more amphiphilic structure, facilitating the interactions at the surface of the mixed micelles, making them potentially more available for cellular uptake [14].

In addition to the effect on total carotenoids, all individual carotenoids were modulated towards higher bioavailability in the presence of WPI. However, these differences were found statistically significant only for Pte, Ptf, α-Car, and β-Car, with strongest responses observed for Pte and Ptf, while plasma Lyc TRL-concentrations did not change between the three tested meals. This is somewhat different from the observations *in vitro*, where proteins rather fostered the bioavailability of individual carotenes compared to xanthophylls [14]. In those studies, the effect of proteins on the micellization of carotenoids was reflected by their polarity; i.e., while proteins enhanced the bioaccessibility of pure β-carotene, a decrease was observed for lutein, while the influence was somewhat limited regarding lycopene [13, 33]. It was postulated that the presence of proteins at the interface may result in a stronger negative interaction of proteins and the more polar xanthophylls, which are preferentially solubilized at the surface of the lipid droplets and later of the mixed micelles [34]. However, more apolar carotenes may also have been bound by the major WPI fraction, the pepsin-resistant β-lactoglobulin, with a high affinity of the carotene to the internal cavity of the β-barrel [35], which could act as a vehicle for hydrophobic compounds with positive effects on carotene solubility. Such interactions may be

specific to a certain structure, i.e., the β-ionone cycle and isoprenoid chain, which may explain the more neutral effect of WPI on lycopene bioaccessibility in the present work. Similar results were found in our previous study regarding the effect of proteins on the bioaccessibility of carotenoids from food matrices [14]. However, co-digested proteins fostered the cellular uptake of carotenes and counteracted the negative effect of proteins on xanthophyll bioaccessibility by improving their cellular uptake, especially in the presence of WPI [14], possibly by producing peptides that had more amphiphilic structure and facilitated the interactions of mixed micelles with Caco-2 cells [36]. However, it cannot be excluded that in the present study, the low amount of xanthophylls present in the juice mixture, compared to carotenes, also aided in a more favorable effect on their absorption.

Interestingly, strongest responses were observed for Pte and Ptf, compared to other carotenoids. These results are in line with our previous findings on bioaccessibility and cellular uptake [14], especially in the presence of WPI. Indeed, *in vitro* studies have shown that these colorless carotenoids, present in tomato and its products, displayed relatively high bioaccessibility [30], and cellular uptake [37], and are more available than lycopene in healthy adults [38]. This was explained by the higher molecular flexibility and twisting ability [39], allowing a better insertion of these carotenoids into the mixed micelles, leading to a greater micellization efficiency [40].

In addition to the effect of tested meal on the AUC for total carotenoids, AUC values for serum TAGs of WPI-supplemented meal were also significantly higher compared to the control meal, and lower from the SPI meal. It is acknowledged that TAGs are absorbed from the

intestine, transported as chylomicrons through the lymphatic system prior to entering the systemic circulation, and are metabolized extravascularly, liberating free fatty acids (FFAs) [41]. During lipid metabolism, FFAs are reincorporated into TAGs in the liver and further transported within lipoproteins. As carotenoid absorption is generally considered an aspect of lipid absorption, it is noteworthy that the lipemic response corresponded with carotenoid absorption [42]. This suggests that absorption of both carotenoids and TAG can be significantly impacted by the presence of proteins, which is further plausible given also that FFA are absorbed from the intestine via mixed micelles. This similarity in absorption patterns was supported by the observed time (T_{max}) to reach the maximum plasma carotenoid and serum TAG concentrations (C_{max}), which followed in both cases, i.e., TAGs and carotenoids, a biphasic curve pattern, with peaks or at least shoulders at approximately t = 2 h and 5 h postprandially (Figure 8.3), similar as observed in previous studies [31, 43]. Furthermore, C_{max} was significantly altered by protein addition, both for TAGs and for total carotenoids, in a very similar fashion as their corresponding AUCs.

While the absorption patterns of carotenoids as influenced by WPI and SPI were similar for men and women, the effect of tested meal did not reach significance in women, which could be due to the generally lower responses of AUC in women. In addition, women displayed higher plasma carotenoid concentrations compared to men, possibly proposing a higher carotenoid status in women, which may have resulted in lower fractional uptake of carotenoids from the test meals. The intestine-specific homeobox (ISX), a transcription factor, can reduce absorption of pro-vitamin A carotenoids, given that the

body status is high [44]. Previous studies have reported similar findings of higher circulating carotenoids levels in women [45-48]. While some investigations postulated that this sex difference might be explained by better dietary habits such as higher fruit and vegetable intake in female participants [49-51], others have explained the difference by higher levels of visceral white adipose tissue in men and different plasma lipoprotein–lipid profiles. A recent investigation reported that increased body weight and waist circumference were associated with lower total plasma carotenoid concentrations, while elevated plasma LDL-cholesterol and HDL-cholesterol concentrations correlated with higher total carotenoids in plasma, due to elevated transport capacity of carotenoids [52]. This would be in line with the present observations (despite the relatively limited sample size in each gender group), as a higher BMI was observed for men that may have been related to higher adiposity in men in the present study, but higher levels of blood lipids in women at the time of recruitment (Table 8.1). These aspects may explain, at least in part, that despite the reduction of the postprandial AUC for total carotenoids in female volunteers by approx. 18% for SPI, and an increase of almost 42% for WPI, this influence was statistically not significant compared to the control meal, unlike in males. However, it is worth mentioning that proteins influenced the lipemic response, as the AUC values for TAGs in females were significantly higher for the WPI-supplemented meal vs. the control meal.

When transferring such findings to daily life and practical recommendations, it is recognized, for instance, that salads should be consumed together with a fat-containing salad dressing, to benefit

from liposoluble micronutrients and secondary plant compounds bound to the plant matrix [53]. Similar considerations may also apply to promoting food combinations with proteins to maximize carotenoid bioavailability. However, our study has the limitation that we used only proteins added in form of powder, and as our earlier *in vitro* studies have indicated that the effect for proteins bound within real food matrices such as cod and turkey are somewhat weaker in their interactions with carotenoids [14], more studies in this domain are warranted to examine effects from other protein sources. Adjusting dietary recommendations for subjects with low vitamin A status or low vitamin A intake such as vegetarians, or people living in developing countries where access to meat and thus preformed vitamin A is often limited, could also be considered, i.e., combining carotenoid intake with well digestible proteins, if available. In addition, minor fractions of the population such as those having digestive disturbances and impeded absorption for liposoluble micro-constituents from the diet [32], may also benefit from the knowledge gained from this trial. Finally, the combination of proteins and carotenoids together in food supplements may deserve further attention [54].

8.5. Conclusions

This study shows, for the first time, that proteins added to a test meal rich in carotenoids, at a protein amount equivalent to 50% of the RDA, could improve carotenoid bioavailability in healthy adults. The results suggest that a well digestible protein such as WPI could be beneficial for carotenoid bioavailability, whereas a less digestible one such as SPI may result in hampered availability. It appears that the

effect of proteins on the bioavailability of carotenoids, and potentially other liposoluble nutrients [55], depends on the type of protein, but likely also on the individual carotenoid species. Further investigations are necessary to fully understand the complex interactions between proteins and carotenoids.

8.6. References

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Chapter 9

General discussion and conclusions

Adapted from experimental chapters (4 to 8) and from:

Influence of proteins on the absorption of lipophilic vitamins and secondary plant metabolites – a review

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Foreword

In this thesis, we set out to investigate the hypothesis that proteins could influence aspects of carotenoid bioavailability, either positively, or negatively. Proteins have been claimed to improve the bioaccessibility of fat-soluble food constituents in several ways. Following their adsorption to lipid droplet surfaces, proteins may stabilize o/w emulsions in the GI tract, this being attributed to the fact that proteins can be highly surface active molecules [1], and that the solubilized particles tend to be negatively charged, preventing the aggregation of lipid droplets [2]. However, protein adsorption to o/w interfaces may also lead to a decrease in the enzymatic degradation of lipids, likely by hindering digestive enzymes to adsorb to the droplet surfaces or binding to enzymes directly, also implying potential negative influences of proteins on the processing of lipid droplets and thus the ensuing micellization process [3]. The experimental work presented in the previous Chapters has provided a set of findings that clarify the impact of different types of dietary proteins on carotenoid bioavailability. A summary of main findings and overall discussion and conclusions of the encountered results is given in the present Chapter.

9.1. Summary of main findings

Carotenoids are recognized for several health benefits, such as their potential role in preventing chronic diseases [4-7]. Indeed, a low intake of carotenoids within a mixed diet represents a major health concern in populations with low preformed vitamin A intake (as in some countries or for vegans/vegetarians), leading to increased susceptibility to infections, growth disorders, skin diseases and night-blindness (nyctalopia) [8]. However, their absorption is quite limited, and studies on the factors influencing their bioavailability have so far remained little explored [9, 10].

Micellization of carotenoids, which includes the release from the food matrix, transfer into lipid droplets and incorporation into mixed micelles, can be considered as determining factors for carotenoid bioavailability. A previous studies showed that the use of proteins as encapsulation materials of carotenoids could be a very effective strategy to improve their chemical stability, solubility, as well as bioaccessibility following *in vitro* GI digestion, pointing out toward increased bioavailability of such combinations [1, 3, 11-13].

The main objective of this thesis falls within this perspective. The general purpose aims to study the influence of dietary proteins on carotenoid digestion aspects of bioavailability, under conditions resembling the *in vivo* environment. More specifically, this study focused on the following point:

1. the interaction of proteins added to test meals with carotenoids during digestion.

2. the effect of co-consumed proteins on physico-chemical properties of the digesta (e.g. surface tension, protein digestion, lipid digestion, micelle size, and zeta-potential).

3. the influence of proteins on carotenoid bioaccessibility and cellular uptake.

4. the impact of gastric lipase on carotenoid emulsification and their transition into mixed micelle in the presence of proteins which digestion begins in the stomach.

5. the differences between various protein sources regarding their potential to influence micelle formation, physico-chemical properties of the digesta, as well as bioaccessibility and cellular uptake of individual carotenoid standards and carotenoid-containing solid and liquid matrices.

6. the *in vivo* validation of the results and hypotheses formed *in vitro*.

The major outcomes derived from these unanswered questions are presented in the next paragraphs.

9.1.1. Harmonized *in vitro* digestion model to study the influence of the co-digestion process of proteins on carotenoid bioaccessibility

First of all, as outlined in chapter 4, we aimed to investigate the effect of a commonly consumed protein source, WPI, on the bioaccessibility of pure β-carotene, under a range of digestive conditions, as the emulsifying properties of proteins were assumed to

be greatly influenced by the GI environment. This investigation was a crucial milestone prior to further investigations.

The addition of WPI to test meals during simulated GI digestion influenced the bioaccessibility of β-carotene positively as well as negatively, depending on the digestion conditions and deviations compared to the standardized INFOGEST consensus model [14]. The findings demonstrated the ability of WPI to slightly enhance the bioaccessibility of β-carotene, by a fractional increase of up to 20%, when applying a higher peristalsis energy, or when reducing the amount of dietary lipids in a meal. A drop of 1/3 in β-carotene bioaccessibility in the presence of WPI was also encountered, following halving the amount of enzymes (pancreatin and pepsin) or doubling the amount of oil, or halving the amount of bile. The following hypotheses can be drawn from these results.

1. Since the harmonized in vitro GI digestion model is carried out for a wide range of purposes and with different endpoints, some adjustments may be needed according to the particular requirements of specific experiments. In our case, we changed the amount of pancreatic enzymes (increasing to 200 U/mL of the final mixture, based on trypsin activity) and peristalsis energy (100 rpm), and observed that these conditions play a decisive role on the evaluation of the fate of β-carotene. This observation can probably be generalized to other carotenoids and possibly to other liposoluble bioactive compounds. It remains arguable which conditions resemble closer the in vivo situation, but it was perceived that the peristalsis surely has been sub-optimal in the standardized INFOGEST in vitro digestion model [14], as it is

difficult to simulate. Likewise, a recent study has proposed to increase the amount of pancreatin if lipid components are studied during in vitro digestion [15]. In fact, our results showed that the influence of proteins strongly depends on the conditions of the environment in which carotenoids are solubilized. These conditions are mainly related to the digestion of macronutrients, i.e. proteins and lipids, being either positive or negative.

2. WPI and possibly other proteins, via their emulsifying properties, may positively modulate carotenoid bioaccessibility, under rather normal and healthy conditions. This positive influence could be ensured either through strong non-covalent interactions with carotenoids within a pocket of hydrophobic residues [16, 17], or via the interaction during the processing of lipid droplets into mixed micelles as proclaimed earlier [1, 18, 19]. In our study, WPI positively affected the stability of o/w emulsions, which resulted in a reduction in the size of lipid droplets after digestion and also in a smaller size of the mixed micelles, although the average absolute zeta-potential of the latter was somewhat reduced.

3. A negative influence of WPI on β-carotene bioaccessibility may be expected, as observed in our *in vitro* study. This was observed when digestion is less complete, especially with high protein concentration, low bile salts and pancreatic enzymes or high lipid amounts, possibly due to the resistance of β-lactoglobulin fraction to pepsinolysis (dimeric globular proteins [20]). Indeed, a high protein concentration may cause the formation of aggregates that could trap β-carotene, and/or may provoke the adsorption of long polypeptide chains on lipid droplets. The latter

case may lead to the formation of a dense and viscoelastic interfacial film, which may obstruct lipolysis, i.e. prevent lipase to adsorb to the droplet surfaces, all of which hindering the processing of lipid droplets into mixed micelles [3].

In summary, slight adjustments of the digestive conditions were required to our specific experiments for the use of the static *in vitro* digestion procedure. Indeed, the digestion of lipids and proteins is crucial to evaluate the bioaccessibility of carotenoids, which is an important component of their bioavailability. Results in our study confirmed the ability of a frequently consumed protein, i.e. WPI, to adsorb to lipid droplet surfaces and contribute to the stabilization of o/w emulsions under physiological conditions, dictating in turn the fate of β-carotene, as a model molecule for lipophilic compounds.

9.1.2. Gastric lipase contribution to improving lipolysis extent and carotenoid bioaccessibility in the presence of proteins in the harmonized INFOGEST in vitro digestion model

Another important aspect related to the methodology was the addition of gastric lipase to the proposed INFOGEST *in vitro* digestion model. Though its presence in the GI digestion is expected to foster emulsification of lipophilic constituents prior to their incorporation into mixed micelles [21], the inclusion of gastric lipase is typically not integrated in *in vitro* models mimicking the digestion process, due to a lack of available HGL. In chapter 5, we aimed to shed more light on the role of gastric lipase on the bioaccessibility of lipophilic compounds,

by comparing the effect of various lipases (*R. niveus*, *R. oryzae*, and RGE) that have also been used in several digestion models, on the bioaccessibility of carotenoids from several food matrices. As protein digestion co-occurs in the stomach and could potentially interact with lipid droplet formation, proteins with different hydrophobicity were co-digested with plant food matrices rich in carotenoids.

In contrast to *in vitro* data reported earlier [22, 23], all tested lipases unexpectedly improved gastric lipolysis by an average of 10-fold, despite their kinetic differences, especially in pH optima. However, the addition of lipases to the simulated digestion had a differential effect on the bioaccessibility of carotenoids from plant food matrices. Most importantly, only RGE improved the overall carotenoid bioaccessibility by almost 2-fold, compared to fungal lipases.

It is acknowledged that bile salts are strong surfactants that compete with lipases for adsorption at the o/w interface, and can prevent the enzymes from reaching their substrate. Due to its amphiphilic structure [24] and high tensioactivity [22], HGL has been reported to be resistant to displacement by bile extracts, which is among the biochemical property that make HGL rather unique among lipases. Interestingly, it was hypothesized that the high tensioactivity of RGE allow a better resistance to bile salts and a higher penetration into phospholipid layer covering TAG droplets (in the gastric phase), triggering therefore the activity of pancreatic lipase during the intestinal passage [25-28]. In contrast, *R. niveus* and *R. oryzae* lipases are more efficiently inhibited by bile salts [22, 29, 30]. A study by Sassene et *al*. confirmed that RGE had similar surface properties to those of HGL, while the lack of specificity of fungal lipases with regard

to the FA chain length of the TAGs does not seem to replicate the digestive properties of HGL [31]. These observations contribute to explain the fact that, in our results, only RGE, being functionally more similar to HGL, significantly contributed to improving both lipolysis extent and carotenoid bioaccessibility in all tested matrices.

In the presence of proteins, the effect of RGE addition seemed to result in a weaker increase in carotenoid bioaccessibility compared to no protein. Nevertheless, the presence of RGE compensated for the negative effect that WPI and SPI showed on carotenoid bioaccessibility, resulting in a positive influence on total carotenoid bioaccessibility in tomato- and carrot juices.

We have reported in Chapter 4 that high protein concentrations, especially with those proteins of high surface hydrophobicity such as the β-lactoglobulin fraction of the WPI [32] or with those having low solubility such as SPI [33, 34], may cause the accumulation of long peptide chains on lipid droplet surfaces. This may hamper the access of lipolytic enzymes to their substrates [35] and affect the processing of carotenoid transfer from lipid droplets into mixed micelles. We hypothesized that RGE, in synergy with pancreatic enzymes, may foster the lipolysis of the otherwise less accessible lipid fractions during the gastric phase, due to the remaining long peptide chains adsorbed at the interface. In addition, RGE may also lead to an improved transfer of the previously trapped carotenoids in hydrophobic pockets of protein residues, into mixed micelles following their release due to subsequent proteolysis. In either cases, the presence of RGE may lead to an increase in the bioaccessibility of carotenoids.

In summary, our results showed that only RGE significantly contributed to improving carotenoid bioaccessibility in all tested matrices, while the presence of proteins mitigated the positive effect of lipases on carotenoid bioaccessibility. These results demonstrate how gastric lipolysis and proteolysis processes are tightly entwined during the gastric digestion. The inclusion of gastric lipase may thus be a more sound physiological improvement. However, it was financially not possible to include that enzyme in the ensuing analysis.

9.1.3. Influence of proteins at various concentrations on the bioaccessibility of pure carotenoids solubilized in oil during simulated GI digestion

Following the methodological investigations performed in chapters 4 and 5, Chapter 6 offered us to focus on the effect of several frequently consumed proteins of various hydrophobicity and food origin (WPI, SPI, SC, and GEL) on the bioaccessibility of commonly ingested carotenoids, i.e. β-carotene, lutein and lycopene (added individually to digesta samples), by means of the INFOGEST European consensus model for static *in vitro* digestion, with slight adjustments (Chapter 4). The chosen carotenoids represent the most commonly consumed carotenoids and also represent different classes (vitamin-A precursors, xanthophylls, and non-provitamin A carotenes).

Similarly to what was observed in previous chapters, proteins added to the simulated GI digestions significantly influenced carotenoid bioaccessibility, either positively or negatively. However, the effects depended not only on the type and concentration of the

protein, but also on the type of carotenoid. The major findings were the following.

1. The presence of proteins doubled the bioaccessibility of β-carotene, but it halved the bioaccessibility of lutein. It is acknowledged that physicochemical properties of carotenoids, such as polarity, can lead to their differential behavior in biological emulsions [36]. The more polar carotenoids, such as lutein, are preferentially solubilized at the lipid droplet surface, while the apolar carotenoids, such β-carotene, are solubilized in the core of the lipid droplets [37]. Therefore, the presence of proteins at the interface may indirectly lead to a negative influence on the bioaccessibility of more polar xanthophylls located close to the surface of the lipid droplets [38], while carotenes may be less disturbed by protein adsorption at the o/w interface. The same reasoning may apply for the surface of mixed micelles. However, direct interactions between proteins and carotenes may also occur, such as by non-covalent interactions within a pocket of hydrophobic residues, which may lead to a positive influence on the bioaccessibility of carotenes (Chapter 4).

2. The effect of SPI depends on its concentration. At 10% of RDA, SPI enhanced the bioaccessibility of β-carotene by almost a third, possibly due to the stabilized emulsions resulting from its high surface hydrophobicity [20]. In contrast, higher concentrations of SPI (i.e. at 25 and 50% of RDA) reduced bioaccessibility with an average decrease of about a half, as observed for lutein. High concentrations of SPI appeared to reduce its digestibility (as shown by SDS-PAGE), and resulted in the formation of aggregates in the

aqueous phase (as observed by microscopy), leading to an increased surface tension and a lower absolute ζ-potential, thus negatively impacting on the processing of lipids and the incorporation of carotenoids into mixed micelles. The applied acidic treatment of the gastric environment was shown to provoke a progressive denaturation of SPI, exposure of its hydrophobic clusters, and aggregation of unfolded and/or denatured proteins [39, 40], constituting one of the major reasons causing low solubility of SPI [34]. However, these effects have been reported to be highly related to the protein concentration applied in an emulsion. In fact, results of a study with SPI-stabilized emulsions indicated that intermolecular association between adsorbed proteins to o/w interfaces was more favorable at higher protein concentrations [41]. These increased intermolecular interactions resulted in reduced repulsive forces between the network of lipid droplets, which led to formation of aggregates, thus negatively impacting on the stability of the emulsions [42, 43]. Tang et *al*. confirmed these observations by showing that the oligomeric glycinin (a hexamer constituting ca. 30% of the total SPI [44]) had a high tendency to aggregate at high protein levels, by forming very thick and inhomogeneous multilayer interfacial films, leading to entrapped/bridged lipid droplets within a network consisting of aggregated particles (Chapter 2) [20]. In contrast, the presence of globulins such as β-conglycinin (a trimer, comprising 30-50% of the total SPI [44]) at low concentration, can form a dense and viscoelastic interfacial film at the interface of lipid droplets,

exhibiting a high coalescence stability, with relatively low tendency to aggregate, due to their highly hydrophilic nature (Chapter 2) [20].

3. GEL and SC showed a better solubility and high stability at wider pH range (high surface charge at pH away from the protein's pI and/or under low ionic conditions [43]), as compared to SPI. Accordingly, their digestion was effective even during the early stage of digestion (as shown by SDS-PAGE), and greater repulsive forces between the lipid particles and a better emulsification were observed (as shown by the higher absolute ζ-potential and the lower surface tension, respectively), which resulted in the formation of emulsions with more dispersed lipid droplets. Consequently, GEL and SC enhanced β-carotene bioaccessibility by almost 2-fold, though an average decrease of about 1/4 was also observed for the bioaccessibility of lutein.

4. In the presence of WPI, despite the improved carotenoid bioaccessibility, a slight negative effect on lipid digestion was found, perhaps due to the high surface hydrophobicity of β-lactoglobulin [32], as well as to its pepsinolysis-resistance (as shown by SDS-PAGE). These properties were reported to allow the adsorption of large peptides to the surface of lipid droplets, which could turn into a disadvantage, such as constituting a biological barrier to digestive enzymes, rendering WPI-stabilized emulsions more resistant to lipolysis. However, this negative effect on lipid hydrolysis disappeared during intestinal digestion, due to a complete digestion of WPI fractions (including β-lactoglobulin), leading to an improvement in the bioaccessibility of β-carotene by more than a third. This positive influence may be supported by the

amphiphilic behavior of WPI, and the abundance of both hydrophobic and hydrophilic groups, decreasing the surface tension and assuring the stability of the emulsions throughout the digestion process [2, 45]. However, it should be mentioned that, as for the other tested proteins, the bioaccessibility of lutein was also negatively impacted by the presence of WPI, in concentration-dependent manner.

In summary, the findings demonstrated the association between the digestion of macronutrients (proteolysis and lipolysis) and their impact on digesta characteristics such as surface tension and macroviscosity, which in turn could influence emulsion stability, repulsive forces and mixed micelle size, all of which influencing on carotenoid bioaccessibility. These results highlight the effects of various proteins on carotenoid micellization, constituting an important stage in their bioavailability.

9.1.4. The effect of proteins on the bioaccessibility and cellular uptake of carotenoids from carotenoid-rich food matrices

The static *in vitro* digestion experiments are often coupled with cell culture studies, such as with Caco-2 cells, to assess intestinal uptake of dietary food constituents including carotenoids. In Chapter 7, we went from isolated carotenoids (and in part isolated proteins) to more realistic, food-based models. For this purpose, we investigated potential interactions of proteins (WPI, SPI, SC, GEL, turkey and cod) co-digested gastro-intestinally at various concentrations with

carotenoids from either tomato juice, spinach, or carrot juice, and digesta were further studied in a Caco-2 cell model of the small intestinal lining.

The results showed that carotenoid bioaccessibility was influenced according to carotenoid hydrophobicity in the presence of proteins during the simulated GI digestion, which resulted in an improved fractional bioaccessibility in matrices containing higher amounts of nonpolar carotenes (such as tomato juice), with up to three-fold improvements, compared to matrices rich in more polar xanthophylls (such as spinach), with a reduction of over 50% in their bioaccessibility. These observations are in line with the findings reported in Chapter 6 with pure carotenoid species (lycopene, β-carotene, and lutein), mostly related to the polarity of each species that led to a different behavior in biological emulsions [36]. In addition, the results showed that the choice of the most suitable protein type could depend on the type of carotenoid to be solubilized. The following examples illustrate that statement.

1. Generally speaking, the presence of proteins improved the bioaccessibility of carotenes by up to 3-fold, while xanthophyll bioaccessibility was rather impaired. Consequently, the addition of proteins to the simulated GI digestion resulted in an improved fractional bioaccessibility in matrices containing higher amounts of nonpolar carotenes such as in tomato and carrot juices, compared to matrices rich in more polar xanthophylls such as in spinach. This confirms the results reported in Chapter 6. The adsorption of proteins at o/w interface may result in negative influences with xanthophylls, limiting their incorporation into the

mixed micelles, while carotenes seem to better benefit from the emulsifying properties of proteins, as they are located in the core of the emulsion.

2. The hydrophobicity of the investigated proteins may play a decisive role regarding the extent of carotenoid micellization. Carotene-protein interactions may occur with proteins with high surface hydrophobicity such as SPI, within hydrophobic pockets, promoting the transfer of the carotenes from the lipid droplets into the mixed micelles (Chapter 4), which could enhance their bioaccessibility [46]. Previous studies have demonstrated the successful use of SPI as a nano-carrier to enhance the stability and bioaccessibility of β-carotene [47, 48]. The authors explained the obtained result by the exposition of hydrophobic sites of SPI (following its denaturation), which fostered intermolecular interactions with apolar β-carotene (i.e., van der Waals forces and hydrophobic interactions) [47, 48]. This is in line with our results, as highest β-carotene bioaccessibility was obtained when co-digested with SPI, compared to other soluble proteins such as WPI and SC, improving micellization by almost three-fold. In contrast, the more polar xanthophylls, may show lower binding affinity to the hydrophobic sites of proteins, perhaps explaining the lowest micellization of lutein+zeaxanthin in the presence of SPI, compared to the more soluble SC and WPI.

3. Proteins from regular food matrices influenced carotenoid bioaccessibility to a lower extent, as compared to matrix-free proteins. This is possibly due to the fact that isolated proteins have weaker network structures and more cleavage sites,

and are hydrolyzed faster and to a greater extent than protein-rich foods [49]. In addition, the co-digested plant food matrices may have an additional impact on the protein network structure of the digested animal matrices, preventing enzymes from accessing protein and thus reducing protein digestibility.

4. The cellular uptake of carotenes appeared to well reflect the impact of proteins on the bioaccessibility of carotenes, i.e., being positive in all investigated matrices. A higher cellular uptake of carotenes was obtained in the presence of co-digested SPI (up to 33% increase in the uptake of β-carotene), followed by WPI and GEL, and finally SC. Interestingly, while the presence of SPI strongly reduced the cellular uptake of xanthophylls, similar as observed for their bioaccessibility, the effect of soluble WPI and SC on the cellular uptake of xanthophylls was somewhat contrarily to bioaccessibility, i.e., their presence had a positive effect on the Caco-2 cellular uptake of lutein+zeaxanthin (up to 12%), as compared to the control condition (no added protein). Overall, the presence of the proteins at the surface of the mixed micelles may lead to enhanced cellular uptake of both carotenes and xanthophylls, by facilitating the interactions of micelles with Caco-2 cells, making them potentially more available for cellular uptake [46].

In summary, the overall effect of proteins was a general improvement of fractional bioaccessibility in matrices containing higher amounts of nonpolar carotenes, compared to matrices rich in more polar xanthophylls. These observed effects on the bioaccessibility of carotenoids also depended on the protein

hydrophobicity, which likely influenced their distribution in the lipid droplets, and thus the extent of carotenoid micellization [36].

9.1.5. The impact of added proteins to a carrot-tomato juice on carotenoid bioavailability in healthy adults

In the previous Chapters, we have drawn novel conclusions on the influence of proteins on the bioaccessibility of carotenoids, using the harmonized static *in vitro* simulation model INFOGEST for studying protein-carotenoid interactions during simulated GI digestion.

In Chapter 8, we aimed to prove or disprove the results obtained *in vitro* on protein-carotenoid interactions. Specifically, a randomized crossover human trial was carried out to test whether proteins from different sources and with different hydrophobicity, can impair postprandial carotenoid absorption, as a marker of bioavailability in healthy humans. A carotenoid-rich test meal, i.e. a mixture of tomato and carrot juice, was served to twenty-four healthy adults (males and females) in the absence or presence of either WPI or SPI at a concentration equivalent to 50% of the RDA. Absorption kinetics of carotenoids were evaluated by measuring the TRL fraction response, representing the newly absorbed carotenoids, at timed intervals up to 10 h after test meal intake.

The findings showed that the two proteins, WPI and SPI, had significantly diverging effects on carotenoid absorption in the total participants, as determined by their AUC of plasma-TRL fraction of total carotenoids, confirming the previous *in vitro* observations. The

WPI-supplemented meal significantly increased the overall AUC of serum TAGs/plasma-TRL fraction of carotenoids (statistically pooled) by 45% compared to the control meal (no additional proteins), and by 57% compared to SPI-supplemented meal, while the latter did not differ significantly, as compared to the control meal.

In the previous *in vitro* trials, we have reported two opposite effects of various proteins on the bioaccessibility of carotenoids, which strongly depended on the type of protein and carotenoid. More digestible proteins, such as WPI, may produce proteins of small molecular size (e.g. casein proteins 20 kDa), especially at the gastric phase, with more rapid diffusion to the o/w interface. In fact, producing peptides of more amphiphilic structure that facilitate the interactions at the surface of the mixed micelles may offer kinetic advantages that go along with improved emulsification of lipophilic food constituents, thus fostering bioaccessibility and the cellular uptake of carotenoids (Chapter 7). However, certain proteins with a high surface hydrophobic nature and high molecular size such as SPI (e.g. soy glycinin 350 kDa), together with their lower gastric digestibility, were suspected to aggregate in the aqueous phase and/or adsorb in the form of long polypeptides at the o/w interface, thus trapping carotenoids and limiting the processing from lipid droplets to mixed micelles (Chapter 6). The *in vivo* results obtained in the present chapter seem to well reflect the *in vitro* effects of WPI and SPI on the bioaccessibility and cellular uptake of carotenoids.

Significant differences were found between SPI and WPI for individual carotenoids, as observed for the postprandial plasma AUC of TRL concentration of phytoene, phytofluene, β-carotene, and α-

carotene. Interestingly, higher bioavailability was found for phytoene and phytofluene compared to other carotenoids. Mapelli-Brahm et *al*. explained that higher molecular flexibility (i.e. apparent shorter chain length) of these colorless carotenoids [50] allows a better insertion into the mixed micelles, leading to relatively higher bioaccessibility [51] and cellular uptake [52].

It should be mentioned that WPI and SPI influenced carotenoid absorption similarly in men and women, but the effect of test meals did not reach a significant level in women. This sex difference was explained by the generally lower responses of AUC in women, due to the lower fractional uptake of carotenoids from the test meals. This reduced uptake may be due to women having displayed already higher plasma carotenoid concentrations compared to men (as observed in plasma carotenoid concentrations at the baseline), similar to previously reported findings of higher circulating carotenoid levels in women [53-56]. In other words, their higher carotenoid status went along with lower fractional absorption of carotenoids from the test meals.

In summary, this is to our knowledge, the first study finding effects of proteins on carotenoid absorption in humans. We were able to verify our initial hypothesis, i.e. proteins do influence aspects of carotenoid bioavailability either positively or negatively. On the one hand, protein adsorption to lipid droplet surfaces, attributed to the fact that proteins can be highly surface active molecules, led to stabilized o/w emulsions in the GI tract by preventing the aggregation of lipid droplets, which foster the transfer of carotenoids from lipid droplets into mixed micelles. On the other hand, protein adsorption to o/w

interfaces may also lead to a decrease in the enzymatic degradation of lipids, likely by hindering digestive enzymes to adsorb to the droplet surfaces or binding to enzymes directly, also implying potential negative influences of proteins on the processing of lipid droplets and thus the ensuing micellization process of carotenoids.

The results from the human study show a similarity to those reported in our *in vitro* trials. The findings suggest that a well digestible and soluble protein such as WPI, could be beneficial for carotenoid bioavailability, whereas a less digestible one such as SPI, may result in hampered overall bioavailability.

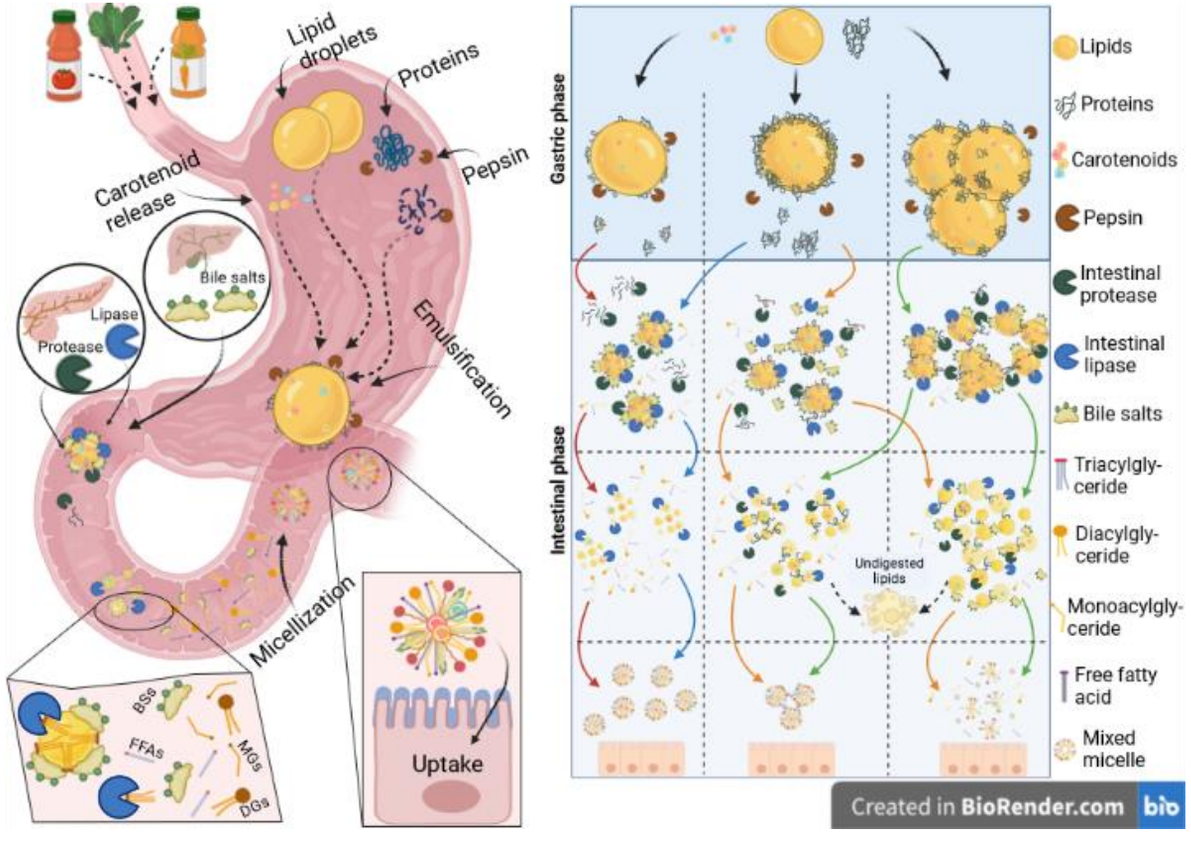

Figure 9.1. Summary of main findings on the influence of dietary proteins on relevant aspects of carotenoid bioavailability. Crucial steps of carotenoid bioaccessibility and cellular uptake in the gut (left panel). Different scenarios of carotenoid bioaccessibility and cellular uptake during various digestive conditions (right panel). **Red arrows.** Normal gastro-intestinal (GI) conditions with high peristalsis (**Chapter 4**) in presence of SC, GEL (**Chapters 6 & 7**), low concentrations of SPI (**Chapters 5, 6 & 7**), well soluble/digestible WPI (**Chapters 4, 5, 6, 7, & 8**) and low lipid amounts (Chapter 4). **Blue arrows.** Presence of gastric lipase (**Chapter 5**). **Orange arrows.** Low concentrations of pancreatic lipase or high amounts of lipids (**Chapter 4**), or presence of low soluble/digestible SPI (**Chapter 8**). **Green arrows.** High concentrations of WPI (**Chapters 4 & 5**) or SPI (**Chapters 5 & 6**).

9.2. General discussion

Although many of the properties of proteins can result in a beneficial effect on carotenoid bioaccessibility, these properties may be more akin to a double edged sword, with also possible negative consequences. In fact, the exposition of hydrophobic groups of the protein i.e. surface hydrophobicity, allowing a greater adsorption at o/w interfaces [57], should be considered with care. As an example, the high hydrophobicity of the β-lactoglobulin fraction of WPI [32], as well as its resistance to pepsinolysis [58], were reported on the one hand to offer stability and protection to the solubilized carotenoids in the acidic environment of the gastric digestion. On the other hand however, the adsorption of large peptides of the β-lactoglobulin fraction at o/w interfaces, could constitute a biological barrier to digestive enzymes [3]. This last scenario was reported in Chapter 6, for the interaction of β-carotene and WPI, which interfered with mixed micelle formation and hampered the bioaccessibility of carotenoids.

The aqueous solubility of proteins is assumed to ensure rapid adsorption to o/w interfaces [57]. The often low aqueous solubility of plant-based proteins as well as their high molecular size, were decisive for their relatively low emulsification performance [43]. Previous studies highlighted in particular the structural rearrangement of SPI occurring in the acidic environment of the gastric digestion, leading to depletion and bridging flocculation of lipid droplets [42]. In Chapter 6, the influence of SPI on the bioaccessibility of carotenoids depended on the protein concentration. At a high concentration (equivalent to 25 and 50% of RDA), the presence of SPI in the simulated digestion

negatively impacted on the processing of lipids and the incorporation of carotenoids into mixed micelles as well as their bioaccessibility. However, at 10% of RDA, SPI enhanced the bioaccessibility of β-carotene by almost a third, possibly due to the stabilized emulsions resulting from the high surface hydrophobicity. Unlike poorly soluble proteins, the relatively high solubility of SC and GEL as well as their stability at a wider pH range, were shown to be involved in the formation of emulsions with more dispersed oil droplets [43], which improved the bioaccessibility of carotenoids by almost 2-fold (Chapter 6).

In addition to solubility and surface hydrophobicity, protein flexibility is believed to be an important dynamic factor governing emulsification ability. It is assumed that flexible proteins are susceptible to protease digestion [59]. In fact, proteins undergo proteolysis at the surface of lipid droplets, which induces the rearrangement of proteins at the o/w interface and increases their surface hydrophobicity, resulting in good emulsifying properties. Caseins, α-lactalbumin and BSA, being well digestible, they are some examples of flexible proteins. A previous study using *in vitro* digestion reported good correlations between the emulsifying activity of these proteins and their flexibility, as detected by their susceptibility to pepsin and α-chymotrypsin digestion [59]. In Chapter 7, we have reported that producing peptides of amphiphilic structure has been especially noticed in SC and WPI. Following their GI digestion, the resulting peptides improved carotene micellization close to 3-fold, though negative interactions with xanthophylls at the O/W interface may occur, which limited their incorporation into the mixed micelles.

Similarly, SC and WPI fostered the cellular uptake of carotenes, and counteracted the somewhat negative effect of these proteins on xanthophyll bioaccessibility, by improving lutein+zeaxanthin cellular uptake. The digestion of WPI and SC may produce peptides that have more amphiphilic structure that can facilitate the interactions at the surface of the mixed micelles, making them potentially more available for cellular uptake [46].

Finally, proteins of small molecular size were described to have higher diffusion rates to the o/w interface, compared to larger protein fractions [60], which should offer kinetic advantages that may go along with enhanced bioaccessibility of emulsified carotenoids.

Other than relying on the aforementioned protein properties, the choice of the most suitable protein type could also depend on the digestive conditions and the type of carotenoid to be solubilized (e.g. different polarity). Based on the results from Chapter 6, similar to those based on drug-micelle interactions (Figure 2.2), very apolar constituents located in the core of mixed micelles may be differently influenced by the presence of proteins, compared to less apolar compounds situated at the outer part of micelles. Regarding the digestive conditions, which can vary significantly from individual to individual [61], less complete digestive conditions such as low concentrations of bile, pancreatin (or surplus of lipids), and also limited peristalsis could rather foster low carotenoid bioaccessibility in the presence of proteins, possibly due to entrapment of carotenoids in the unprocessed lipid phase, as reported in Chapter 4.

9.3. Conclusions

Among the various mechanisms by which dietary proteins impact on carotenoid bioavailability, the present research indicates that positive effects are essentially linked to their emulsifying properties. Several protein properties have been described to play a role in the capacity of proteins to adsorb and form an interfacial film at lipid droplet surfaces to ensure emulsion stability [62]. These properties of proteins include sufficient surface hydrophobicity, low molecular size, high amphiphilicity, protein solubility, flexibility, digestibility and surface charge (pI and ionic strength). These properties could translate into main effects influencing the bioaccessibility of carotenoids, namely involving the processing of lipid droplets, which start in the stomach (Figure 2.1), and ensuing incorporation into mixed micelles as well as their stability in the small intestine (Figure 2.2). However, the influence of proteins on carotenoid bioaccessibility could also be linked to direct carotenoid-protein interactions, via hydrophobic pockets within peptides, which ensure the solubility of these fat-soluble compounds in the GI tract and their prevention of oxidation [16, 17].

In the present thesis, the findings suggest that a well digestible and soluble protein such as WPI, could be beneficial for carotenoid bioavailability, whereas a less digestible one such as SPI, may result in hampered overall bioavailability. Relying on the aforementioned protein properties, as well as the results obtained from the present work, the tendency of many animal-derived proteins to be soluble over a wider pH range, to be of a relatively smaller size, and to be more

flexible than plant-based proteins, appear to make those animal proteins a better choice for improving the bioavailability of carotenoids. Their properties allow for a more rapid diffusion to the o/w interface in order to stabilize the lipid phase within an emulsion [62], resulting in smaller droplet size, eventually facilitating the transition of lipid droplets and contained liposoluble bioactive compounds such as carotenoids into mixed micelles [2].

9.4. References

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Chapter 10

Perspectives

Adapted from: Influence of proteins on the absorption of lipophilic vitamins and secondary plant metabolites – a review

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10.1. Considerations regarding additional analytical experiments

The digestion of macronutrients such as of proteins and lipids seems paramount for the extent of carotenoid bioaccessibility. In the present work, the quantification of protein hydrolysis has not been performed, we have carried out SDS-PAGE to visualize the hydrolysis of investigated proteins in most of the experimental chapters. It would be interesting in the future to follow the fate of smaller protein fragments by coupling SDS-PAGE visualization with an optical densitometry, or use more sophisticated methods such as LC-MS/MS to quantify the released amino acids or remaining small petptides in the digesta. This could allow for a more detailed association analyses between protein digestion and carotenoid bioaccessibility. Similarly, the lipid digestion results obtained by fatty acid titration in Chapter 4 were not fully informative, it would be interesting to follow free fatty acid release in the digesta, e.g. by GC-MS (after methylation of the fatty acids), which was not carried out in the present study, though the additional benefit without determining mono-and diglycerides may have been limited. Both LC-MS/MS and GC-MS/MS were not available for our project.

Regarding cellular uptake, the Caco-2 cell model allows studying the uptake of carotenoids under reasonable physiological conditions, as most transport proteins present in the enterocytes are expressed in these cells. Thus, this model allows to obtain a better understanding of the bioavailability of carotenoids [1]. In Chapter 7, we measured the uptake of carotenoids by Caco-2 cells, following a 4 h

incubation with a digesta. Despite washing cells with PBS prior to cell lysis and carotenoid extraction from Caco-2 cells, carotenoids may remain attached (i.e. adsorbed) to the cell surface, which could lead to errors in the assessment of the uptake of carotenoids by Caco-2 cells. To clarify any remaining doubt, it could be advised in the future to take some confocal laser scanning microscopy images of the apical membrane of Caco-2 cells, in order to visualize the absence or presence of carotenoids on the cell surface, which is possible thanks to their natural fluoresence. Photos of the interior cell can also be taken in order to show carotenoid fluoresence inside the celles, representing carotenoids taken up by Caco-2 cells.

In the human study in chapter, we have determined the AUC of plasma-TRL fraction of carotenoids, representing the newly absorbed carotenoids after test meal intake. However, to achieve an even more accurate assessment of carotenoid absorption, the bioconversion of provitamin A carotenoids (β-carotene, β-cryproxanthin and α-carotene) into vitamin A may be considered. Vitamin A palmitate could thus be attempted to option to overcome such a challenge in our study, would be the use of test meals containing intrinsically labelled carotenoids. For instance, the administered plant food matrices can be intrinsically labelled with either the addition of a carbon-stable isotope presented in the gas atmosphere as $^{13}CO_2$, or through the addition of a hydrogen-stable isotope presented to the roots in the form of heavy water ^2H_2O [2]. This allows the formation of the carotenoids in their normal cellular compartments, and the isotopic label enables identification of those serum carotenoids as well as their derived retinol, which originate from tomato and carrot juice mixture.

However, this would entail considerable costs and times (for plant growing) and likely further ethical considerations. Extrinsic labelling with already labelled and added carotenoids is also possible, but bioavailability is expected to differ from intrinsic carotenoids.

10.2. Considerations of other protein sources

Due to consumer concerns about ethical, environmental (i.e. sustainability), and health issues (e.g. allergic reactions and also associated chronic diseases) associated to some extent with animal-based proteins, the use of plant proteins has been receiving more interest recently, especially from pharmaceutical and food industries, for encapsulation and delivery of lipophilic compounds. Being cheaper, renewable, and biodegradable, legume proteins for example are a rich source of proteins such as albumins, globulins, and prolamins, though with relatively low solubility and poorer functionality compared to animal-based proteins [3, 4].

It should be stated that large differences in the digestibility of different plant-based proteins have been reported. For instance, SPI has been shown to have a lower digestibility than faba bean proteins [5], which may result in different effects on the bioavailability of carotenoids compared to those reported in our human study. In addition, the extent of proteolysis of faba bean proteins after GI digestion was reported to be very similar to that of whey proteins [6]. Another research group has recently assessed the *in vitro* apparent protein digestibility of whey and faba bean protein isolates (defined as nitrogen contained in the peptides lower than 10 kDa and free amino

acids of the soluble fraction) and calculated the protein digestibility-corrected amino acid scores (PDCAAS, by adapting the methodology of Food and Agriculture Organization of the United Nations and World Health Organization [7]). Results indicated similar protein digestibility and PDCAAS (ca. 90% and 75%, respectively) for whey and faba bean proteins, suggesting that faba bean proteins could be a good candidate for whey protein substitution for certain foods [8], at least if the emphasis is put on digestibility. By extrapolating the results obtained in the present thesis in terms of influence on carotenoid bioavailability, to these proteins, one could speculate that the more digestible faba bean protein that behaves in a somewhat similar way than whey protein during digestion, could be a good alternative to the animal-based protein source, in terms of positive influence on the bioaccessibility of carotenoids. However, to our knowledge, faba bean proteins have not been studied regarding their effect on carotenoid bioavailability.

In line with consumer demands for animal protein alternatives, mycoproteins (also known as fungal proteins) have been widely used, for instance as Quorn products for their good imitation of meat structure. As the name suggests, this protein-rich product is made by fermentation processes from a specific type of fungus, *Fusarium venenatum* [9]. Its dry weight contains approximately 45% protein, 25% fiber, and 13% fat (typically low in saturated fat) [10], thus showing desirable nutritional properties. In fact, a study involving 100 healthy adult volunteers has confirmed that the biological value of that mycoprotein was similar to milk protein, i.e. 85%, or superior to it when supplemented with methionine, i.e. 92% [11]. Protein

digestibility and PDCAAS were reported to be of 91% and 78%, respectively [9], which suggests that these mycoproteins, might be a good alternative to animal proteins, also to positively modulate the absorption of carotenoids. However, the high fiber content of these products may result in detrimental effects regarding carotenoids, as dietary fibers are well known to lower their bioavailability [12].

Fish and other seafood are also playing a key role as main protein sources. Although the available *in vitro* data suggest that the bioaccessibility of carotenoids such as astaxanthin from salmon is relatively high, it is difficult to conclude whether the high bioaccessibility is due to the naturally lipid-rich matrix, its protein content, or even the nature of the xanthophyll [13]. However, our *in vitro* results from Chapter 7 showed that cod proteins had only a limited impact on the bioaccessibility of carotenoids, unlike other chosen protein isolates. A range of fish protein powder products with higher protein content than the original fish flesh, are now developed as functional food, with a protein content of at least 70% [14]. The *in vivo* digestibility of raw fish flesh proteins is in the range of 90–98 %, while protein efficiency ratio (an index of protein quality, based on the weight gain of a test subject divided by its intake of proteins during the test period) is slightly above that of casein, the major milk protein [14]. To our knowledge, there are no data yet concerning the influence of the fish protein powder products, as encapsulation material or in added form, on the absorption of carotenoids. However, the high digestibility of fish protein powder could be a good indicator to hypothesize that these proteins may have also a positive impact on the bioavailability of carotenoids.

10.3. Global nutritional aspects

There is a wide range of proteins from natural sources or further processed for the purpose of human consumption, but only a limited range of well-studied proteins, with respect to their emulsifying properties, is available. Therefore, finding suitable proteins in terms of their influence on the bioavailability of liposoluble bioactive compounds such as carotenoids, constitutes an additional challenge.

The knowledge obtained from this thesis may benefit the growing global carotenoid market size, which was USD 1.44 billion in 2019 and is projected to reach USD 1.9 billion by 2028 [15]. In fact, many marketed dietary supplements and nutraceuticals do contain carotenoids of various species (astaxanthin, β-carotene, lutein, zeaxanthin, lycopene, canthaxanthin, and others) and sources (synthetic and natural). Most marketed carotenoid supplements are available in the form of softgels, with carotenoids being dissolved in different types of oils (among many others: extra virgin olive, soybean, and sunflower oil). As industries increasingly offer lipid-reduced products or alternatives, proteins could therefore be good candidates. Similarly, many functional foods are enriched/fortified with a variety of carotenoid species, though not all compounds have gained a substantiated EFSA (European Food Safety Authority) health claim that allows for a somewhat improved advertising of the products. Nevertheless, the knowledge acquired from this thesis on the interaction of proteins with carotenoids, and on how to improve the bioavailability of these nonpolar phytochemicals, would constitute

valuable information. In fact, the obtained insights may be of interest to spark improvements on the formulation of carotenoids in such dietary supplements and functional food items, e.g. with respect to protein quantity and/or type. This might be a potential asset toward claiming enhanced bioavailability, though admittedly this will likely depend on the interaction of all individual compounds of each functional food item.

The insights of the present thesis would also be of relevance for health related associations, in terms of a more optimized dietary composition of various food items. For instance, many consumers may prefer low-fat salad dressing substitute. A high-protein dressing might be a good alternative [16, 17], being rich in either whey or soy proteins, resulting in a salad dressing with protein content ranging from 14 to 30 %. One could hypothesize that WPI-based salad dressings may help to improve bioavailability of apolar carotenoids from the various vegetable matrices used in a salad, especially when these bioactive plant compounds are consumed with a low-fat food matrix. In this regard, several of the fat-free dressings may be superior than their reputation regarding their quality to enhance the absorption of lipid-rich food constituents [18].

Beyond the use of proteins to improve the bioavailability of bioactive compounds, replacing lipids with proteins could contribute also to other beneficial effects from a nutritional perspective. In fact, the ability of dairy peptides, such those from WPI, to improve glucose homeostasis and elicits a strong effect on satiety is generally well recognized [19-21], which would be an additional argument, from a nutritional point of view, in favor of adding WPI to aid the absorption

of carotenoids. For some consumers, such as vegans/vegetarians, the ingestion of Quorn mycoprotein was also reported to reduce energy intake and insulinaemia [9, 22-24]. Despite the fact that the influence of Quorn mycoprotein on the absorption of carotenoids remains to be confirmed, being a good alternative to animal proteins such as WPI due to its good digestibility, could suggest that Quorn mycoprotein could have similar beneficial effects on the bioavailability of carotenoids, compared to those reported with WPI in our *in vivo* trial.

In line with nutritional perspective, the information obtained from the present thesis would be of particular interest to some consumers with low intake of animal products. These consumers include vegetarians and vegans (looking for a more sustainable and nutritious source of protein), or people living in rather poor areas (such as in developing countries where the access to meat and thus preformed vitamin A is often limited), as they do rely on vitamin A intake via carotenoids. It should be stated that vitamin A deficiency is still among the major nutrition-related deficiencies globally, affecting over 250 million schoolchildren worldwide [25]. Vitamin A deficiency leads in an increased risk of infections, diarrhea, and blindness, being a public health concern in 45 out of the 122 countries investigated in the World Health Organization report [25]. As not only a sufficient intake, but also a good bioavailability of provitamin A carotenoids is paramount, an optimal combination of fruits and vegetables or their timed intake with non-animal proteins such as those from fungi (e.g. Quorn mycoprotein [9, 22]) or legumes (e.g. faba bean protein [6, 8]), which showed similar behaviour to WPI during digestion, might be an

effective way to enhance carotenoid uptake and assure a high vitamin A status.

As the low water solubility of carotenoids requiring incorporation/solubilization into lipid droplets and the formation of mixed micelles [26, 27], is a common similarity that is shared by fat-soluble vitamins (A, D, E, and K), apolar polyphenols e.g. curcumin or resveratrol, as well as plant sterols/stanols, the results obtained from this thesis may, to some degree, be extended toward the bioavailability of these liposoluble micronutrients and phytochemicals, though this is somewhat hypothetical and needs to be shown in ensuing research.

A further, more general benefit of enhancing carotenoid bioavailability from the diet may lie in their contribution to combatting major chronic diseases, such as metabolic syndrome [28], cardiovascular diseases including type 2 diabetes [29], and also age-related macular degeneration (the major cause of vision loss in the elderly [30]), via their anti-inflammatory, antioxidant, immuno-regulatory and anti-cancer properties [31-33], though further research in this domain is surely warranted.

10.4. Future research directions

An array of studies have been conducted, though typically on a rather limited number of proteins, encompassing studies with animal models such as in mice [34-36], a few human studies [37-39], and a large number of *in vitro* investigations (Table 2.2). In general, the latter support results on living beings, i.e. protein encapsulation was highlighted to offer stability and protection of sensitive molecules

against oxidation, and enhanced the aqueous solubility and bioavailability of liposoluble constituents such as liposoluble vitamins, as well as secondary plant compounds such as carotenoids. However, a number of knowledge gaps remain.

One of the important aspects to consider is that limited data exists on direct interactions between dietary proteins and carotenoids during digestion. Orange carotenoid protein, glutathione-S-transferase pi isoform 1, and crustacyanin are examples of carotenoid-binding proteins within living cells that have been well characterized [40], and protein-carotenoid complexes may well form during GI digestion. A study by Mensi et *al*. has shown this type of interaction *in vitro*, i.e. the ability of β-lactoglobulin to bind β-carotene within its interior cavity [41], which may act as a vehicle of hydrophobic carotenoids in an aqueous environment during digestion, but this remains to be demonstrated *in vivo*.

Similarly, only very little is known about how the cellular uptake is affected by the interaction of proteins with carotenoids. Lu et *al*. proposed that protein emulsifiers such as WPI and SC may improve carotenoid uptake by facilitating the interaction of micelles with Caco-2 cells [42], but detailed mechanistic studies in this area do not exist.

A further neglected factor of carotenoid bioavailability and their further metabolism is the human colon. It is uncertain if it can contribute to carotenoid absorption. It is known that microbiota is capable to catalyze a large number of chemical reactions on a diverse array of non-absorbed foods [43], e.g. fermenting fiber or certain polyphenols, producing short-chain fatty-acids and other degradation

products [44]. However, regarding carotenoids, not much is known on the potential bioavailable fraction or type of metabolites/degradation products, such as further cis/trans isomers, apocarotenals, or similar, which may likewise be bioactive, given that absorption occurs in the colon. A recent study has also indicated a potential prebiotic like effect of carotenoids on the gut microbiota [45]. The interaction of carotenoids with the colon constitutes an important gap deserving further research.

Finally, the release kinetics of lipophilic food constituents from encapsulated materials are still poorly understood, but can be expected to be crucial for developing effective delivery systems for such compounds. Thus, designing studies to understand the release kinetics of lipophilic compounds under physiological relevant digestive conditions is paramount. This will allow for a better understanding on the influence of various proteins on the timely release behavior of apolar food compounds such as carotenoids, and their GI processing from their presence within lipid droplets to mixed micelles in the gut [46].

Therefore, further *in vitro* studies as well as human clinical trials are desired to better explore protein structure-function relationships and their interactions with lipophilic food constituents to overcome the above mentioned limitations and gaps for the use of proteins to enhance the bioavailability of carotenoids, and enabling proteins to be used in a wide range of fat-soluble bioactive compounds.

10.5. References

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Carotenoids are secondary plant metabolites with antioxidant and anti-inflammatory properties. Some can be converted into vitamin-A. Their dietary intake and tissue concentrations have been related to the reduced incidence of several chronic diseases. Due to their low water solubility requiring emulsification prior to absorption, carotenoids are of low and varying bioavailability. While dietary fiber, lipids, and minerals were shown to influence the solubilization of carotenoids, a prerequisite for their bioavailability, the interactions between proteins and carotenoids during gastro-intestinal (GI) digestion have, to our knowledge, never been studied systematically. The purpose of the PhD thesis was thus to study interactions of carotenoids and frequently consumed proteins during digestion that could impact aspects of carotenoid bioavailability. Preceding a confirmatory human trial, *in vitro* studies were conducted, by means of GI digestion coupled to Caco-2 cellular uptake models to investigate bioaccessibility and cellular uptake, respectively.

When investigating effects of whey and soy protein isolate (WPI, SPI), gelatin and sodium caseinate on individual carotenoids, carotene bioaccessibility increased by almost 2-fold, while xanthophylls were reduced in their bioaccessibility to about half. Similarly, for real food matrices, bioaccessibility and uptake were more enhanced for apolar carotenes vs. xanthophylls. In the human trial, WPI improved by about 1/3 the bioavailability of carotenoids from the tomato/carrot juice, while SPI had a borderline negative effect, confirming *in vitro* results.

These findings may aid consumers in choosing meal compositions, especially persons relying on carotenoids for meeting vitamin A requirements, such as vegetarians, or those living in areas with low available pre-formed vitamin A. The results may also be interesting for food/supplement producers, offering insights into formulations that assure high carotenoid bioavailability.

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