Methods for Assessing Aspects of Carotenoid Bioavailability

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Abstract: Carotenoids are a group of C-40 isoprenoid-based molecules with >600 representatives in nature, of which approximately 30 are of importance within our daily diet. This class of phytochemicals has recently attracted much attention due to potential health beneficial effects associated with carotenoid consumption, including reduction of cardiovascular diseases, protection from age-related macular degeneration, various types of cancer, and perhaps, bone health. Therefore, an increasing number of studies have been carried out, focusing on carotenoid bioavailability from the diet, which is typically low, in the magnitude of 1-50%, successive metabolization and measuring carotenoid status. However, up to date, there is no clear consensus on how to measure carotenoid bioavailability and status. A number of methods have been developed to measure certain aspects of bioavailability, including *in vitro* studies assessing matrix release and micellarization, i.e. bioaccessibility, uptake or transport into cells simulating the human small intestine (e.g. Caco-2 cells), animal experiments, and also human studies. However, these techniques do not necessarily yield well-correlated results. In living beings, carotenoids may be determined in different tissues, including plasma, plasma triacyl-rich lipoprotein fraction reflecting newly absorbed carotenoids, or various target tissues where carotenoids do accumulate to some degree, including the retina, liver, or adipose tissue. Isotopic methods employing stable or radioactive labeled carotenoids have been developed to differentiate between endogenous and exogenous carotenoids and to estimate utilization from single meals. The relation between carotenoid intake, uptake, absorption, distribution, metabolization/ excretion, and status demand a good understanding of the existing methods to assess these various aspects of bioavailability across different models.

Keywords: Carotenoids, bioavailability, bioaccessibility, absorption, status assessment.

INTRODUCTION

Carotenoids are C-40 polyene structures, which cannot be synthesized by humans or animals, therefore relying on their uptake from diet. Carotenoids and their derivatives have many fold functions during development [1], for the immune response [2] and the vision cycle [3, 4]. There is also growing evidence for their preventive role in a number of chronic or age-related diseases, partly but not only due to their antioxidant properties. For example, carotenoids have been proposed to impede the onset and proliferation of several types of cancer, including cancer of the lung [5, 6], prostate [7, 8], and breast [9,10]. It has been suggested that some carotenoids, especially when consumed in the form of fruits and vegetables, alleviate cardiovascular disease [11, 12], and may play a beneficial role for bone health and density [13, 14]. Lutein and zeaxanthin seem to be of further importance for the proper functioning of the human retina of the eye [15, 16]. As a result, there is increasing interest in the relation between carotenoid intake from the diet, bioavail-ability, carotenoid status, and the relation to chronic diseases.

While profound data on carotenoid content in foods are available [17-20] and knowledge on dietary intake of carotenoids has been rapidly increasing [14, 18, 21], knowledge on their bioavailability, i.e. the amount of carotenoids that are absorbable and can be used for specific physiological functions, is still incomplete. Carotenoid bioavailability is influenced by a variety of factors often termed as SLAMENGHI [22, 23], including the a) species of carotenoid, b) molecular or chemical linkage, c) amount of carotenoids ingested, d) effects of food-matrix, e) effectors of absorption and bioconversion (i.e. presence of enhancers/ inhibitors), f) nutrient status of the host, g) genetic factors h) host-related factors and i) and the interaction of the above factors.

This discrepancy is, at least partly, due to a lack of standardized methods to assess bioavailability or aspects of it, such as carotenoid release from the matrix, solubility in the gut, uptake by the mucosa, sequestration into chylomicrons, distribution in the bloodstream and incorporation into other tissues (Fig. **1**). Even though a number of methods have been proposed to determine aspects of carotenoid bioavailability, there is often limited comparability between results obtained, as these are prone to vary largely between the type of carotenoid studied, food matrix investigated, test system or species employed, tissues sampled, and carotenoid detection methods. The same can be said for the determination of carotenoid status - a measure of how well the body has been in supply of a specific carotenoid. The most appropriate approach to investigate carotenoid bioavailability is naturally the human body. However, as human studies can be very expensive, time consuming and ethically disputable, many investigations have focused on more simple models, even if not all aspects of bioavailability can be sufficiently

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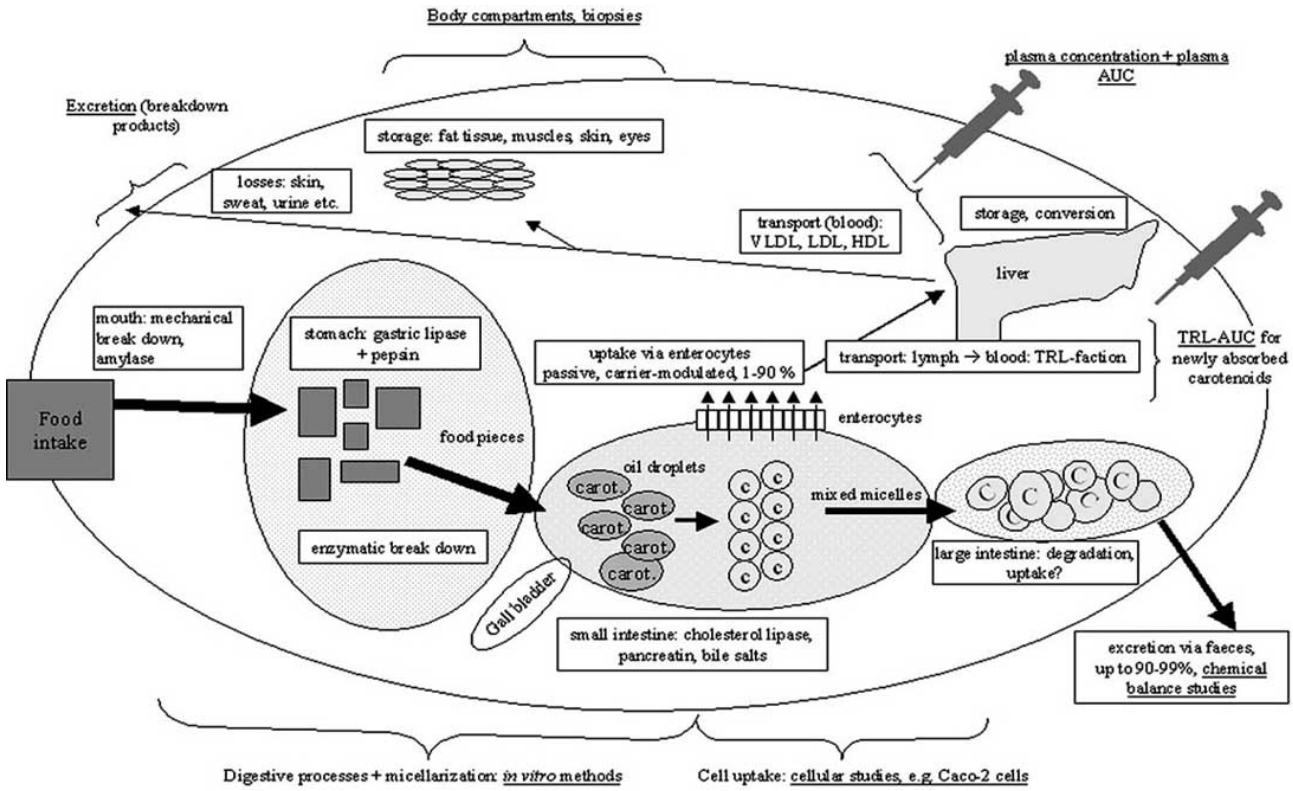

Fig. (1). General overview on carotenoid digestion, uptake, and distribution, together with potential techniques to determine aspects of carotenoid bioavailability.

simulated, and results not directly transferred to humans. Among these techniques are *in vitro* models, allowing to study solubility aspects such as micellarization of carotenoids [24, 25], following gastrointestinal digestion. These experiments are often coupled to cell culture experiments such as Caco-2, simulating the small intestinal environment [26, 27] allowing to study uptake and transport mechanisms. Rapidity and ease of use make these *in vitro* techniques a valuable screening tool in carotenoid utilization. A variety of animal species, from isolated gut sacs [28] to complete animal models, especially rats and mice [29, 30], have been used to assess carotenoid bioavailability, as they allow assessing also transportation in the bloodstream and tissue distribution. However, the physiology of many of these animal species differs considerably from humans, e.g. in the intestinal flora, conversion of provitamin A into retinol, and distribution between lipoprotein fractions in blood plasma, making it difficult to compare results across models and even studies and to infer to humans. In addition, ethical concerns have been increasingly confined their usage. The aim of this review is to summarize present methods available to assess carotenoid bioavailability and status and to discuss their limitations and comparability, ranging from *in vitro* screening techniques to *in vivo* animal and human investigations.

CAROTENOID INTAKE, ABSORPTION, AND DISTRIBUTION

In order to compare various techniques for measuring aspects of carotenoid bioavailability, it is important to comprehend the pathways of carotenoids following digestion, uptake, absorption, and distribution in the human body. Considering the aqueous environment in the digestive tract, challenges arise during the transport of lipophilic carotenoids from the food matrix to the enterocytes, including release from the matrix, transfer to lipid droplets, and incorporation into mixed micelles, emulsions of lipid droplets.

In a first step, carotenoids have to be released from food particles during mechanic and enzymatic breakdown starting in the mouth. Following this step, proteins are hydrolyzed by pepsin in the stomach and about 10 to 30% of the ingested triacylglyerols are cleaved by gastric lipase, yielding diacylglycerol and free fatty acids [31]. While most carotenoids are still matrix-bound at this stage, free carotenoids are dissolved during the formation of lipid droplets, mostly consisting of di- and triacylglyerols, free fatty acids and cholesterolesters [32]. The predigested food is then stepwise released via the pylorus into the duodenum, where the pH is increased by sodium bicarbonate. The lipid droplets are further processed under the influence of pancreatic lipase and the release of other lipid soluble compounds from the food matrix such as cholesterol, resulting in the formation of mixed micelles of ca. 8 nm diameter [33, 34], containing additional phospholipids, monoglycerides, fat-soluble vitamins (A, D, E, K), and bile acids required for emulsification.

Differences between various carotenoids exist during micellarization. Rather unpolar carotenoids such as the carotenes or remaining xanthophyll esters move preferably into the core of the micelles, while polar carotenoids may rest at the surface [35]. However, some carotenoid esters are

cleaved by cholesterol esterase [36], acting at the interphase of the micelles and the aqueous phase. Earlier studies suggested carotenoid uptake by the enterocytes through passive diffusion via the aqueous layer and cell membrane [28, 37, 38], while recent data has favored a facilitated uptake via membrane transporters, such as scavenger receptor class B, type 1 (SR-B1) [39-44]. These processes have been suggested to occur mainly in the small intestine, with carotenoid uptake in the large intestine assumed to be low, even though carotenoids can reach the large intestine intact and may be available for absorption [45, 46]. During gastrointestinal passage carotenoids however can be further degraded, such as by bacteria in the large intestine, perhaps by as much as 80% [46], further decreasing bioavailability. In fact, carotenoid absorption is usually ranging for beta-carotene from 3% [47] and 22% [48] up to 90% [49], while, on average, more polar xanthophylls seem to be slightly better available [23, 50], with exception of epoxyxanthophylls such as violaxanthin, which seem to be of lower bioavailability [51, 52].

However, during the subsequent transfer through the enterocytes, provitamin A carotenoids are partially cleaved by 15-15'monooxygenase (BCO1) [53, 54], converted into retinal by retinal reductase, reduced to retinol, and esterified, preferably into retinyl palmitate such as by lecithin:retinol-acyltransferase (LRAT) [55]. In addition to this central cleavage, asymmetric cleavage, such as through 9'10' monooxygenase (BCO2) [56], can occur, leading to the formation of beta-apo-10'-carotenal and beta-ionone. This enzyme has also been shown to cleave non-provitamin A carotenoids such as lycopene [57, 58]. A part of the carotenoids might also be released back into the gut, such as by cell sloth [34]. Intact carotenoids, together with retinyl esters, can then be secreted from the Golgi apparatus of the enterocytes into the lymph in form of triacylglyerol-rich chylomicrons, reaching the blood stream via the thoracic duct [34]. Passing the liver, carotenoids are either stored in the membrane fraction, cytoplasm and lipid droplets of the hepatocytes [59-61], or re-enter the bloodstream within low-density lipoprotein (LDL) and high density lipoprotein (HDL) particles, with hydrocarbon species preferably trans-ported by LDL and VLDL (very low density lipoproteins), and xanthophylls about equally by HDL and LDL [reviewed by 62]. Carotenoids can then be transported to different tissues, especially to those with a high expression of LDL-receptors [63], such as adipose [64], prostate [65] or adrenal tissue [66]. Some carotenoids, especially lutein and zeaxanthin do accumulate in the macula of the retina of the eye [16]. However, in general terms, it seems that carotenoids are rather generally and evenly distributed and stored throughout the entire human body and that there exists no primary target tissue. The further fate of carotenoids is less well understood. It is noteworthy that carotenoids undergo a certain degree of isomerisation during absorption and distribution in the human body, with cis:trans ratios differing between different carotenoids and tissues. While a large part of all-trans lycopene seems to be isomerized into cis-lycopenes [65, 67, 68], beta-carotene is predominantly present in the all-trans form in humans [63]. Also, cis:trans ratios could differ between body tissues, as isomerisation has been suggested to progress continuously after ingestion, until reaching an equilibrium [67, 68]. Excretion of the carotenoids or their degradation products [69] occurs predominantly via bile and pancreas into the feces, urine excretion being negligible [70], with exception of retinol, which may be increased up to 1000 fold during infection, up to 10 μmol/d in urine [71].

CAROTENOID DETECTION

Most carotenoids, with exception of some precursors such as phytoene and phytofluene absorb visible light between 400 and 500 nm and possess high molecular absorption coefficients, around 125.000-155.000 [72, 73], allowing for rapid and relatively sensitive quantification via VIS spectrometry [74, 75]. Without prior separation, only total carotenoid concentration can be determined at best, being often impeded due to the presence of other chromophores such as chlorophylls in plant extracts [76] or, in more polar extracts, also vitamins such as riboflavin. Nonetheless, different spectrophotometric protocols have been proposed for carotenoid determination [77, 78]. Being more time and cost intensive, reverse-phase high-performance liquid chromatography (RP-HPLC) coupled to UV/VIS photodiode array detection (DAD) is widely used, as it allows for the simultaneous separation, detection and quantification of individual carotenoids in aqueous solutions [79, 80]. Due to the chemical properties of the carotenoids, C-18, and, more recently, C-30 stationary phases have been frequently employed, as they allow for the discrimination between geometrical isomers [81, 82] and separation of very similar carotenoids, especially lutein and zeaxanthin, which is difficult to achieve on a C-18 phase. Although being a valuable tool for carotenoid detection in food samples, RP-HPLC coupled to UV-VIS has its limitations for detecting low abundant carotenoids or metabolites, such as found in human/animal tissues or plasma. HPLC-DAD techniques typically show detection limits around 10-200 ng/mL [83, 84], depending among other on the matrix. Therefore, more sensitive detectors have been coupled to HPLC, such as electrochemical detection, being considerably more sensitive compared to UV-VIS (>100 times), allowing the detection of 0.1 ng/mL carotenoid standards [85]. As carotenoid determination by fluorescence is rather of poor sensitivity and selectivity [86] this technique has not been used for carotenoid detection. Coupling liquid chromatography to mass spectrometry can be highly sensitive, offering additional information about the molecule monitored, i.e. parent mass or fragment products. Especially atmospheric pressure chemical ionization (APCI) coupling methods have been developed, both for HPLC-MS [87-89] and tandem HPLC-MS-MS, the latter allowing quantification of levels down to 0.1 ng/mL in human plasma [90].

METHODS FOR ASSESSING ASPECTS OF CAROTENOID BIOAVAILABILITY

In Vitro Digestion and Micellarization

In 1981, Miller and colleagues [91] described a method for estimating iron availability *in vitro*, providing a relatively rapid, simple, low cost approach for mimicking gastric and small intestinal stages of digestion. Simulating human

digestion in the upper parts of the GI tract, the so-called bioaccessibility, the amount of a nutrient or non-nutrient that can be released from the matrix and is available for absorption, can be determined. Typically, gastric digestion is simulated at 37°C with pepsin at pH 2-3 in a water bath, followed by pH adjustment to pH 7-7.5, and addition of a mixture of pancreatin and bile acids (Fig. **2**). Occasionally, an additional step simulating chewing and enzyme digestion during the oral phase with saliva containing α-amylase is included [92]. This *in vitro* technique has been used later for a variety of compounds, including various elements [93, 94], amino acids [95], phytosterols [96], and carotenoids (Table **1**). In order to determine the amount of a compound that is released from the matrix, the amount of carotenoids emulsified into the aqueous phase, i.e. the percentage of carotenoids present in mixed micelles, is quantified. Often, micellarization experiments have been coupled to cell lines resembling the small intestinal mucosa (see following chapter).

However, the percentage of micellarization yields quite relative values, depending, among other factors, on the concentration of carotenoids in the test meal and its composition, especially the amount and type of lipids present [97], pH, enzyme concentration, bile acids added, and digestion time [26], making it difficult to standardize experiments and to compare results across studies (Table **1**). Another drawback of this model is being a static one, i.e. concentrations of food and enzymes being constant reaching an equilibrium during the gastric and small intestinal phase, which is not the case *in vivo*. Even though dynamic models have been developed [98, 99], these models have been hardly used due to their increased complexity. Finally, even though micellarization is the first important step for carotenoid absorption, a decrease of micellarization may not necessarily result in lower uptake by the mucosa (see following chapter). It has been speculated that the positioning of carotenoids within the mixed micelles, i.e. at the surface vs. the core, which is typically not determined during micellarization experiments, could impact the following transfer and uptake of carotenoids into the enterocytes [35,100]. In general, micellarization of carotenoids varied from approx. 1-2% for all-trans lycopene [97, 101] to 80% for trans-lutein [24].

Besides these drawbacks, the *in vitro* digestion method has been frequently used as a screening tool for studying the impact of several dietary factors on carotenoid release and solubility. It has been implemented for the investigation of various food matrices, including raw, mixed salads and vegetables [97, 102-106], citrus juice and applesauce [27, 107], baby food [26], to complex, boiled meals based e.g. on potato and beef [44]. It has further been used to study to the

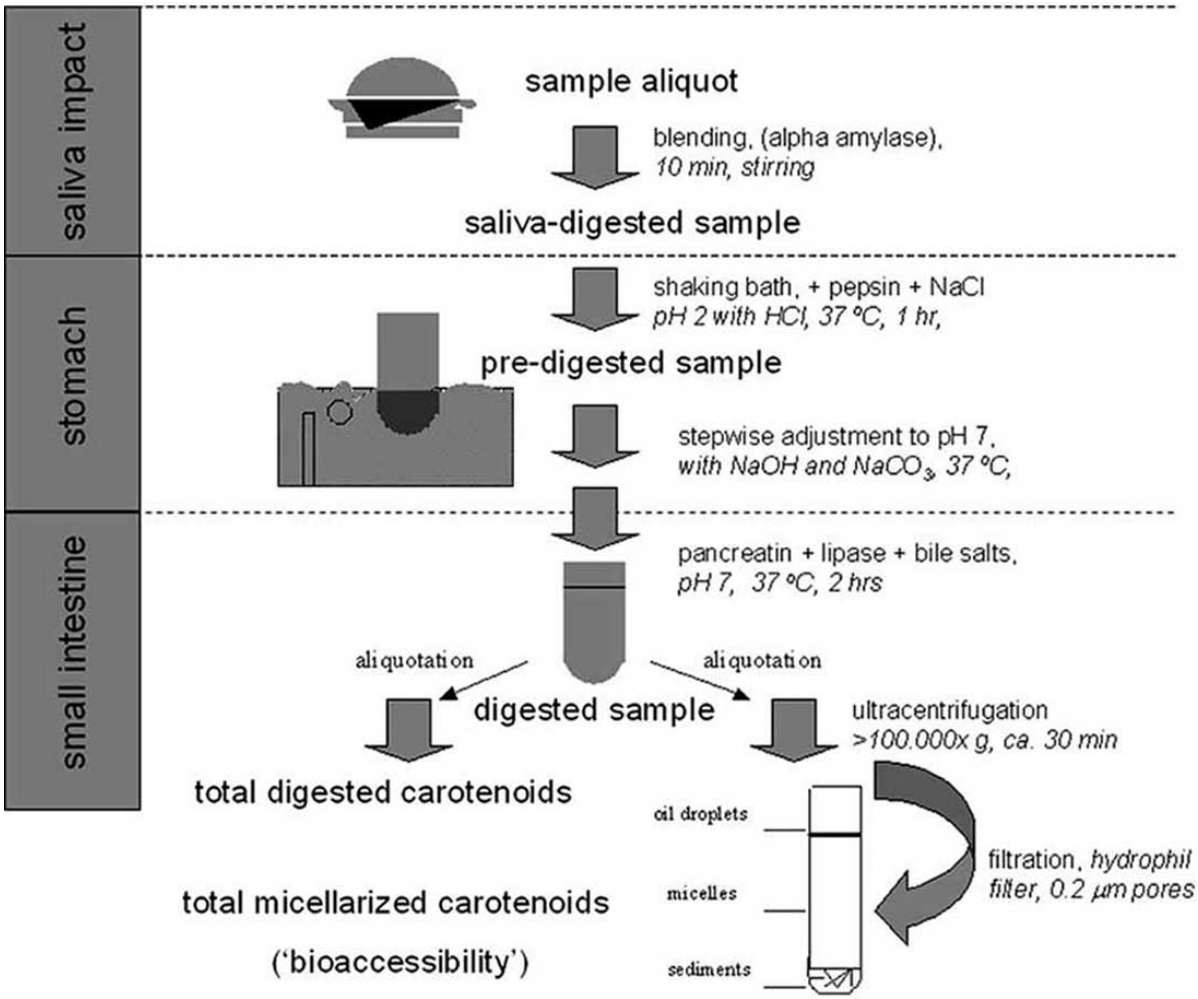

Fig. (2). Overview on assessing carotenoid *in vitro* bioaccessibility through micellarization. The given protocol is used for *in vitro* digestion experiments in the authors' laboratory.

Table 1. Various Conditions Used for Carotenoid Micellarization Experiments Across a Selection Different Studies

Study	Carotenoid Studied*	Conc. Carotenoids in Final Digesta (μg/mL)♣	Conc. Pancreatin in Final Digesta (mg/mL)	Conc. Bile Salts in Final Digesta (mg/mL)	pH During Gastric; Intestinal Phase	Digestion Time Gastric + Intestine (min)	Total Micellarization in the Aqueous Phase (%)
Miller *et al.* 1981 [91]	n.a.[+]	n.a.	ca. 0.3	ca. 2	2; 7.5	120+150	n.a.
Garret *et al.* 1999 [26]	α-car, β-car, lutein, lycopene	0.9 1.9 0.8 2.0	0.4	2.4	2; 7.5	60+120	13 15 25 <1
Hedren *et al.* 2002 [104]	β-car, α-car	ca 3.5 ca 1.0	0.6	3.8, 7.6	2; 7.5	60+30	3-39*** 60% ¬
Chithumroon-chockchai *et al.* 2004 [24]	lutein, zea, β-car	4.2 0.1 0.9	0.3	0.4 GDC[&]; 0.2 TDC; 0.4 TC;	2; 7.5	60+120	55-80>> 55-75>> 25
Chithumroon-chockchai *et al.* 2006 [102]	zea, mono- and diester	1-7	0.3	0.4 GDC[&]; 0.2 TDC; 0.4 TC;	2; 7.5	60+120	24-68
Fernández-García *et al.* 2008 [310]	lycopene	9-10	ca. 0.5	3	7.4	30+120	Values only given as final concentrations
Granado *et al.* 2006 [92]	β-car, lutein	1.2 2.1	50	9	1.1; 7.8	60+120	81 95
Reboul *et al.* 2006** [44]	α-car, β-car, lutein, lycopene	n.d. *	0.3	9	4; 6	30+30	8.9 4.4 37.6 1.1
Dhuique-Mayer *et al.* 2007 [107]	β-crypto, β-car	6.5 1.8	0.2	5	4; 6	30+30	15-40 26-33
Hornero-Mendéz *et al.* 2007 [117]	β-car, α-car	n.d.	0.2	1.3	2; 7.5	60+120	3-80***

Abbreviations used: α-car = α-carotene; β-car = β-carotene; β-crypto = β-cryptoxanthin; zea = zeaxanthin; ♣all values approximations; [+]n.a.= not applicable; *: n.d. = no data; **part of the study designated for method development; *** depending on processing steps; [&]GDC: glycode-oxycholate; TDC: taurodeoxy-cholate; TC: taurocholate; α-carotene micellarization was found app. 60% lower than β-carotene; >> depending on the matrix (spinach vs. supplement)

presence of different triglycerides [97], bile salts and enzymes [26] and different carotenoid species present [101] on micellarization.

Which parameters do impact the micellarization of carotenoids within this model? Usually, test meals are ground or blended in order to simulate homogenization during the oral digestion phase. In the original publication [91], the following gastric phase was set to two hours at pH 2, however, this incubation time has been reduced in more recent studies to one hour or 30 minutes [26, 44], even though the original time probably reflects more accurately passage time through the stomach [108], and were altered to decrease analysis time. As the pH of the stomach can vary from pH 0.5 to ca. pH 7 in healthy subjects, with 3-7 more typically following ingestion of a test meal [reviewed by 109,110], this parameter has also been varied, from pH 1.1 [92] to 3 [97, 106,111] and even 4 [44]. The pH optimum of pepsin albeit is around pH 1.5-2 [108]. pH could also impact carotenoid concentration directly, through the formation of breakdown products at more extreme pH. Asai and colleagues [111] e.g. found degradation of neoxanthin and violaxanthin into neochrome and luteoxanthin/auroxanthin, respectively, when pH was lowered from 3 to 2. Pepsin concentrations have likewise been varied, ranging from 1.3 mg/mL [107] to ca. 25 mg/mL [92] within samples to be digested. It appears

however that the addition of pepsin does not play a crucial role for micellarization (unpublished results), at least not for plant based test meals with low amounts of proteins in the matrix [26, 104].

To simulate the transition from the stomach to the duodenum, the pH of the predigested meal is, mostly stepwise increased by $NaHCO_3$, and lipase within pancreatin and bile extracts [26, 91] or pure bile salts are added if addition of crude bile has to be avoided, e.g. when measuring cholesterol micellarization [96], or for increasing efficiency of micellarization [24, 101]. The following intestinal phase, originally lasting 2 hours [91], has been retained in most studies, but in some was shortened to 30 minutes [107], which might risk incomplete carotenoid micellarization, albeit micellarization of beta-carotene in the above study was, with ca. 25% not lower compared to other studies [e.g. 112]. On the other hand, prolonged incubation times risk carotenoid degradation, for alpha-carotene and lutein as shown by Garrett and coworkers [26, 113], when increased to 6h and 24h respectively, however, no significant losses were detected for beta-carotene.

In contrast to the relatively low impact of pepsin on the micellarization efficiency of carotenoids, pancreatin and bile salts seem to play a predominant role for micellarization. Own preliminary results for beta-carotene (unpublished) indicated a reduction of 90% micellarization when pancreatin was entirely omitted during this digestion stage, whereas the absences of bile salts led to almost no detectable micellarization, as these are required for emulsifying mixed micelles in the aqueous phase. Without pancreatin, the digestion of the triglycerides is incomplete, resulting in lower concentrations of di-and especially monoglycerides and reduced micelle formation. These results are in line with results obtained from other studies [26, 104, 114], where the omission of bile salts or the complete small intestinal phase almost completely inhibited micellarization of beta-carotene, although Hedren *et al*. [104] found only a 10% reduction of micellarization of beta-carotene when pancreatin was omitted.

pH during the intestinal phase could also impact micellarization, as a too low pH (<4.5) has been reported to result in decreased solubilization of beta-carotene in mixed micelles [33], however, a slight acidity showed to improve micellarization [33, 62]. The reasons remain speculative, but one might hypothesize that the protonization of the bile salts at lower pH could improve the transfer of the apolar carotenoids during the formation of mixed micelles, while a too low pH does compromise the integrity of the micelles.

Another underestimated step is the separation of the micelle fraction from the digesta, achieved typically by ultracentrifugation [115], even though shown to be replaceable by 5000xg and 45min [101] followed by filtration of the digesta for separation of aqueous phase (micelles) from oil droplets and sediments, typically achieved by 0.2 μm pore cellulose filters. Own preliminary results (unpublished) showed limited influence of filtration on the presence of xanthophylls and beta-carotene, while for lycopene, a drastically reduced micellarization was shown [26], from ca. 5 to 0.5%, the reasons being unclear. It is possible that filtration goes along with low absolute losses of carotenoids, more strongly affecting low micellarized carotenoids.

How do *in vitro* studies compare to cellular or human studies? In a study by Reboul *et al*. [44], micellarization of alpha- and beta-carotene, lutein and lycopene *in vitro* was compared to micelles obtained from an earlier *in vivo* human study by the same group [116]. Overall, a high and significant correlation (R=0.90) was found, with very similar micellarization efficiency, with exception for lutein, which was much better micellarized *in vitro* (37% vs. 7.7%, Table **2**) although the authors suggest an underestimated bioavailability from spinach in the human study, due to analytical problems. This implicates that the *in vitro* digestion step reflects to a large extend digestion *in vivo* bioavailability. Reboul *et al*. [44] further compared *in vitro* bioaccessibility ratios of lycopene, beta-carotene, and lutein from different test meals to bioavailability ratios obtained from blood plasma measurements (AUC) in human studies investigating similar test meals. Albeit even though up to 8-fold differences were found between the bioavailability and bioaccessibility ratios, overall correlation was high and significant (R=0.98), indicating that on average, similar results can be obtained for micellarization and absorption, and is among the most important steps determining bioavailability. Discrepancies between *in vitro* studies and *in vivo* studies however can occur, especially when beta-carotene conversion into retinol is not taken into account [92].

However, by this *in vitro* technique, it has been possible to improve our understanding in processes involved in carotenoid matrix release and formation of mixed micelles during digestion. For example, it appears that xanthophylls, due to their higher polarity, possess a higher tendency for incorporation into mixed micelles compared to carotenes, with lycopene showing lowest incorporation efficiency [24, 26, 35, 38, 44, 107, 114]. Micellarization efficiency can be increased, especially for the carotenes, with the addition of various types of lipids [26, 97, 104, 106, 117, 118]. On the other hand, micellarization has been suggested to be lowered by the presence of higher amounts of dietary fibers from whole fruits and vegetables, probably impeding carotenoid release from the matrix or slowing down formation of mixed micelles and their transport to the mucosa, perhaps due to increased viscosity [106, 112, 119].

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

Cell Based Models

Both cellular uptake or transport models have been increasingly used during the past years to study carotenoid availability, often coupled to a preceding *in vitro* digestion to determine dietary uptake. This combination, being far less expensive and time consuming than animal and human studies, has been widely used in pharmacologic studies for drug testing [120], but also for a number of other compounds, including carotenoids [27, 32, 41, 121-123]. While

uptake models are cheaper and simpler, with only the apical side and the cells as a compartment, transport models with both basolateral and apical side compartment have been developed, allowing the studying of transport fluxes and sequestration through the cell monolayer [122-125]. Several cell models for the investigation of carotenoid uptake have been proposed, i.e. rat hepatic stellate cells (HSC-T6) [126] transfected renal COS-7 cells from monkeys [42], exfoliated human colonic epithelial cells [127], human retinal pigment epithelial cells (ARPE-19) [128], human lung fibroblasts (WI-38) [129] and the human Caco-2 cell line, which, once differentiated, mimics the enterocytes of the small intestine to a large extent, and has been the most used cell line for studying carotenoid uptake. This cell line shows the remarkable behavior of spontaneously differentiating into mature, absorptive epithelial cells following a proliferation stop due to contact inhibition. The differentiated cells show cellular polarization, development of tight junctions and a brush border membrane [130] and the typical colonocyte/ enterocyte hybrid phenotype after 2-3 weeks post confluency. These cells then express marker genes typical for differentiated enterocytes, e.g. alkaline phosphatase [131], sucrose isomaltase [132] and other metabolic enzymes including some cytochrome P450 isotypes [133]. In addition, a number of typical transport proteins are expressed, including SR-B1 [39-43], Niewmann-Pick disease type C1 gene-like (NPC1L1), and ATP-binding cassette transporter subclass member 1 ABCA1, participating in retinol-efflux [125], probably impacting carotenoid transport in these cells. The human Caco-2 cell line was originally established in the 1970's from a colon carcinoma from a 72-year old white Caucasian patient [134] and subsequently cultured. In addition to the parental cell line, several subclones have been developed, showing altered gene expression, such as the different expression pattern of multi drug resistance transporters [135].

As is the case for the micellarization models, also the Caco-2 cellular models have been suffering from problems of comparability between different research studies. The use of different subclones such as TC-7 [40], HTB-37 [114] or CRL-2102 for retinol [136], different time points of post-confluency (2-21days), the way of carotenoid introduction to the cells, such as various concentrations, digesta or artificial micelles, as well as different culture conditions, e.g. percentage of added bovine serum, make it difficult to compare results between across studies and laboratories (Table **2**) [24, 38, 105-107, 126, 135], and standardization procedures are highly desirable.

Another limitation of Caco-2 cells is that these cells do simulate only enterocyte-like cells, while other cells naturally present in the small intestine, such as mucus-producing and M cells, are not represented [137]. Also, paracellular transport capacity in Caco-2 cells is known to be comparatively low [138], and could be increased by co-culturing of Caco-2 with the mucus producing HT29-5M21 cells [137], even though this transport is probably of minor importance for carotenoid uptake, due to their lipophility and requirement for lipoprotein incorporation for further transport. As with micellarization, there is also the lack of dynamic simulation. With the established stationary model, the uptake from one meal during a certain time is usually investigated, while effects from subsequent meals such as altered sequestration of chylomicrons, cannot be simulated [101]. It has been shown that the uptake of carotenoids such as lutein does depend on the incubation time of Caco-2 cells to digesta, with increasing uptake over a time course between 4 and 20h, following a linear pattern [24]. Similar results were obtained by Garret *et al*. [26] for alpha-and beta-carotene and lutein for up to 6h, with the majority of studies allowing 4 h (Table **2**). Concentrations and ratios of micelle fraction to cells will also determine carotenoid uptake. For e.g. beta-carotene, a linear relation between its concentration in micelles and Caco-2 cell uptake was found, at least for physiologic relevant concentrations [139], a fact that may allow further standardization if also shown for other carotenoids.

Smaller cellular surfaces such as on microplates have become more customary due to a higher number of parallel experiments possible, up to 12 well plates [26]. However, the comparatively large volume and amount of carotenoids needed for HPLC detection, most commonly used for these experiments, precludes currently the miniaturization below 24 plate-wells. Further research and improvements of the cellular model will be needed for improved mimicking the *in vivo* situation.

Besides these apparent limitations, the Caco-2 cellular system has been a valuable model for screening trials of carotenoid uptake in many studies. These included either investigating transport of pure compounds in form of solutions or artificial micelles [39,105,125] or the uptake from predigested meals, i.e. natural micelles [44,97, 101,114,140]. While results from *in vitro* digestion alone, i.e. micellarization seem relatively consistent (Table **1**), at least in terms of general trends of bioaccessibility, results obtained from cellular uptake studies of different carotenoid species and test meals are less conclusive, probably due to the larger number of varying parameters between different studies, due to increased complexity of the cell vs. the *in vitro* digestion alone. For example, a number of studies have suggested a higher uptake of xanthophylls into Caco-2 cells compared to carotenes [103, 124] while other studies suggested the exact opposite [38, 105, 113, 126].

Different uptake mechanisms between carotenes and xanthophylls into the cells have been suggested. During and colleagues [39] found alpha- and beta-carotene uptake into Caco-2 cells inhibited up to 50% when SR-B1 transporter was blocked, while xanthophyll transport, e.g. for lutein and cryptoxanthin, was only slightly affected (7-20% decrease). Although lycopene was only reduced about 20% in this study, Moussa and colleagues [41], found a decrease of 50-60%, using the same TC-7 clone, being in line with results of the same study employing mice overexpressing SR-B1, where lycopene absorption became detectable in plasma at 10-times higher concentration compared to control animals. This preference of SR-B1 for carotene uptake might, at least partly, counterbalance the lower micellarization of carotenes or even result in higher cellular uptake compared to the xanthophylls. However, in ARPE-19 retinal cells, zeaxanthin uptake was similarly impaired as beta-carotene, when SR-B1 expression was knocked down [128], indicating that different

Table 2. Examples of Studies Employing Caco-2 Cells for Determination of Carotenoid Uptake or Transport, and Parameters of Exposure

Study	Carotenoid Studied	Carotenoid Matrix and Amount Added	Cell Type	Amount Carotenoid Added to Cells (μg)	Type of Well and Surface	Cell-Post-Confluency (d)	Passage Number (#)	Exposure Time/Time of Harvest (h)	Bio-Availability (%)
Garret *et al.* 1999 [26]	lutein, lycopene, α-car, β-car	digesta 1:3 diluted in DMEM$^+$, 1mL	n.d.	0.28 0.65 0.31 0.65	up, 12 well dishes, 4 cm^2	11-14	23-37	6	25 negligible 45 25
Sugawara *et al.* 2001 [38]	asta, α-car, β-car, cantha, capsa, β-crypto, fuco, lutein, lycopene, neo, viola, zea	AM in DMEM$^+$, 1mL	n.d.	0.6 0.6 0.6 0.6 0.6 0.6 0.6 0.6 0.2 0.6 0.6 0.6	up, 12 well dishes, 4 cm^2	20-22	25-50	0-5-6	n.d.
Chitchumroo-nchockchai *et al.* 2004 [24]	lutein	AM, 1:4 diluted in DMEM, 1.5 mL apical, (2.5 mL DMEM basolateral)	HTB-37	2.3	TW, 6-well dishes, 9.6 cm^2	21-25	26-34	6 al, 20 bl	3 (basol.); 37 (cells);
Liu *et al.* 2004 [105]	lutein, zea, β-car	standards in WME" medium + digesta 1:4 WME medium	n.d.	0.6-4.5	up, 6-well dishes, 9.6 cm^2	2 and 14	n.d.	1-12	n.d.
During *et al.* 2005 [39]	all-tr-β-car 9-cis-β-car 13-cis-βcar α-car, lutein lycopene	AM in DMEM, 2 mL apical, (2.5 mL DMEM basolateral)	TC-7	1.1	TW, 6-well dishes, 9.6 cm^2	21	n.d.	16	11 2 3 10 7 3 (cells)
Reboul *et al.* 2005 [40]	lutein	AM: 1 mL apical, (2 mL basolateral FBSF**)	TC-7	0.46	TW, 6 well dish, 9.6 cm^2	21	40-60	0.5 al, 0.5 bl	n.d.
Ferruzzi *et al.* 2006 [27]	all-tr-β-car, 9-cis-β-car, 13-cis-βcar, 15-cis-βcar	digesta 1:4 diluted in DMEM, 2 mL	HTB-37	12	up, 6 well dish, 9.6 cm^2	10-13	23-36	4, har 18 h	8-11 16-23 12-34 15-24

(Table 2) Contd….

Study	Carotenoid Studied	Carotenoid Matrix and Amount Added	Cell Type	Amount Carotenoid Added to Cells (μg)	Type of Well and Surface	Cell-Post-Confluency (d)	Passage Number (#)	Exposure Time/Time of Harvest (h)	Bio-Availability (%)
Failla *et al*. 2008 [208]	α-car, β-car, lycopene	digesta 1:4 diluted in DMEM, 12.5 mL	HTB-37	0.04 0.13 0.05	up, 75 cm^2 flask	11-14	26-37	4, har 20 h	28 27 13

Abbreviations used: " William's Medium E, *all values are approximates, ** fetal bovine free serum medium, n.d. = no data, up = uptake, TW = transwell, har = harvest, al = apical; bl = basolateral, asta = astaxanthin, capsa = capsanthin, α-car = α-carotene, β-car = β-carotene, α-crypto = α-cryptoxanthin, β-crypto = β-cryptoxanthin, cantha = canthaxanthin, viola = violaxanthin, fuco = fucoxanthin, neo = neoxanthin, zea = zeaxanthin.

tissues may express different subtypes of transporters with different substrate specificities. The availability of xanthophylls could further differ depending on their appearance in the food matrix either free or as esters, the latter ones mostly being cleaved prior to absorption [141]. Zeaxanthin and cryptoxanthin esters for example were shown to be less micellarised and taken up compared to the free form in a number of studies [102, 107]. However, other *in vitro*, animal and human studies tend to suggest equal availability of cryptoxanthin, lutein and capsanthin esters compared to the free form [141-145]. While esters could be less easily emulsified due to extreme apolarity, it is possible that they are better protected in the core of the micelles compared to free carotenoids, in addition, it was shown that esters are cleaved during absorption by e.g. carboxyl-ester-lipase and become available for absorption [36, 101, 107], resulting in similar absorption compared to the free xanthophylls. While carotenoid esters have also been shown to be taken up by the intestinal cells intact [102, 107], it remains uncertain whether they can be further cleaved within the intestinal cells and absorbed.

Furthermore, it is difficult to compare micellarization of provitamin-A carotenoids between *in vitro* micellarization experiments and Caco-2 cell uptake studies, as within the cells, especially provitamin-A carotenoids are partly broken down by enzymatic cleavage into retinal or apo-carotenals. If these metabolites are not considered, there is a high potential for finding large discrepancies between micellarization and uptake studies. However, differences exist between the parental cell line, showing no monooxygenase activity, and several subclones, such as the TC-7 and PF11 cells [146].

In summary, the combination of *in vitro* digestion and cell studies yields an important screening tool, allowing for taking into account many processes that occur *in vivo*, and being of suitable high throughput for screening in multi-well plates. Given ethical concerns performing animal and human studies, cell studies have become increasingly popular. Unfortunately, the inter-laboratory differences are large and, up to date, perturb the comparison of results to a large extent. In addition, there is, to the author's knowledge, no study on the correlation between results from cell culture experiments and animal/human investigations.

Animal-Derived Models

Looking for alternative models to the time- and labor-intensive human studies albeit still allowing studying complex absorption and metabolization processes of beta-carotene, El-Gorab *et al*. [28] employed isolated everted gut sacs from the rat small intestine. During this procedure, a segment of the gut was removed, inverted, and kept ex vivo in mucosal fluids under sufficient oxygen to keep the tissue viable. Another procedure was based on using isolated gut sacs *in vivo*, i.e. small intestinal loops, requiring comparable complicated operations at the rat [37,147], or ferrets [148]. During these early studies, basic understanding on carotenoid absorption, transport, and metabolization was improved, obtaining results on the influence and composition of enzymes and bile salts and their effect on beta-carotene absorption and conversion to vitamin A [28]. Although improved during the last years for pharmacological studies, especially the everted gut sac technique [149], these models were, to a large extent, replaced by cellular studies. As metabolization pathways differ strongly between species, for example, much higher conversion of beta-carotene to retinyl esters in rats compared to humans [34], these animal techniques were of limited predictive value for studying carotenoid pathways in humans. Given also ethical considerations, and in addition considering that these models have to be prepared and maintained with considerable expertise and skill, this system has hardly been used recently. Alternatives to the everted gut sac technique have been developed, such as employing brush border membrane vesicles (BBMV) derived from rats and gerbils, allowing for relatively cost effective and rapid uptake studies [150, 151]. However, preparation of these brush boarder membrane vesicles still requires living animals as a source of cells, and variations between species still limit interpretability of results.

Animal Studies

Animals have been used for a long time in order to understand aspects of carotenoid uptake, absorption, distribution and metabolization. Advantages of using animals over humans include improved coordination and standardization of experiments, accessibility of tissues not easily sampled from humans, such as liver or other inner organs, using radioactive labels, and high dosing options. According to Lee and colleagues [30], the ideal animal shows physiological properties similar to humans, including the intact absorption of a variety of carotenoids, partial cleavage of carotenoids, especially the pro-vitamin A carotenoids into retinol, and their distribution among tissues and plasma. Additionally, factors such as affordability and good mana-

geability in laboratory environment including repeatability and rapidness of trials should be given. The perfect model does, naturally, not exist. However, finding an appropriate animal for specific questions functioned as a good compromise for many studies (Table **3**).

The majority of studies used serum/plasma, especially the area under curve (AUC) technique to determine carotenoid bioavailability from single [111, 152-156], or multiple doses [29, 157], while triacylrich-lipoprotein fraction has only been investigated in lymph-duct cannulated rats [158, 159], as it is analytically challenging to sample a sufficient large volume, especially in small animals. However, also fecal-oral balance techniques [160] and isotopic techniques for tissue retention [153-155] were used. Biopsies to study tissue concentrations of carotenoids have likewise been conducted, especially from calf livers [161].

Avian Species

Carotenoid bioavailability and vitamin A conversion have been extensively studied in chicken (*Gallus gallus domesticus*), mainly due to the relatively high availability, affordable price, high number of offspring, reduced ethical concerns as opposed to higher mammals, but also due to commercial interests such as embryonic development, vitamin and health status of animals, and quality of the eggs, e.g. egg yolk color [162]. Due to the phylogenic distance to mammals however, one should be careful to extrapolate results directly to humans. Birds show anatomical differences in the intestine and lymphatics [163], do have extremely efficient metabolization of pro-vitamin A carotenoids into retinol [164, 165], and distinctions in the structure and enzyme activity, i.e. the paraoxonase structure and activity, in HDL/LDL [166].

Recent studies have focused on non-provitamin A carotenoids, demonstrating that astaxanthin accumulates in many tissues, i.e. kidney, liver, adipose tissue and skin, with about 70% of the total astaxanthin present in plasma in the HDL fraction [167], a percentage somewhat higher than that reported for xanthophylls in humans, around 45% [168, 169]. An increase of tissue carotenoids (especially lutein) in liver and plasma was found in a recent study on the red-legged partridge (*Alectoris rufa*), revealing an impact of the androgen status on e.g. carotenoid bioavailability, at increased testosterone levels [170]. Contrary results were found in a rat study, showing increased lycopene storage in liver and adrenals after castration [171].

Chickens with a sex-linked Wisconsin hypoalpha mutation (WHAM) with low expression of the ABCA1 transporter, leading to low levels of circulating HDL, were used as a model analogous to the human Tangier Disease to study levels of circulating lutein and zeaxanthin [172]. While intestinal and hepatic carotenoid uptake was not impaired, similar as in Caco-2 cells where ABCA1 (as well as other transporters including SR-B1) was blocked by ezetimibe [39], only low levels of carotenoids were detected in other non-hepatic tissues, i.e. retina and the skin, indicating the importance of HDL for carotenoid tissue distribution.

Studies have also suggested that chicken could take up both free and esterified lutein and capsanthin to a similar extent [143], due to their ability to cleave diesters enzymatically [173], results that were found also in some [142, 174], albeit not all human studies [23].

The impact of feed, especially of purified fiber (lignin, hemicellulose, citrus pectin) as a food additive, was investigated, resulting in reduced beta-carotene utilization measured as vitamin A concentration in the liver of chicken [175], similar as suggested for humans during short-term studies [176, 177].

Rats and Mice

Both mice (*Mus musculus*) and rats (especially *Rattus norwegicus forma domestica*) are well characterized, and have been used predominantly as models for human chronic diseases, especially in cancer research, vitamin A deficiency and research on immune functions [178-180]. These rodents are easy to handle, have high numbers of offspring and housing is relatively affordable. On the other hand, both species show high discrepancies concerning the uptake, absorption and metabolism of carotenoids, leading to difficulties when comparing results to humans. First, rats and mice are white-fleshed animals, categorized as carotenoid non-responders, needing unphysiological high dietary levels of beta-carotene to obtain similar plasma and tissue concentrations compared to humans, mainly due to a high conversion rate into retinyl esters in the brush border of the enterocytes [30, 155, 181, 182]. Pharmacological doses especially of xanthophylls however might be studied. Carotenoid doses of 0.1 and 1% of total food content, and single doses of 500mg/kg body weight in form of duodenal infusions of the non-provitamin A carotenoids astaxanthin, canthaxanthin and lycopene were well absorbed, detectable in plasma, and not cleaved to a measurable extent [154, 155, 157-159].

Recent studies employing highly sensitive equipment, such as mass spectrometry, focused on physiologically, lower levels following dietary intervention. In rats, uncleaved beta-cryptoxanthin was found in the plasma and liver [29]. In mice, physiologically concentrations of neoxanthin and its furanoid-derivatives (R/S)-neochrome were shown to reach the plasma and were stored in the liver [111]. Fucoxanthin (a major carotenoid in brown algae of nutritional importance in Asia) metabolites were also detected in plasma and liver [111, 183], following physiological doses (23µg or ca. 1.2 mg/kg), and several other tissues, including the adipose tissue, following daily fucoxanthin intake for one week [184]. This is in contrast to several human studies, where neither violaxanthin, neoxanthin, taraxanthin nor their metabolites could be detected in blood [51, 52].

It is interesting to note that castrated rats showed reverse effects of testosterone compared to birds, i.e. low levels of testosterone increased the amount lycopene stored in the liver, despite low food intake of the rats following surgery [185]. Testosterone supplementation to castrated rats increased levels of androgen and diminished lycopene liver concentrations to the level of control rats. Similarly, diet-induced low circulating androgen levels (food restriction) resulted in increased liver lycopene concentrations in rats [171]. It was speculated that decreased lipoprotein secretion resulted in increased lycopene retention by the liver.

Table 3. Selection of Different Animal Models Used for Investigating Aspects of Carotenoid Bioavailability and Status

Animals Involved	Study	Carotenoids Investigated	Test Meals	Aspects Studied	Tissues Sampled	Results	Critics and Limitations
mice	Umegaki *et al.* 1995 [182]	β-car*	soybean oil, algae extract	effects of sodium cholate and β-carotene supplement.	blood, liver and bone marrow	sodium cholate increases uptake	no significant uptake of intact β-carotene at physiological levels, but extensive cleavage to retinyl esters
	Asai *et al.* 2004 [111]	neo*	artificial micelles	metabolism	plasma, liver	isomerisation in the stomach	
	Huang *et al.* 2006 [203]	lycopene	corn oil	uptake and distribution	various tissues	liver main store for lycopene	
rats	Hollander *et al.* 1978 [37]	β-car*	artificial micelles	pH, chain lengths of fats, bile	intestinal loops	higher uptake at lower pH; highest absorption with oleoric acid	no significant uptake of intact β-carotene at physiological levels,, but extensive cleavage to retinyl-esters; metabolism and storage different
	Grolier *et al.* 1998 [238]	α-car, β-car*	supplemented, sterilized diet	micro flora	plasma, liver	micro flora reduces carotenoid status	
	Mathews-Roth *et al.* 1990 [154]	cantha* lycopene	olive oil olive oil	uptake and distribution	various tissues	liver main store	
	Boileau *et al* 2001 [171]	lycopene	fortified meal	distribution	liver	influence of androgenes	
	Breithaupt *et al.* 2007 [29]	α-crypto, β-crypto*	fortified corn meal	uptake and distribution	blood, liver	β-crypto* uptake higher than α-crypto	
gerbil	Deming *et al.* 2000 [187]	β-car*, cis-isomers	carrot powder	conversion	various tissues	dietary fiber and fat influence postabsorptive conversion	physiology different from humans; surgical operations hardly possible; β-car conversion rate higher than in humans
	Deming *et al.* 2002 [186]	β-caro*	cottonseed oil	uptake and conversion	serum, stomach and intestine	trans better available than cis isomers	
	Davis *et al.* 2008 [311]	β-crypto. β-car*	fortified corn meal	uptake and conversion	serum, liver	provitamin A carotenoids are available from fortified maize	
	Escaron *et al.* 2006 [202]	lutein, β-caro*	cottonseed oil	uptake	serum, stomach and intestine	negative interactions between different carotenoids	
	Mills *et al.* 2007 [190]	lycopene	freeze-dried red carrots and tomato paste in cottonseed oil	uptake	serum, liver	matrix effects: tomato paste a better source	
ferret	Wang *et al.* 1992 [156]	β-car*	AM*	uptake and conversion	lymph, small intestine	uptake of intact β-carotene	high retinyl ester concentration in plasma; β-car conversion rate lower than in humans
	Wang *et al.* 1995 [148]	β-car*	AM*	uptake and conversion	lymph	vitamin E enhances β-carotene uptake and conversion in intestine	

(Table 3) Contd…..

Animals Involved	Study	Carotenoids Investigated	Test Meals	Aspects Studied	Tissues Sampled	Results	Critics and Limitations
	Zhou *et al.* 1996 [213]	α-car, β-car*	beatlets + different carrot preparations	uptake and distribution	serum + various organs	matrix effects: processing increases availability	
	Erdman *et al.* 1998 [274]	β-car*, cis-isomers	pellet food + algae extract + beadlets	uptake and conversion	serum, stomach and intestine	higher uptake and storage of trans isomers	
	Boileau *et al.* 1999 [204]	trans and cis lycopene	AM*	uptake	lymph	cis isomers better taken up than trans isomers	
poultry	Erdman *et al.* 1986 [175]	β-car*	flour containing fiber	conversion	serum, liver	some types of fiber reduce β-carotene utilization	anatomical (i.e. crop) and physiological (i.e. LDL/HDL distribution) differences
	Schaeffer *et al.* 1986 [312]	lutein	supplemented corn/soy meal	uptake + inhibition	serum, liver, intestine, toe web	negative effects of ochratoxin	
	Takahashi *et al.* 2004 [167]	astaxa*	carotenoid containing water	uptake and distribution	plasma and several tissues	main deposition in fat tissue, intestine, spleen, and liver	
pre-ruminant calves	Chew *et al.* 1993 [313]	β-caro*	beatlet	uptake	plasma and blood cells	β-carotene accumulates in lymphocytes	generally to expensive (housing etc). physiological (LDL/HDL ratio) differences
	Bierer *et al.* 1995 [215]	α-caro, β-caro, cantha* lutein lycopene	beatlet, olive oil	uptake	serum	matrix effects (crystalline form); higher clearance rate of more polar species	
pigs	Schweigert *et al.* 1995 [314]	β-car*	olive oil	uptake, distribution and conversion	plasma, several tissues	intact β-carotene is absorbed and stored in lung, liver and kidney	"white-fleshed" animals, different tissue distribution
dogs	Korytko *et al.* 2003 [152]	lycopene	gelatine capsule	uptake, distribution	plasma, several tissues	cis isomers better taken up than trans isomers	uptake and metabolism of β-car different
	Chew *et al.* 2000 [221]	β-car*	fortified diet	uptake, distribution	plasma, leucocytes	immune response enhanced	
monkeys	Krinsky *et al.* 1990 [155]	β-car*	olive oil	uptake and metabolism	serum, several tissues	intact β-carotene is absorbed and stored liver	very rare and expensive
	Mathews-Roth *et al.* 1990 [154]	cantha* lycopene	olive oil	uptake, distribution	serum, several tissues	carotenoids are absorbed, stored and not metabolized	
	Snodderly *et al.* 1990 [227]	lutein, zea, crypto*	dry food diet	uptake, distribution	plasma	cis isomers better taken up than trans isomers	very rare and expensive

*Abbreviations used: AM = artificial micelles; asta = astaxanthin; α-car = α-carotene; β-car = β-carotene; α-crypto = α-cryptoxanthin; β-crypto = β-cryptoxanthin; cantha = canthaxanthin; zea = zeaxanthin.

Gerbils

As an alterative model to mice and rats, Mongolian gerbils (*Meriones unguiculatus*) have been used, being a practical species for laboratory research for various reasons, i.e. high offspring rate, and, due to their small size and weight of ca. 70-80 g for an adult male, ease of maintenance in a laboratory, allowing to keep high numbers for improved statistical strength [30, 186-190]. Mongolian gerbils have a cholesterol and lipid metabolism similar to humans [191], and absorb a number of carotenoids intact at physiological levels, including beta-carotene and lycopene, similar to humans [30]. As they also convert dietary provitamin-A carotenoids to a similar extent to retinol as humans [191], with beta-carotene concentrations in liver highly dependent on dietary uptake [30, 186, 192], this species has been used for a number of studies investigating carotenoid utilization.

A major disadvantage can be their small size and weight, impeding the sampling of large volumes of tissues, or to conduct any operations. In addition, in contrast to other animal species, including rats, Mongolian gerbils seem to be relatively resistant to vitamin A deficiency, taking months to develop symptoms, regardless of the original liver and nutritional vitamin A status [30, 193, 194]. Humans have shown symptoms of vitamin A deficiency such as impaired dark adaptation after several months [195] – being in contrast to gerbils if one considers the short live time of the animals. Furthermore, in contrast to results obtained from *in vitro* and *in vivo* studies in humans, increases in dietary fat did not lead to altered beta-carotene absorption, but enhanced postabsorptive vitamin A conversion and lowered liver beta-carotene content [187], suggesting major differences in the physiology compared to humans. Studies further suggested a similar bioavailability from different vegetables sources compared to supplements, with the plant matrix (maize, carrots) having only a minor impact on provitamin A status [187, 196-198]. These results appear different from human studies, where a complex vegetable matrix often resulted in lower bioavailability of lutein and beta-carotene compared to supplements and oily suspensions [199, 200]. However, the beta-carotene bioavailability in gerbils was not directly determined, only the vitamin A status was assessed, which is, besides carotenoid uptake, known to depend on multiple additional factors such as enzyme cleavage activity. Similar as in human studies, high amounts of soluble dietary fiber on the other hand, i.e. pectin, seemed to have a negative effect on beta-carotene bioconversion and vitamin A status [176, 187].

Lutein uptake was also found to be different from humans. Tanumihardjo and coworkers [201, 202] investigated lutein uptake compared to beta-carotene and found that lutein was only marginally circulated and stored. In contrast, beta-carotene showed much higher bioavailability and was 7 and 45-times more concentrated in the liver, when given alone or in combination with lutein, respectively. They concluded that the different lipoprotein profile of gerbils compared to humans might be the cause of differences with gerbils possessing higher concentrations of HDL than LDL, albeit, given similar high affinity of HDL to the xanthophylls as in humans, this should rather favor xanthophyll transport.

Lycopene on the other hand seems to be well absorbed by gerbils as shown by Huang and colleagues [203]. In this comparative study on mice, rats and gerbils, the latter responded highest to dietary lycopene, accumulating in plasma, liver and kidney. Nonetheless, the lycopene storage pattern was similar to mice and rats, with the main storage tissue being the liver.

Ferrets

Ferrets (*Mustela putoris puro*) are larger and heavier when compared to rats, ca. 1200 g, allowing for easier surgical operations such as cannulation of the lymphatic ducts or the portal vein [30, 204]. However, as they require additional space and are more cost intensive in comparison to rodents. For studies investigating the relation of carotenoids to human diseases, rats may therefore be preferred. Physiologically, ferrets seem to be an adequate model for carotenoid absorption, absorbing beta-carotene intact, cleaving the molecule similarly to humans and showing a human-like tissue distribution, such as in the liver, lung and adipose tissue [30, 156, 181, 204]. Also alpha-carotene, lycopene and canthaxanthin [205, 206] were absorbed intact. Several studies investigated the uptake of carotenoid isomers, showing that all-trans beta-carotene is preferentially absorbed over 9- and 11-cis beta-carotene, while for lycopene the cis-isomers are favored [204, 207], being similar to human metabolism [68] and to *in vitro* studies [126, 208]. However, higher retinyl ester concentrations have been reported in plasma as opposed to humans [209], and a conversion rate of beta-carotene to vitamin A equivalents of >15:1 [210], which is somewhat higher than the values suggested for humans, 6:1 [211] or 12:1 [212]. A further study investigated the influence of food matrix using ferrets [213], finding higher availability of beta-carotene from commercial beadlets than beta-carotene from isolated chromoplasts and from carrot juice. The authors suggested that the crystalline form present in chromoplasts, decreased the bioavailability compared to the beadlets, which was similarly found *in vitro* [24, 104].

Large Mammals and Primates

For a number of studies, either non-human primates were chosen due to phylogenetic proximity to humans or large mammals, because their physiology, such as intestine (pigs) or cardio-vascular system (pigs, calves), closer resembles the human body as, e.g. rodents. Major restrictions, especially for a larger numbers of these animals are higher costs for maintenance and care, their availability, especially of primates, and ethical restrictions.

Lee *et al*. [30] suggested preruminant calves (*Bos primigenius Taurus*) to be a good model for carotenoid absorption and metabolism, as calves are at this stage monogastric, can absorb beta-carotene intact, use it as a source of vitamin A [214], and show similar beta-carotene tissue distribution patterns, including the 9-cis-isomer, compared to humans [30]. Calves have also been reported to absorb canthaxanthin, lutein, lycopene, and alpha-carotene intact [215]. The additional advantage of allowing for taking sufficiently large sample sizes from various tissues is counterbalanced by the difficulty of housing a statistical sufficient number of calves.

Pigs (*Sus domestica*) are generally regarded as a good model for human nutrition [216], due to similar size and weight, similar physiology and gastrointestinal tract [217]. However, intact carotenoid absorption was only very low following oral uptake of [C^{14}] beta-carotene, accumulated in the lung and in smaller amounts in the liver, while plasma concentration remained barely detectable [153] and the carotenoid metabolism differed significantly from humans [218], impeding comparisons to humans.

The effect of long-term supplementation with beta-carotene and lutein was studied in dogs (*Canis lupus familiaris*), focusing on changes of the immune system [219-221]. In a study investigating lycopene uptake, Korytko *et al*. [152] found mainly cis-isomers accumulating in tissues and plasma, although the supplement contained predominantly of all-trans lycopene, indicating a preferred uptake of the cis-isomers also seen in cell culture experiments [208] or conversion from trans to cis forms, as reported in human studies [65, 222]. Dogs may therefore constitute a good model for the investigation of the relation between prostate cancer and lycopene, due to the similar accumulation of cis isomers in the prostate.

The Rhesus Macaque (*Macaca mulatta*) has been a popular model for health-related studies, especially for studying cardiovascular diseases, due to highest similarities to humans compared to other model animals. However, they are extremely expensive and are of limited availability. The Rhesus monkeys show large differences between responding and non-responding individuals as well as human-like plasma levels and tissue distribution of beta-carotene and retinol [155]. The same authors investigated the uptake of [^{14}C] canthaxanthin and [^{14}C] lycopene, detecting similar absorption and distribution pattern compared to beta-carotene [154]. Following ingestion of a single bolus dose, the authors also found lycopene plasma levels remaining almost constant for 48hrs after reaching a plateau at 8hrs, while canthaxanthin was rapidly cleared. This clearance appeared similar to what was reported for humans, where canthaxanthin and lycopene peaked between 10-24 hrs and decreased slowly thereafter [223-225].

The Macaque monkeys (*Macaca fascicularis*) were often used to study the distribution of carotenoids in the macula lutea of the eye, due to similar distribution of zeaxanthin and lutein compared to humans, with zeaxanthin being the dominant species [226]. Compared to humans, macaque monkeys show lower beta-carotene concentrations in the plasma, presumably due to higher cleavage efficacy, as consumption was suggested to be similar [155, 227]. It was also suggested that macaque monkeys preferably take up certain carotenoid isomers, i.e. accumulating 13-cis isomers of lutein and zeaxanthin in plasma, while others, such as the 9-cis-zeaxanthin isomer, although being present in the diet, could not be detected [154, 155, 227]. This isomer formation or accumulation appears to be different for humans, albeit 13-cis isomers of lutein and zeaxanthin were also detected in lower concentrations in humans [228, 229].

In summary, the broad variety of available animal models allows for choosing a reasonable model for the specific questions targeted. While rodents are possibly not the best model to study carotenoid absorption and bioavailability, due to low intact absorption in these animals, ferrets, gerbils and preruminant calves have sufficient similarity to study uptake and absorption of carotenoids, while large mammals often offer no significant further advantage and are more difficult to house.

Human Studies

Human studies constitute the most appropriate way to obtain definite knowledge on carotenoid utilization in humans, in spite of the fact that other methods may also yield decent estimates of bioavailability aspects in humans. However, when compared to *in vitro* techniques and most animal studies, human studies are generally costly, more difficult to standardize due to variances e.g. in individual lifestyles, and ethical restrictions limit the accessibility to various tissues, duration of the study, and also recruiting large number of subjects. An overview on various techniques to assess carotenoid bioavailability in humans is given in Table **4**.

Depending on the underlying question, various study regimens may be followed, including single dose or single meal administrations, investigating rather pharmacological aspects such as absorption kinetics; or rather mid-to long-term dietary intervention studies to detect average utilization during a prolonged time period. For these purposes, various techniques can be employed, including the sampling of various biological fluids such as blood plasma used for both single dosing and longer-term intervention with carotenoids [230], triacyl-rich lipoprotein (TRL) fraction of the plasma representing newly absorbed carotenoids [47], sampling tissues such as adipocytes [231], buccal cells [232], and feces representing a major excretion pathway [70]. To distinguish endogenous carotenoids from carotenoids administered, isotopically labeled carotenoids may be used [233, 234]. Thus, duration of study, dosing, type of carotenoid and underlying research questions may require various investigation strategies. These include appropriate hypothesis forming, a priori calculations including estimating the required number of subjects for statistical reasons, and gender choice, as sex-specific differences may exist for the distribution and utilization of some carotenoids. For example, lutein distribution in the macular pigment (MP) and adipose tissues was found different between men and women in a small scale study, with women showing a negative relation between lutein concentration in adipose tissue and MP, i.e. tissues competing for lutein [235], perhaps due to altered metabolism due to higher amount body fat of women. An impact of testosterone has also been suggested, leading to differences of at least lycopene storage in various tissues including liver [236], which was also suggested in an animal study [185].

Oral-Fecal Balance Technique

The oral-fecal dietary or chemical balance technique estimates absorption as the difference between ingested amount carotenoids and excreted dose in feces, if sufficient collection time is allowed, i.e. up to 6 d following ingestion of the meal, to ensure complete GI passage. Even though this technique has been partly successfully used to determine mineral absorption [237], it is not the method of choice for determination of carotenoid uptake by the human body, for

Table 4. Comparison of Various Techniques to Measure Aspects of Carotenoid Bioavailability in Humans

Type of Method	Aspect Studied	Type of Intervention	Time and Type of Sampling	Advantage	Disadvantage	Studies
Fecal-oral balance (chemical/mass balance)	relative and absolute absorption	single dose/meal	all fecal material up to several days following single dose	non invasive	endogenous losses decrease apparent absorption, degradation in intestine increases apparent absorption	[239,241,242]
Plasma sampling	relative absorption	longer dietary intervention	single or few points	simple to perform	only relative absorption can be studied	[174,230,243, 246,247,250]
Plasma-AUC, single dosing	relative or absolute absorption	single dose/meal	multiple points from 1h to several days post-prandial	interindividual absorption patterns taken into account	relatively long-lasting, requiring analysis of large n of samples	[52,100,144,176,243,255,260, 315-318]
Plasma-AUC, prolonged dietary intervention	relative absorption	longer dietary intervention	multiple points over several days	cancels out daily plasma variations within subjects	large n of samples, may more typically reflect real dietary uptake due to continuous intake	[262]
Plasma-TRL	relative or absolute absorption	single dose/meal	multiple, hourly points for up to ca. 12h	reflects newly absorbed carotenoids, absorption can be determined,	analytically laborious, analysis of large n of samples	[263,268]
Tissue sampling	relative absorption, status	single dose or prolonged interventi-on	following several days after consumption or onset of study, assuming equilibrium of tissue and plasma: e.g. adipose tissue, liver, buccal cells	may reflect status, investigation at physiologically target site, e.g. macula	invasive, increased matrix effects may perturb carotenoid measurements, single endpoint may be subject to conc. variations over time	[8,64,65,231, 248,278,279]
Isotopic studies	all of the above, kinetic modeling/ compartmental analysis	single dose or prolonged interventi-on	all of above	differentiation endogenous and dietary carotenoid, allowing for compartment modeling	sophisticated analysis methods required	[262,294,300, 301,308]

several reasons. First, carotenoids are likely, at least in part, to be affected by the GI microflora, as shown in rats [238], perhaps due to degradation [239], which would result in falsely overestimating absorption of carotenoids. On the other hand, endogenous losses from bile, pancreas or cell sloth increasing the amount of excreted carotenoid would result in too low carotenoid absorption. In addition, collection of faecal material is cumbersome for subjects and researcher, and detection of carotenoids from large quantities of faecal material difficult, requiring e.g. chromatographic purification with alumina [239]. Furthermore, unless isotopically labelled carotenoids are employed, determination of carotenoid absorption from single meals would require following strict dietary regimens for several days in order to avoid carotenoids from subsequent meals to disturb the balance. Nevertheless, a few older studies have used this technique to estimate carotenoid absorption [240]. Mass balance technique is still used in subjects where large intestinal degradation through bacteria can be assumed to be low, such as in ileostomists, by collecting the ileal chyme. For example, Faulks *et al*. [241], studied absorption of lutein and beta-carotene from whole leaf vs. chopped spinach in ileostomists, finding higher lutein absorption from whole-leaf spinach, which was explained by prolonged GI passage time; no effect was found for beta-carotene. A similar study design with ileostomists investigated absorption of beta-carotene from cooked pureed carrots versus chopped, raw carrots [242], finding 65 vs. 41% absorption, suggesting a rather negative effect of the matrix on carotenoid bioavailability, in line with other human studies [199, 230, 243].

Blood Plasma and Serum

The carotenoids beta-carotene, lycopene, lutein, alpha-carotene and beta-cryptoxanthin are the most abundant ones in human blood plasma [69, 244, 245], with blood serum concentrations of approx 0.9, 0.7, 0.5, 0.2 and 0.2 nmol/mL respectively [245], and can therefore be comparatively easy detected following e.g. liquid/liquid extraction. Plasma concentrations of these carotenoids have been reported to respond to dietary changes, and might therefore constitute an indicator of current carotenoid intake or status of carotenoid supply. Low plasma carotenoid concentrations have been found following prolonged low intake of carotenoids, such as in early depletion studies [195], and higher serum/plasma

concentrations following intervention diets rich in carotenoids [246, 247], allowing for estimating relative carotenoid bioavailabilites. For example, following dietary intervention studies over 4 wks [230] or 8 wks [246] it was demonstrated that cis-isomers of lycopene had higher bioavailability compared to the all-trans form. These interventions are usually conducted following a 1-3 wk washout, i.e. a standardized diet low in carotenoids, which is considered an advantage as it aids in standardizing starting plasma carotenoid levels and reduces variability in response to the intervention [248]. Carotenoid concentrations following intervention are then compared to baseline values after washout and often to another intervention, either in a parallel group [247, 249], or, following another washout period, in the same subject in a crossover-fashion [51, 174, 250], each subject being his/her own control. The latter design is more time consuming and increases the risk of drop outs, but reducing variability of results.

Plasma concentrations can also be measured following single bolus doses, however, as abundant carotenoids can be rapidly transferred from plasma to other tissues such as liver or adipose tissue [48, 251], it is difficult to assess bioavailability from single doses based on individual plasma measurements, and measuring area under plasma curves (see following chapter) are preferred in order to reduce variability due to individual plasma kinetics. Following individual doses, plasma responses of carotenoids typically peak at ca. 24 h [252, 253], even though later peak plasma responses, up to 3 d [254] postprandial were reported, probably due to differences in the study design such as subsequent diet, especially lipid intake, reaching plasma concentrations of up to 1.5 nmol/mL in some subjects.

Area Under Curve (AUC) from Blood Plasma/Serum

In order to determine carotenoid absorption from single bolus doses or single test meals based on plasma samples, the area-under-curve technique has been frequently employed. This technique requires collecting blood samples at regular intervals, i.e. typically hourly during the first hours, with 12-36 h sampling intervals for several successive days [100, 255, 256] following test meal intake, with shorter measurements of up to 24h often failing to completely follow the decrease of carotenoid concentration in serum or plasma [52, 142, 242]. Prior to dietary intervention, a washout period with low intake of carotenoids may be involved, and carotenoid concentrations in serum or plasma are assessed, and any increase over baseline concentration during the monitored time is integrated. This technique allowed for the determination of relative absorption and bioavailability [252]. Absolute absorption can also be done by e.g. determining the ratio of the AUC of a carotenoid administered intra-venous vs. orally. However, even though this is a frequent approach for determination of bioavailability in pharmacological studies [257, 258] or in mineral bioavailability [259], it appeared difficult to dissolve or emulsify carotenoids for intra-venous injection. However, the AUC technique cannot be used if the factor affecting bioavailability studied does also affect clearance rate from plasma [100], changing AUC in addition to absorption.

This serum AUC technique was used to compare lutein and zeaxanthin absorption following intake of a single dose of carotenoids vs. their esters. Esters were found to be somewhat better bioavailable (i.e. higher AUC) compared the unesterified form [100, 144], albeit different formulations were served in one study [100]. Additionally, the 24h blood collection in the other study might have been too short for complete estimation of AUC. AUC in plasma over 20h was also used to show that some types of dietary fiber, such as pectin, had a negative effect on carotenoid (lycopene and lutein) absorption [176], which had been earlier demonstrated in animals, i.e. chickens [175], however, it is again possible that the limited 20h time course compromised the estimation of the AUC correctly. It was likewise used to show higher carotenoid (lutein) response when supplemented in corn oil compared to spinach and broccoli over 104 hours [260]. The effect of adding lipids to carotenoid containing meals or components, especially to lycopene, was also found in other studies based on plasma measurements following an intervention over several days [261], or monitoring plasma TRL (see following chapter).

AUC can also be used for longer dietary intervention studies, albeit results are then difficult to interpret pharmacokinetically. For example, Horvitz and colleagues [262] investigated the serum AUC bioavailability of lycopene during 11d intervention, finding increased plasma levels from tomato paste compared to unhomogenized red carrots, being in line with animal studies [190].

Plasma TRL Fraction and Lipoproteins

Another method involving AUC measurements is the determination of carotenoids in the triacylglyerol-rich lipoprotein (TRL) fraction of the serum/plasma [263-265]. The TRL fraction is mainly assessed in bioavailability studies to measure newly absorbed carotenoids or retinyl esters [47] from single meals or doses, as it is thought that this chylomicron rich pool does not rapidly exchange carotenoids with the HDL/LDL fraction of the blood. If factors such as the half-life of carotenoids in this fraction and plasma volume is estimated, fractional absorption from single test meals can be assessed [47]. Based on TRL-fraction investigations, absorption was shown to follow a biphasic pattern of appearance, between 2-3 and 4-5 hrs postprandial, such as for beta-carotene [222] or lycopene [47, 67, 222, 266, 267], as sequestration from the enterocyte into lipoproteins is thought to be increased during subsequent lipid uptake from the diet. For determination of beta-carotene absorption, retinyl esters have to be assessed in addition to free beta-carotene, otherwise absorption will be underestimated [116]. When using this technique, typically hourly time points following test meal intake incorporating blood draws are carried out, over time periods of 8 to 12 h [263, 268], including baseline level measurements prior to test-meal intake. AUC is then determined for each carotenoid after subtraction of baseline values.

Drawbacks of using this technique is that compared to the plasma, the TRL fraction gives no insight into further metabolism and long-term distribution of carotenoids, relatively large plasma volumes (usually >2.5 mL) may be needed for TRL separation, and obtaining TRL fraction from plasma can be time-consuming, as usually ultra-centrifugation over prolonged time (0.5 to several hours) is involved [47, 263]. As TRL appearance is impacted by the

following test meals, larger variations across studies compared to e.g. long-term feeding studies and plasma measurements, may be expected. Therefore, long-term absorption measured by plasma appearance may differ from TRL studies due to body adjustment by the diet. On the other hand, the TRL measurements might be less subjective to host related differences such as antioxidant status, which has been discussed to impact serum-concentrations during long-term studies. A good example, based on both TRL and plasma measurements might be the studies suggesting that high doses of one carotenoid could impede absorption of another [256, 258, 268, 269], whereas this effect was generally not observed for long-term intervention studies [270-272]. Whereas it seems plausible that carotenoids compete and inhibit each other during uptake and absorption, the simultaneous intake over prolonged periods of time could also result in carotenoid sparing effects in plasma, due to additive antioxidative effects, prolonging the half-life of carotenoids in the circulation and/or tissues.

TRL fractions have been investigated in a number of studies. Although the micellarization for (13-cis) beta-carotene was shown to be significantly higher compared to the all-trans form [116], the uptake of the all-trans compared to (9-cis) beta-carotene, was favored in humans [267], being in line with other human studies based on plasma measurements following single dosing [273] or prolonged dietary intervention [249], and with studies in gerbils and ferrets [186, 274], again indicating differences in the cellular uptake or absorption between various carotenoids.

Even though chylomicrons have been investigated most often, other lipoprotein fractions have also been studied. Serum LDL and HDL concentrations for several carotenoids were studied, including the less frequently investigated phytofluene, to detect differences in bioavailability [275]. Dose-corrected concentrations of phytofluene comparable to the other carotenoids was shown, and lower beta-carotene bioavailability based on lower incorporation into apoB containing lipoproteins (LDL, IDL, VLDL), following a 4wk feeding trial with a plant stanol low fat rich yoghurt. However, as these lipoproteins do not reflect newly absorbed carotenoids only, but a mixture of endogenous and recently absorbed carotenoids, it appears harder to draw conclusions on bioavailability following dietary intervention.

Tissue Sampling

Collecting tissues from human samples can be invasive and is ethically restricted. In addition, since many of these tissues can, if at all, only be sampled in small amounts, refined analytical techniques have to be used for accurate detection of carotenoids. Ideally, the tissue collected should be easily accessible in sufficient quantity or measurable, accumulate a large portion of circulating carotenoids, and correlate well with average, especially long-term carotenoid intake. Carotenoids have been reported to be preliminary stored in adipose tissue and the liver. Absorbed beta-carotene, for example was shown to be stored 80% in adipose tissue and 10% in the liver [276]. Due to the impediment of requiring biopsies, only a few studies have sampled liver [277] or adipose tissue [64, 231, 278, 279]. Concentrations of total carotenoids in liver between 0-280 nmol/g have been detected [277], around 0-30 nmol/g in adipose tissue in one [251] but up to 6000 nmol/g in the abdominal adipose tissue in another study [64]. Other tissues that have been collected or investigated included prostate tissue from patients scheduled for prostatetectonomy [8, 65], which was suggested to be a good lycopene target tissue due to enrichment of lycopene up to 10 times above that of plasma, up to around 3 nmol/g [65], the relatively easily accessible buccal cells [248, 280, 281] with concentrations up to ca. 4 nmol/μg protein, the non-collectible macula pigment [282, 283] and skin, where beta-carotene was found to be accumulated with a delay of approx. 2 wks following dietary intake [284]. High concentrations, at least for lycopene were also found in testes and adrenals, up to nine-times higher compared to liver [285], where lycopene seems to be especially located in the membrane and nuclear fraction [286].

Measurement of carotenoid tissue concentrations is of special interest as a marker reflecting rather long-term intake of carotenoids. With respect to the correlation of carotenoid intake from the diet and a number of chronic diseases, it is desired to assess the long-term status of carotenoid reflecting intake and supply. However, this determination is impeded by a number of factors. Firstly, no specific target tissue has been detected for carotenoids, which would allow rapid sampling and possessing a strong association with long-term dietary intake. As mentioned, relatively high concentrations of carotenoids may be found especially in liver, adipose tissue or, for lycopene, prostate, and lutein/zeaxanthin in the macula lutea of the eye, all of which are not easily accessible. A further impediment is that different carotenoids are metabolized and stored differently. Provitamin A carotenoids alpha- and beta-carotene are, as well as cryptoxanthin, partly metabolized into retinyl-esters, e.g. 19% after 56 days following a single beta-carotene dose [287]. The retinyl esters are then stored predominantly (>90%) in the liver and kidneys [234], while others, including lutein and zeaxanthin, accumulate in the macula of the human eye, and lycopene, as stated earlier, in prostate, testes, and breast tissue [65].

Furthermore, even with high carotenoid accumulation in some tissues, the correlation to dietary intake might still remain low. Correlations between either carotenoid intake or plasma concentrations with adipose tissue [279] and skin [278] have been found, suggesting that these tissues could be used to investigate carotenoid utilization and perhaps status. Similarly, in a recent study by Chung *et al.* [64], significant correlations were found between dietary intake and concentrations of individual and total carotenoids in adipose tissue, including abdomen, buttocks, and inner thigh, albeit correlations differed depending on site. A positive correlation (0.56) between plasma and adipose tissue for beta-carotene has been reported [288], however, in a cross-sectional study, only poor or no correlation between beta-carotene and lycopene in adipose tissue and intake from certain fruits and vegetables was found [289]. In another cross-sectional study with over 500 subjects, low but significant correlations between intake and adipose tissues for alpha-and beta-carotene, and beta-cryptoxanthin but not lutein/zeaxanthin were detected [279] in women, but not in men, while plasma levels and intake correlated higher and significantly for most carotenoids in both genders. However, correlation between plasma and adipose tissue correlated somewhat better,

indicating overall strength of correlation with the intake to decline from plasma to tissues.

No correlation between e.g. lycopene mucosal buccal cell concentration and serum was found in several studies [248, 290, 291], but not all studies [278, 280]. Interestingly, while a low correlation between lutein in the macula and serum has been reported, a negative correlation of lutein in the macula and adipose tissue in women was found [235], suggesting that tissues competed for lutein. A significant correlation between the MP optical density and plateau plasma concentration was also found also by Bone *et al.* [283]. It seems that carotenoids are rather rapidly exchanged between serum and tissues, impeding their use as a long-term status marker of dietary intake.

Taken together, there is controversial data on whether and to what extent tissue sampling correlate with dietary intake, or plasma carotenoid concentrations, and for the individual a single measurement based on one sample might not be sufficient. While plasma appears to be relatively well correlated with carotenoid intake, reflecting short-term changes of the diet, this is less so for the tissues, which do exchange slowly, perhaps constituting a more long-term marker of carotenoid supply.

Studies Employing Carotenoid Isotopes

Employing isotopically labelled carotenoids has the advantage that these carotenoids become physically distinguishable from its native, chemically identical forms, yielding the basis for mathematical modeling [48]. Additionally, it allows their usage as labels, i.e. the carotenoids can be blended into a food and the fate of this specifically carotenoid can be followed after intake, and differentiated from endogenously present carotenoids of the host, or carotenoids from subsequent meals. This allows studying uptake from e.g. single test meals, or comparing several subsequent test meals in one subject for the same carotenoid, given a different labeling technique for the two carotenoid sources, similar as carried out in a previous study administering D6-beta-carotene and ^{14}C-beta-carotene simultaneously [292]. The use of stable isotopes for studying carotenoid utilization has been recently reviewed [233, 234] summarizing results from a dozen human studies. Since then, almost an additional dozen studies have utilized isotopically labeled carotenoids [223, 292-299].

The ideal label can be given in very tiny amounts, acting as a true tracer, i.e. not altering normal metabolism, is safe, affordable, and easily detectable. This ideal situation does usually not exist, and compromises have to be sought. Carotenoids can be labeled with either stable isotopes or radioisotopes. Sufficiently long-living radioisotopes can be determined by scintillators, which are generally affordable, and sensitive [233], i.e. doses down to 0.5-1 nmol can be used for administration and detection [300]. In addition, as the so labeled carotenoid does not need to be detected by mass discriminating techniques, metabolization or breakdown of the compound of interest does not perturb absorption and bioavailability results. ^{14}C and ^{3}H with a half life of 5730 and 12 years, respectively, have been used [154, 155, 301-306], however, also ^{17}O and ^{18}O may theoretically be used, but, to the authors' knowledge, have not been incorporated into xanthophylls for bioavailability studies, even though they have been used to study carotenoid metabolization in bacteria [307]. The main disadvantages of radioisoptes are ethical considerations, which have often prohibited their use.

Stable isotopes for carotenoid labeling include ^{13}C or ^{2}H, with sufficiently low natural abundance of 1.1% and 0.015 %. Vitamin A and carotenoids labeled with 3-10x^{13}C and 5-8x^{2}H are available from different suppliers, including e.g. Cambridge Isotope Laboratory (Andover, MA, USA) and ARC Laboratories (Apeldoorn, the Netherlands). Stable isotopes are typically detected with sophisticated and expensive mass spectrometry techniques, including HPLC-MS [287, 295, 297, 298, 308], HPLC-MS-MS [305], or, for highest sensitivity for femtomole range detection, accelerator mass spectrometry [234]. However, an HPLC method to separate ^{2}H8beta-carotene, has been reported, while separation of ^{2}H6beta-carotene from beta-carotene was not successful [305], explained by increased polarity when carrying deuterated atoms. As a consequence, with using recent state of the art techniques, the earlier drawback of stable isotopes requiring large doses for sufficient sensitivity is dwindling. Thus, doses as low as 55 μg ^{13}C10beta-carotene were administered when using HPLC-MS [308], and HPLC-MS-MS [305], and amounts as low as 1 nmol C-labeled (both radioactive or not) beta-carotene by using accelerator-MS [300] can be employed. Using stable isotope techniques, TRL-AUC fraction for beta-carotene has been investigated [309] to demonstrate that free and plant sterol esters can have a negative impact on carotenoid absorption, as has been compartment modeling for studying beta-carotene utilization [48].

Another problem consists of the way the label is introduced to a food matrix or supplement. If the label should perfectly mimic behavior of carotenoids natively present in the food, it should be present in the very same form within the matrix, such as in chloroplasts or chromoplasts. This however requires very expensive and time consuming intrinsic labeling, and has not been carried out often, such as by growing kale in the presence of labelled CO_2 [295, 298]. In addition, this labeling also leads to labeling of many other compounds, such as different carotenoids with identical mass in the food matrix, which may impede analytical detection. Extrinsic labels simply, such as labeled carotenoid standards added in pure form and blended with the test meal on the other hand could work for simple matrices such as food supplements, assuming equilibrium between the added carotenoids and carotenoids normally or natively present, are more affordable, can be dosed easily, but do not account for more complex matrix binding effects. Nevertheless, a number of studies have been conducted, investigating uptake and distribution of carotenoids dissolved in oil ingested together with a the test meal [287, 300, 305, 308].

CONCLUSIONS

In summary, there exist a large variety of models to assess several aspects determining carotenoid bioavailability. While simple and cost effective micellarization studies showed, in general, decent agreement to cell, animal, and

human studies, this model is limited by not being able to assess further 'downstream' pathways affecting bioavailability, i.e. uptake, absorption, and biodistribution, which may significantly counterbalance decreased micellarization, as with carotenes or xanthophyll-esters. Cell studies are somewhat more complex, allowing taking into account transport mechanisms into and across the intestinal epithelium, which are probably, together with micellarization, the most important steps in determining bioavailability. Nevertheless, transport in lipoproteins, biodistribution, and long term additive or synergistic effects, such as antioxidant mechanism resulting in carotenoid sparing effects, cannot be simulated by cell models and have to rely on animals. However, as there are considerable differences between animals and humans in carotenoid uptake and metabolization, especially for the pro-vitamin A carotenoids, the human study is being still considered the gold-standard.

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