CHAPTER 14

In Vitro Digestion Protocols. The Benchmark for Estimation of In Vivo Data

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Abstract

This chapter examines the current knowledge obtained by the application of *in vitro* digestion protocols (IVDs) to measure the micellarization of carotenoid-rich food sources. The discussion starts with the definition of the term bioavailability, which generates scientific and technological information necessary to support the health-through-food idea. An outlook of the use of the bioaccessibility function in food and nutrition science and the creativity directed to the development of IVDs is exposed from a practical point of view, rationalising the inputs from the *in vivo* physiology of human digestion, and the key developments implemented, crucial for the successful use of IVDs. Later, the events correlated with the digestion of food lipids are outlined, keeping the *in vivo* applicability of *in vitro* data as the thematic thread of the story. The effectors in micellarization of carotenoids are subsequently presented, accounting the significance of food structure, food matrix and processing features, physiological issues, and other host-related aspects. The correspondence between the earlier results obtained from *in vivo* studies with the recent outcomes from IVDs is emphasised. The IVDs developed for carotenoids are discussed, introducing elements and references for comparison between them and with the harmonised IVD lately launched.

14.1 The Application of Good Practices in the Use of the Term Bioavailability

We, fortunately, live in an age that science has become a critical pillar of society. The efforts of the scientific community are aimed to improve living conditions in a sustainable world, both environmentally and economically, reaching all members of humanity and nature ecosystems. This model of science in society has required to shorten the distance between the laboratory bench and people's attitudes towards the scientific knowledge. To focus our vision of actual needs that require taking action, the diffusion of the scientific developments is compulsory and, conversely, the feedback from the public is essential. In this sort of dialogue, science must communicate fundamentals and key concepts clearly and accurately, without oversimplification, to create critical thinking skills in society. The use of language must highlight the potential benefits and risks of the scientific innovations with a speech that finally allows the plural and diverse people to take more informed decisions. Among the essential public long-term debates such as the climate change, economics versus sustainable development, synthetic biology and ethics, and others, the application of healthy lifestyle and nutrition habits in society, and how the different actors (scientific community, policy-makers and health agencies, food industry and consumers) contribute to the achievement of this target has recently demanded a continuous attention. Indeed, it has been necessary to create a concerted framework with several regulatory issues aimed to provide confidence of the society in the scientific advancements and evidence in relation with the impact of food in human health, mainly through the activity of certain nutrients and non-nutrient substances in decreasing the risk of development of certain diseases.^{1,2}

One of the key issues that may significantly support the confidence of consumers in the strategy of health-through-food is the bioavailability concept. The word *bioavailability* is probably not infiltrated in the popular vocabulary, as different scientific terms are used to indicate the idea of bioavailability, and the final association that consumers make of this concept is related with other words such as safety, quality, efficacy, absorption and even digestibility.³ However, other familiar concepts are also linked with bioavailability, *e.g.* design, processing, and activity, although the association with those terms is less frequent. Therefore, it is difficult to build a definition of bioavailability without excluding some of the ideas that are also enclosed in this concept.

Depending on the limit we apply or the scientific expertise we manage, there are different working definitions. Additionally, the considered definition will direct to the use of diverse experimental approaches, the existence (or not) of harmonised protocols and the scope of the data obtained with them. Hence, the Food and Drug Administration defines bioavailability from the pharmacological point of view as the rate and extent to which the

therapeutic moiety is absorbed and becomes available at the site of drug action. This is measured as the area-under-the-curve of the fraction of the dose that reaches the systemic circulation after oral administration. When contaminants in soil are the target of the study, the bioavailability is defined as the degree to which chemicals present in the soil matrix may be absorbed or metabolised by human or ecological receptors or are available for interaction with biological systems.⁴ In human nutrition, bioavailability is regarded as the fraction of the nutrient or bioactive compound ingested that is available for use in physiologic functions or to be stored.⁵ In the same field, bioavailability could be defined as the proportion of a given nutrient in a given food or diet that the body can actually use.⁶ Consequently, the term bioavailability may include the sequence of digestibility or availability for absorption, absorption itself, first-pass metabolism, tissue distribution and storage, and bioactivity or metabolism. Other definitions, even in the nutritional arena, do not reach that complete sequence, and the bioavailability includes digestibility or availability for absorption, and absorption, while in some cases bioavailability refers to digestibility or availability for absorption.^{7,8}

The idea of bioavailability should encompass its concept and definitions, but when applied to nutrition, it is the understanding and operational ability to show that the nutrient or non-nutrient compound provides a health benefit through measuring the digestion and assimilation and, once absorbed, how it performs a function in the body by observing some endpoint of the metabolic pathway used as biomarker for bioavailability.^{9,10}

14.1.1 The Need of Splitting the Term Bioavailability into Bioaccessibility and Bioactivity. A Practicable Achievement vs a Paramount Effort

The achievement of bioavailability, as it has been defined above, means carrying out a paramount effort. It is necessary to implicate broad fundamentals with different technical approaches, and the amount of experiments necessary to cover the diverse aspects of bioavailability is considerable. Moreover, some of the specific issues within the concept of bioavailability require samples of biological tissues (humans or animals) to measure the delivery of food compounds on sites of specific organs and to identify or isolate biomarkers of biological action, what conveys practical and ethical difficulties.¹⁰ Considering the complete sequence of events enclosed in the bioavailability definition, it is conceivable to split the term into aims or concepts that resolve the accomplishment of bioavailability from a realisable point of view. Thus, the concept of bioavailability includes two functions, bioaccessibility and bioactivity (Figure 14.1), see also Chapter 13. Bioaccessibility includes the entire sequence of events that take place during the digestive transformation of food into

material that can be assimilated by the body, the absorption/assimilation into the cells of the intestinal epithelium and the presystemic metabolism.¹⁰ Bioactivity is the measurement of those events linked to how the bioactive compound is transported in the systemic circulation, reaches the target tissue and interacts with other molecules and follows some metabolism yielding a biomarker and a physiological response. This distinction of bioaccessibility and bioactivity is rationalised by the existence of systematic experimental *in vitro* and *ex vivo* techniques for the analysis of bioaccessibility (see Figure 14.1 and section 14.2), while bioactivity determination requires individual and specific experimental approaches that need to be adjusted to every *bioactivity claim*, and consequently, there is no common systematic methodology.¹¹ Indeed, some bioactive compounds that perform their activity in the stomach or gut do not require the estimation of all the events linked to bioaccessibility, *i.e.* only the function bioactivity needs to be measured to obtain the bioavailability. This is the case of non-digestible polysaccharides and oligosaccharides,¹² the antioxidant, antimutagenic and antigenotoxic activities described for chlorophyll derivatives,¹³ or the lipid hydroperoxidase activity of myoglobin, and phenolic antioxidants.^{14,15} Conversely, the bioaccessibility provides enough evidence to support some issues without measuring the bioactivity. Thus, both the claims related with the nutritional content of food¹⁶ (see section 14.1.2) or comparison of the food composition between different foodstuffs are sustained by the measurement of bioaccessibility, because we have already the knowledge concerning to the bioactivity of nutrients so that it is not necessary to determine it.

[Figure 14.1 near here]

Within this scenario, the reader should consider that extensive research focussed on bioavailability is actually aimed to bioaccessibility, *i.e.*, both terms were often used indistinctly. However, it is important to note that the term bioavailability includes bioactivity, while bioaccessibility is often referred as the fraction of a compound that, once released from the food matrix in the gastrointestinal tract, is available for intestinal absorption,⁶ what makes bioaccessibility becoming equal to digestibility or micellarization (Figure 14.1). These terms are a measure of the degree of digesta that has become ready for absorption, but micellarization applies specifically to lipophilic compounds that must incorporate to mixed micelles. Hence, mixed micelles are molecular aggregates capable of crossing the hydrophilic environment of the intestinal lumen overlying the intestinal cells where dissociation of micellar components occurs. Although bioaccessibility matches with micellarization, studies where *in vitro* micellarization protocols are coupled with *in vitro* absorption in cell culture are

also frequently available. Measurement of bioaccessibility in such way yields more reliable data, although still the possible effect of the first-pass metabolism is not considered. Only a critical read of the references, particularly of the methodologies applied to each case, will show whether bioaccessibility was made equal to micellarization, micellarization plus cellular absorption or the three steps described in Figure 14.1.

14.1.2 Applications of the Bioaccessibility Concept in Food and Nutrition Science

The claims related with nutrient content of food should ascertain the bioaccessibility of the nutrient for which the claim is posted among other guidelines.¹⁶ That is, the objective of food labelling is not solely to provide information to the consumer of the macro- and micro-nutrients facts, and the reached percentage of daily value based on the recommended daily intake, but also to give reliability in that the nutritional benefits are achieved. This is a critical issue in the design of food formulations that incorporate nutrients or bioactive compounds in food matrices where the bioaccessibility has not been considered a principal component in the resulting nutritional value of the food.¹⁷ The events taking place during digestion, the solubility or the release of compounds, the probable complex interactions between different classes of ingredients, the efficiency of the activity of gastrointestinal enzymes, the cellular permeability or transport mechanisms are significant influencing factors that limit or enhance the final *in vivo* bioaccessibility.¹⁸ Thus, the presence of some ingredients limits the bioaccessibility of other ones, and the contrary effect, an enhancement of the bioaccessibility has also been previously described.¹⁹⁻²¹ This issue was the motivation for developing the *nutraceutical bioavailability scheme* that characterises the abovementioned factors and how they could assist in the design of food matrices to improve the bioaccessibility.²² Systematic approaches in the outline of delivery systems, where the goal is to maximise the bioaccessibility of low soluble lipophilic compounds have been published.²³⁻²⁵

14.2 In Vitro Digestion Protocols: The Imitation Game

The application of *in vitro* digestion methods has represented a significant advance in knowledge not only in the digestive fate of nutrients and non-nutrients but also in the biological and physiological features of the gastrointestinal tract. As any *in vitro* technique, the *in vitro* digestion methods try to reproduce the physicochemical processes taking place during human digestion in a controlled environment out of the organism. Therefore, the number of variables and *in vivo* factors are considerably reduced, so that the effect of a set of critical variables in the digestibility is tested, and mechanisms of action might be inferred. On the other hand, to obtain results comparable with the *in vivo* approach, it is necessary to have

an in-depth knowledge of the biological and physiological barriers that limit intestinal absorption, the composition of salivary, gastric and intestinal fluids, the enzymatic activities and changing pH conditions, the transient time and gastrointestinal motility, and the intricate interactions established between food components.¹⁸ At this point, it is when the reader should discriminate that complex *in vitro* digestion methods will mimic the physiology of human digestion, while the straightforward procedures will produce a considerable amount of data within a high-throughput screening process. In any of the situations, complexity vs simplicity, the researcher should consider that the decision-making process on the method design to apply is related with the kind of target compounds which micellarization is determined, and the question that needs to be answered. Thus, the experimental protocols applied to the study of the digestibility of minerals are different from those applied to estimate the micellarization of lipophilic molecules.²⁶

The efforts made to the design of protocols for the *in vitro* digestion of peptides and proteins are commonly concentrated in the gastric phase of the digestion, while the study of the conditions reached during the intestinal phase is the target in the case of digestibility of lipophilic compounds. The conclusion is that there is no standard protocol, but a consensus of minimum benchmarks and conditions that should be applied to obtain a satisfactory grade of confidence in the results, to allow the comparison of results between different laboratories, and to establish further improvements in the technique.

14.2.1 Key Developments in the Design of *In Vitro* Digestion Protocols

The scenario for the development of *in vitro* digestion protocols has been changing and challenging, with some doses of controversy and conclusive efforts to refine the methodology. Again, it is necessary to remark that the results obtained from the *in vitro* procedures should be close to those obtained from *in vivo* approaches. Thus, the inclusion or exclusion of some of the sequential steps and the parameters outlined in common experimental protocols to assess micellarization should be carefully observed, as they may introduce significant effects in the results.

The physiology of human digestion is the golden standard to be reproduced by the *in vitro* protocol that transforms the original food into a digesta or chyme ready for absorption. This complex task might be carried out with static or dynamic approaches. Static methods are designed like a batch processing, introducing the sequence of buccal and gastric stages, and then the intestinal phase. At each step, the food material is mixed with fluids which composition could resemble that of the digestive fluids. Hence, their chemical composition includes a complex mixture of organic and inorganic constituents,²⁷ although it is a common

practice to use a simpler combination of chemicals (see section 14.2.3). The sample to solution ratio is a critical factor to control that may vary between 1:2 and 1:150 g mL⁻¹ as described for *in vitro* protocols applied to the bioaccessibility of metals.²⁸ The temperature applied during the complete process is 37 °C, with shaking or agitation of the mixed food materials and fluids during defined periods of time (2 min for the oral stage, 8 min-2 h for gastric phase and 2-4 h for intestinal digestion). Once the analyses of the content of the target compound or nutrient in both the starting material for digestion and the digest, the micellarization index is calculated with the equation 14.1.

$$\text{Micellarization index} = \frac{\text{Amount of target compound or nutrient in micelles}}{\text{Amount of target compound or nutrient in the starting material}} \times 100 \quad 14.1$$

One of the key developments in the design of *in vitro* digestion protocols was the introduction of dynamic conditions.²⁹ This approach allows the control of the transient time of food, the gastric and gut emptying and the composition of digestive fluids, along with other experimental conditions to simulate with high degree of precision the *in vivo* gastrointestinal process. This multi-compartmental design is complemented with another system that simulates the conditions reached in the large intestine, so that a tool to analyse the fermentation of digesta by the intestinal microbiota is available.^{30,31}

Standardization has been another relevant issue in the design of *in vitro* digestion protocols. Different procedures have been developed to obtain reference values of the micellarization index under experimental conditions that may be reproduced in any laboratory. This is the case of the analysis of digestibility of metals from contaminated soils, aimed to assess the risk of toxicity. Five standard protocols have been designed to achieve this issue.³²⁻³⁶ However, a comparative study revealed that the protocols yield different digestibility indexes, even when the same certified material was used.³⁷ Moreover, this comparative study showed that the gastric pH value is the critical factor in the mobilisation of metals from the contaminated soil so that the differences in the protocols regarding the conditions of the gastric phase should be carefully evaluated. Another significant achievement in the study of metal digestibility was the introduction of dialysis membranes in the intestinal phase. Hence, an equilibrium is established between the chyme and the dialysate and the freely dissolved metals and small complexes diffuse across the membrane.²⁶ In the case of digestibility of iron, the results obtained with this improvement in the design of *in vitro* digestion protocols are highly correlated with human iron availability trials.^{38,39}

A look at the literature regarding the published *in vitro* digestion protocols suggests that the experimental procedures were almost tailored at each laboratory in function of the physicochemical characteristics of the target compounds, the kind of question to be answered or the mechanism to be figured out.⁴⁰⁻⁴² Protocols have been published for the *in vitro* digestion of minerals, carbohydrates, proteins, triacylglycerides, vitamins, phytochemicals (e.g. chlorophylls, carotenoids, phytosterols), with significant changes in the experimental conditions and with different research purposes.⁴³⁻⁵² The existence of such amount of different procedures and the quite different digestibility indexes they provide (even within the same group of target compounds) pointed to the need for a harmonised *in vitro* digestion protocol. The consensus protocol could help to obtain comparable data for interlaboratory control of samples (useful in the assessment of toxicity risk), to draw general conclusions about the influence of critical factors affecting digestibility of nutrients and nutrient-drug interactions, and to become a tool in the design of efficient new food formulations and delivery systems of nutraceuticals.⁴² The elaboration and harmonisation of *in vitro* digestion protocols was the major aim of the INFOGEST project, resulting in the publication of a static protocol easy to set-up in any laboratory, with experimental conditions that were agreed after a careful revision of the literature by experts in the field, and allowing the comparison of data obtained by different research groups.^{53,54} The harmonised protocol is focussed in protein hydrolysis and points to several practical issues that should be considered in the application of digestive conditions, although the basic set of conditions close to the *in vivo* situation is established and referred by the authors as the *smallest common denominator*. Moreover, the protocol includes standard methodologies to measure the activity of gastric and intestinal enzymes, so that the comparability between different laboratories is easier to reach, removing confounding factors. However, caution should be observed when applying this harmonised protocol to measure the digestibility of lipophilic compounds, e.g. carotenoids, because the correlation of the *in vitro* results with the published *in vivo* data is still lacking.⁵⁵

The use of microscopic and light scattering techniques has provided very useful information to understand the effect of the conditions applied at each digestive step, e.g. in the size distribution of microemulsions, the changes produced in the structure and composition of the cell wall and the subcellular organelles and other macromolecular structures (chloroplasts, chromoplasts, milk fat globules) in plant food materials, and how they evolve during the simulated transit through the digestive tract. This information is important in the design of food delivery systems.^{42,56-63}

Regarding the measurement of the micellarization index of lipophilic compounds, a critical improvement to obtain reliable results in line with the *in vivo* digestion was the set-up

of experimental conditions to isolate the micellar fraction. Micelles are water-soluble molecular aggregates produced by the self-assembly of amphiphilic compounds after the enzymatic activity of pancreatic secretions and the emulsifying action of bile salts with the lipid fraction of the ingesta. These globular macro-structures with a diameter range between 2-20 nm present a shell region built with polar lipids and bile salts that interact with the chyme, while the apolar content is stored in the core. Micellarization of the digested lipids (*e.g.* free fatty acids and cholesterol, monoacylglycerides, phospholipids, lipophilic vitamins, chlorophyll derivatives, carotenoids) is the strategy that nature has developed to transport the digested lipids from the chyme to the unstirred water layer of the small intestine (see section 14.2.2). Consequently, to obtain the micellarization index of lipophilic compounds, it is necessary to isolate the micellar fraction once the *in vitro* digestion protocol has finished. The conditions were established with intestinal aspirates from human beings following the feeding of a triacylglycerol-rich meal.⁶⁴ The *ex vivo* chemical and physicochemical analyses of the different layers obtained after ultracentrifugation of the aspirates revealed the composition and size distribution of the micelles, which were isolated from the undigested lipids and rest of chyme fluids.

Another significant advancement in the design of *in vitro* digestion protocols has been to tailor the experimental conditions according to the gastrointestinal capability of different population groups, mainly the new-borns and infants,⁶⁵⁻⁶⁸ and the elderly.⁶⁹⁻⁷¹ More recently, the potential need of *in vitro* digestion procedures adapted to mimic the *in vivo* circumstance of patients with hepatobiliary disorders, or those subjected to surgery (gastric or intestinal bypass) and medication, or people affected by chronic illnesses like Chron disease or other inflammatory diseases of the gut, and diabetics among others has been emphasized.⁷² The current diversification of methods by different target populations comes to provide a detailed answer to food industry in the development of tailored food products, while the potential requirements demonstrate the on-going efforts that the design of *in vitro* digestion methods demands.

Last but not least, there has been a considerable effort in the acquisition of standardized gastrointestinal fluids with the aim of improving the relevance of the *in vitro* methods and consequently, their similarity with the *in vivo* approach, and to allow the comparison of data from different laboratories minimising the possibility of finding contradictory results. The use of standards or reference materials of gastrointestinal fluids is a required scenario when the *in vitro* digestion protocol is coupled with cell culture models to establish absorption rates and transport mechanisms. Data from cellular models are highly sensitive to small differences in the experimental conditions so that the interlaboratory

comparison is challenging. Additionally, the implementation of simulated gastrointestinal fluids may increase the relevance of data and the *in vivo* predictive capability of the *in vitro* procedures, since physiological conditions are mimicked.⁷³ Some references are available to acknowledge the composition of standard gastrointestinal fluids even simulating fasted and fed state conditions.^{74,75}

14.2.2 Digestion of Lipophilic Compounds or How to Cross the Water Barrier

Digestion and absorption of lipids involve a sequence of physicochemical processes, *i.e.* *release from food matrix, transfer to lipid droplets, emulsification* and *micellarization*, which render hydrophobic compounds in a fashion capable, *i.e.* mixed micelles, to be transported through the hydrophilic environment of the intestinal lumen, which cross the unstirred water layer overlying the intestinal cells and diffuse through the mucin layer to the apical side of enterocytes, where dissociation of micellar components occurs, and starts the interaction of dietary lipids with the brush border proteins for transfer into the cell (see Chapter 13). In this complex process, several parameters are key players, from the natural or processing-induced physicochemical barriers and their effect in the process of lipids release and formation of lipid droplets, the action of digestive secretions that include enzymes, bile salts and other emulsifying agents, to the kinetics of gastric emptying and gastroduodenal motility.⁷⁶ Additionally, the understanding of the regulation of cellular uptake of dietary lipids is only completed once the genetic factors, mainly defects and polymorphisms, are ascertained, as well as the functional consequences produced in the proteins they encode. A vast open research field that started to deal with this issue a few decades ago is still very active, gaining insights on significant determinants of the intestinal lipid flux, the lipid homeostasis, cardiometabolic disorders and atherogenesis while supporting the efforts in finding nutritional strategies and genetic developments to face congenital lipid mal-absorptions.⁷⁷⁻⁸⁹

It is noteworthy to claim again the attention of the reader about the difficult task to mimic the events taking place during food digestion in the human gastrointestinal tract with an experimental approach that is aimed to keep it relatively simple and flexible. Nevertheless, it is necessary to include a minimum set of standardized parameters that reflect the actual *in vivo* digestive conditions. Indeed, such knowledge allows making a critical revision of the *in vitro* digestion protocols, what it is particularly useful for comparisons between data obtained from different experimental conditions. Moreover, if the objective is to design an *in vitro* digestion protocol, the *in vivo* conditions are the starting point. Therefore, the reader is referred to literature containing biochemical data of gastrointestinal secretions, motility, and kinetics of digestion.^{77,90-95}

14.2.3 Methods Applied to Measure the Micellarization of Carotenoids: A Practical Approach

14.2.3.1 Critical Factors that Affect the Micellarization of Carotenoids

The considerable research literature regarding the measurement of carotenoids appearing in serum after the ingestion of different carotenoid-rich sources, *e.g.* fruit and vegetables, juices, oils, either in human beings or animals, was the starting point to learn that efficiency of the carotenoid bioaccessibility is controlled by several exogenous and *in vivo* factors that may limit or enhance their final disposition in systemic circulation and inner tissues. Thus, the study of Castenmiller and West⁹⁶ systematically reviewed the factors that influence carotenoid bioavailability, which were condensed in the mnemotechnic term *SLAMENGHI* where each letter represents one variable. It is necessary to remark again that the influence of these factors was initially ascertained from *in vivo* experiments, while our more recent advancements have made use of *in vitro* techniques to determine the considerable number of factors that affect bioaccessibility with similar confidence to the *in vivo* approach, a subject that is addressed below.

In this chapter, the factors affecting bioaccessibility of carotenoids are grouped within food structure, food matrix and processing features, physiological issues, and genetic and host-related aspects, which have been the common thread in recent reviews.^{97,98} Not all of them can be uncovered through *in vitro* approaches because these techniques cannot reproduce the high complexity of human digestion, while the influence of genetic and host-related factors is not accessible. Thus, a general outlook on the effects in micellarization and cellular absorption of carotenoids from food matrices, in addition to some physiological issues, is provided below, and summarised in Figure 14.2, emphasising the correspondence between the earlier results obtained from *in vivo* studies with the recent outcomes from *in vitro* models of digestion.

[Figure 14.2 near here]

Food processing usually is the starting point to observe the efficiency of micellarization of carotenoids because it is a factor with special weight in the digestive process. Raw carotenoid-rich food sources such as fruits and vegetables generally provide low digestibility efficiency, while application of different food processing techniques may significantly increase the outcome of carotenoid absorption. Simple processes, *e.g.* chopping,

cutting, mashing, blending or juicing, mechanically disrupt the original matrix and release the cellular contents to a homogenised environment, overcoming the adverse effect of the natural barriers (organelle and cellular membranes, cell wall), as noted in Chapters 2 and 13. Thus, the *release from food matrix* and *transfer to lipid droplets*, the first step in the digestive process of lipophilic compounds, is considerably enhanced. In fact, several *in vivo* studies revealed the influence of mechanical processing in the plasma circulating carotenoids, which were significantly increased when processed carotenoid-rich sources were consumed in comparison with the corresponding raw foodstuffs.⁹⁹⁻¹⁰² However, processing liberates other food components that were not previously in contact with carotenoids, *e.g.* oxidative enzymes, reactive oxygen species, organic acids and pectin, resulting in a deleterious effect on the pigment content or carotenoid complexation that hinders the *in vivo* accumulation of carotenoids.¹⁰³⁻¹⁰⁶

Application of technological treatments (thermal and non-thermal processes) and cooking procedures (boiling, roasting, frying, microwave heating) softens the original plant matrix and alleviates the transfer of carotenoids to lipid droplets in the intestinal lumen¹⁰⁷⁻¹⁰⁹ but, again, the enhancement in micellarization should be evaluated together with the probable carotenoid degradation and isomerization reactions due to temperature regimes. These *in vivo* studies already pointed to the consequences of the cellular matrix of the plant material and the carotenoid deposition state, in the (poorly) release and solubilization of carotenoids when raw fruits and green leafy vegetables were consumed, and the (often) positive effect of processing in carotenoid absorption. The same conclusions are reached through the application of *in vitro* digestion protocols,^{110,111} so that a positive correlation has been observed between the *in vivo* and *in vitro* studies.

Novel techniques of food processing based in non-thermal methodologies should be considered as a plausible alternative to the conventional thermal treatments for optimising retention of organoleptic attributes of food with increased convenience and extended shelf-life in addition to the preservation of nutrient content.¹¹² These techniques include high-pressure processing, ultrasonic technology, and application of pulsed electric fields that came to lessen the extent of thermal degradation processes affecting nutrients and bioactive food compounds, and the generation of off-flavours. However, the *in vitro* studies dealing with the effect of these techniques in the micellarization of carotenoids show contradictory results, as recently reviewed.^{107,113} Apparently, non-thermal processing creates new textures and fibre networks,^{114,115} or the processing conditions are not adequate to significantly disrupt the natural structures where carotenoids deposited, that is, the meaning of processing techniques as an *effect* in carotenoid micellarization is below the significance of the food matrix (either

natural or processing-related) factor.¹¹⁶⁻¹¹⁹ Hence, considerable effort has been recently made to establish the correlation between micellarization of carotenoids and structural characteristics of the food products,¹¹⁷ including pectin properties, the strength of the fibre network, microstructure, and particle size. The reader should realise that the effects of these novel technologies in the micellarization of carotenoids have been established with data obtained from *in vitro* studies. To the best of my knowledge, no *in vivo* studies have been carried out so far to measure the impact of such innovative food preservation techniques on the absorption of carotenoids, and particularly the lack of human studies was recently highlighted.¹²⁰

Fibre encompasses effects related to both food processing and food structure. The fibre content of food modulates lipid digestion by interactions with lipophilic compounds and the pancreatic secretions, essential to achieve *emulsification* and *micellarization* of the dietary lipids. Moreover, the processing-related changes in the structure of fibre produce modifications of the rheological properties of the chyme, which may limit the release of the lipids. *In vivo* studies have dealt with this issue, showing that dietary fibres reduce the absorption of carotenoids.¹²¹⁻¹²⁴ The same trend is reproduced with *in vitro* digestion protocols, providing knowledge on the molecular mechanisms for the interaction between soluble fibre and dietary lipids, the chemical features related to the functionality and structure of fibre, its influence in size distribution of lipid droplets and viscosity of the digest, as well as its impact in assembling of micellar aggregates.⁶¹⁻⁶³ Considerable efforts are now being made to learn the side-effects of dietary components co-ingested with fibre, being the amount and type of lipids one of the significant contributors to modulate the effect of dietary fibres. Hence, the discussion is still open about the effect of fibre on the assimilation of carotenoids (Figure 14.2). The amount and type of fat is another critical factor to consider, as observed *in vivo* (see Chapter 13) and also in the *in vitro* experiments with carotenoids.^{89,102,125-130} *In vitro* digestion protocols coupled to cellular models to test the micellarization and absorption efficiency of carotenoids, often assumed as bioaccessibility, have provided pieces of evidence at the molecular level of the mechanisms that are implicated in the mechanical and biochemical events during digestion of lipids affecting the *transfer to lipid droplets*, *emulsification* and *micellarization*, and the subsequent routes of *cellular absorption* and *trafficking of lipids*.^{50,131-136} These studies addressed different physicochemical parameters that sustain the close interactions between the different classes of dietary lipids, sometimes establishing synergistic effects, sometimes competing during the sequential steps of digestion and hampering the final bioaccessibility value. Additionally, this knowledge opened the door to further developments in this field, particularly in the design of food delivery systems and

food structures that encompasses a complete set of variables and strategies to enhance carotenoid bioavailability/bioaccessibility.¹³⁷

As a rule, both the addition of fat and mechanical processing enhance the carotenoid digestibility, while exclusive thermal processing contributes to a lower degree, and novel technologies may induce different effects in the final result. Indeed, it is difficult to isolate the influence of one factor on the others as most of the studies include several of the mentioned factors in the experimental design so that drawing a definitive conclusion is not possible. However, the meaning of fat addition in the digestion of carotenoids is so prominent that it is a common practice to add some amount of oil^{50,135,138} or oil-in-water emulsion^{139,140} at the intestinal phase of the *in vitro* protocol to enhance carotenoid bioaccessibility. It is noteworthy to note that when the bioaccessibility of carotenoids from lipid-rich food sources (egg yolk, oils, dairy products) is measured, several of the factors mentioned above have less incidence in the result.¹⁴¹

Despite the efforts made to develop *in vitro* dynamic methods mirroring *in vivo* parameters of the gastrointestinal motility, dynamic changes in secretions and biochemical conditions of the gastric and luminal environments, and the kinetics of emptying and mass transport phenomena, they have rarely been applied to measure the micellarization of carotenoids. Some studies that measured the micellarization of lycopene^{142,143} and of egg-yolk carotenoids¹⁴⁴ used the dynamic TNO-gastrointestinal tract system, obtaining consistent results with *in vivo* data. Despite the valuable information that the dynamic *in vitro* methods could provide, the practical simplicity of the static *in vitro* protocols, their straightforward coupling with cellular uptake or transport models of the intestinal epithelium, and their reliability in the prediction of *in vivo* data, definitively hinder the application of dynamic procedures.

The isolation of mixed micelles after the *in vitro* digestion of carotenoid-rich foods either from static or dynamic protocols (*d-micelles*) for delivery of these pigments to cell culture was the logical development to mimic the physiological conditions better in comparison with experiments that used techniques for making model micelles (*m-micelles*).^{145,146} Although the *m-micelles* represent a strategy to accurately control the composition of the molecular aggregates, attaining a fair manipulation of the particle size as well, *d-micelles* are a more natural approximation, resembling the vast chemical composition that *real* micelles have. Therefore, linking the digestive processing of foods to isolate *d-micelles* followed by absorption/transport experiments with cell culture models comprises the analysis of the effect in bioaccessibility of the food matrix and processing features, roughly physiological issues, and allow the study of some genetic and host-related aspects because

cellular absorption and trafficking of lipids is genetically regulated as noted above.¹⁴⁷⁻¹⁴⁹ The reader should realise that within such experimental design the factors influencing bioaccessibility, as well as absorption and, partly, first-pass metabolism are accomplished. Thus, this research line that implements the sequence *in vitro* digestion of food, isolation of d-micelles and delivery to cell culture models has been very active, becoming a fundamental piece of evidence not only of the molecular events that take place both at the apical and basolateral cellular membrane of the enterocytes, but also to correlate the *in vitro* observations with data obtained from *in vivo* models.

14.2.3.2 Correlation of Carotenoid *in vitro* Digestion Data with the *in vivo* Absorption Studies

The *in vitro* digestion protocols have remained under continuous scrutiny since their introduction¹⁵⁰ as a predictive tool for the outcomes of the food digestion *in vivo*. The valuable efforts made to develop a protocol mirroring the human digestion physiology, although a nearly impossible task, were a requirement to obtain both reproducible and physiologically relevant data and draw conclusions applicable to the real *in vivo* scenario. In addition, the critical comparison of *in vitro* data with the *in vivo* results provides hints to reinforce the application of a protocol or to introduce modifications therein to obtain a better correlation. It is important to highlight that the search of correlation between *in vitro* and *in vivo* has not only been focussed in parallel numerical data but also seeking whether the *in vitro* protocol could be used as a surrogate for prediction of *effects* rather than figures of micellarization percentage (Table 14.1). Thus, some references made use of direct comparison of *in vivo* absorption of carotenoids¹⁵¹ with the carotenoid *in vitro* micellarization,¹⁵² obtaining a significant relationship between mean *in vitro* digestion values of β -carotene, α -carotene and lycopene, except for lutein, which was five-fold higher *in vitro* than *in vivo*. Nevertheless, authors concluded that the *in vitro* model used in the study is suitable for numerically predicting bioavailability of carotenoids in foods. The same report¹⁵² made use of a different approach consisting in the comparison of the relative absorption values published in human studies with the relative carotenoid digestion measured by the *in vitro* protocol, obtaining a significant relationship, despite the wide variability of the *in vivo* data. Other different approach applied for comparison between *in vitro* and *in vivo* trial (performed once), used the carotenoid micellar contents from the *in vitro* method and increment in carotenoid serum content or carotenoid area-under-the-curve of the postprandial response curve in the *in vivo* data. However, the aim of such comparison was not numerical, but to test whether the effect observed *in vivo* (higher or lower absorption depending on different food features) could be predicted with the *in vitro* protocol. In fact, the effects of cell-wall rupture and

heating on carotenoid release from carrot tissue observed *in vivo*¹⁵³ were predicted by the *in vitro* study.¹⁵⁴ Moreover, the *in vivo* multiple-dose effect after the intake of lutein-fortified fermented milk at low (4 mg of lutein esters) or high (8 mg of lutein esters) amount was consistently observed with the *in vitro* simulation, including the similar extent of hydrolysis of lutein esters for both doses.¹⁵⁵ Similarly, the effect of phytosterols (occurrence or deficiency) in the absorption of β -cryptoxanthin esters from milk-based fruit drinks (phytosterols did not significantly affect the serum response of β -cryptoxanthin) was consistent with the *in vitro* observations.¹⁵⁶ Conversely, the effect of milk or milk plus iron on the absorption of carotenoids from fruit juices was not entirely in agreement with the *in vitro* observations that showed a significant enhancement of the extent of xanthophyll ester hydrolysis and their subsequent micellarization when the juices were modified with milk, a fact not observed *in vivo*. The authors stated that methodological and biological constraints could play a significant role in the outcome of the *in vivo* study and that the lack of agreement does not mean a shortage of the predictive value of the *in vitro* models.¹⁵⁷

[Table 14.1 near here]

Other studies summarised in Table 14.1 came to support that although *in vitro* models might not be fully predictive, the *in vitro* protocols show a high analytical value to estimate the actual trend of the effects. Another issue that deserves attention is that the *in vitro* protocols tested for correlation with *in vivo* studies do not include the complementary experimental design for delivery of isolated d-micelles to cell culture models. The absence of such *filtering effect* could have been the source of the partially predictive value of the *in vitro* protocols, which often tend to overestimate both the numerical value of the carotenoid micellarization index and the intensity of the tested effect. Although the carotenoid micellarization index is frequently identified as the potential value of bioaccessibility because it is the main impacting element, the reader should not forget that as shown in Figure 14.1, carotenoid bioaccessibility is the result of micellarization, absorption, transport and first-pass metabolism.

14.2.3.3 Description of *in vitro* Digestion Protocols Applied to Measure the Micellarization of Carotenoids

A summary of the available *in vitro* digestion protocols applied to measure the micellarization of carotenoids was published¹⁰ and recently discussed.^{41,170} The method of Garrett et al.¹⁵⁰ was the first specific protocol developed for carotenoids, which is based on the protocol

described by Miller et al.²⁶ but introducing the necessary modifications to isolate the micellar fraction⁶⁴ and coupling with the Caco-2 cell culture model for estimation of the carotenoid absorption. Initially aimed to measure bioaccessibility of carotenoids from baby meals, this method has been applied to different test meals and supplements.^{171,172} The method has evolved introducing some changes, mainly the experimental conditions for the isolation of d-micelles¹⁷³ and the addition of lipase and carboxyl ester lipase to improve the activity of hydrolytic enzymes.¹⁷⁴ Later, Hedrén et al.¹¹⁰ validated some critical steps of the *in vitro* protocol (addition of antioxidants, inner atmosphere, amount of bile salts and pancreatic enzymes, shaking conditions) and used a soft centrifugation procedure to obtain the maximum amount of carotenoids released (not micellized) from the food matrix, while this new protocol was not coupled with the Caco-2 cell culture model. The method published by Liu et al.¹⁷⁵ revisited the procedure of Miller et al.²⁶ with particular emphasis to assess the uptake of carotenoids by the Caco-2 model, both from pure compounds and whole foods, establishing culture conditions, time kinetics and the delivery system for the cellular uptake (micelles were not isolated, but the diluted digesta was directly delivered to the Caco-2 cells).

Subsequently, Reboul et al.¹⁵² considered the *in vivo* conditions observed during human digestion of carotenoid-rich food sources¹⁵¹ and adjusted the original version of the method of Garrett et al.¹⁵⁰ to obtain a better approach to real physiological conditions, but discarding the coupling with the Caco-2 cell culture model. Successively, Granado-Lorencio et al.¹²³ started from the static gastrointestinal procedure published by Oomen et al.¹⁷⁶ and introduced modifications related with the addition of enzymes for hydrolysis of xanthophyll esters, as well as the conditions applied for isolation of the aqueous-micellar phase, without coupling with the Caco-2 cell culture model. Similarly, the protocol published by O'Connell et al.¹⁷⁷ adapted the original version of Garrett et al.¹⁵⁰, without adding any external lipid source and reported opposite trends than earlier *in vitro* studies, but like those observed *in vivo*.

Figure 14.3 summarises the conditions applied in these static *in vitro* protocols, so that the reader could observe most of the significant modifications introduced over time (pH values, incubation times, concentration of digestive enzymes, and the method for the isolation of mixed micelles, coupling with Caco-2 cell culture model) and could consider their possible impact in the results. Additionally, the INFOGEST consensus protocol published by Minekus et al.⁵³ is also represented in Figure 14.3, which has been adapted and applied to determine the micellarization of carotenoids in fruits and beverages.^{55,178-180}

[Figure 14.3 near here]

An important question is what method should be used and whether further modifications will be proposed. Although several methods are available to measure the bioaccessibility of carotenoids, the efforts made to develop a harmonised *in vitro* protocol should now be exploited, so that the consensus protocol⁵³ could run as the reference to test any proposal of modification, and to produce *in vitro* data for validation with *in vivo* studies. In fact, the consensus protocol⁵³ has been compared with a modified version^{178,181} of the *in vitro* protocol of Garrett et al.¹⁵⁰ (basically discarding the coupling with the Caco-2 cell culture model and increasing the concentration of digestive enzymes). Both procedures were applied for screening the micellarization of carotenoids in fruits, vegetables and animal products resulting in similar results of both stability and micellarization index of carotenoids, independently of the protocol used for estimation. Additionally, the consensus protocol was systematically applied for measuring the micellarization of β -cryptoxanthin and β -carotene from the main fruits contributing to the intake of these carotenoids in Spain.⁵⁵ The *in vitro* data were considered not high enough to explain the serum concentrations according to food dietary intervention studies. On the other hand, the authors claimed that the consensus protocol indicates that the contribution of β -cryptoxanthin to the vitamin A intake might be higher than expected *in vivo*.

14.2.4 The Direction over the Magnitude of *In Vitro* Digestion Protocols

The extensive application of *in vitro* digestion protocols to measure the micellarization of carotenoids in the context of nutrition research has produced a considerable amount of data. It is a constant, and frequently necessary, practice to compare the micellarization results obtained by different laboratories because the association of data (numbers) reinforces the reliability on the experimental approach and generates feedback to introduce changes in the protocol (see section 14.2.3.3). However, it is tricky to deal with numbers in this issue since the harmonised protocol has been recently launched, so that the data obtained so far made use of different protocols with diverse experimental conditions (see Figure 14.3).

However, in addition to the lack of a consensus protocol, some other facts may limit the utility of data comparison. The variability in the fruit ripening stage, the pattern of the initial food processing for sampling, the addition of effectors for absorption, the efficiency of the extraction process applied for quantification of the carotenoids in the food and in the micelles, and even how the results are attained comprise key information that should be clearly stated in any report.^{55,182} Nevertheless, some references contain compiled data of micellarization of carotenoids from different food sources.^{166,182,183} However, the experimental

conditions of the applied *in vitro* protocol, the source and handling of the food, and the aim of the study must be carefully thought before obtaining working conclusions from data evaluation.¹⁸⁴ Table 14.2 provides information regarding the *in vitro* digestion protocols applied to investigate effectors in micellarization of carotenoids organised by food structure and/or food matrix matters, food processing effects and amount and type of fat. Moreover, the lessons learned from the published studies dealing with these effectors, a brief description of the used food materials and the *in vitro* protocols applied, and some quality controls implemented during the experimental design are included. Hence, the reader has some critical inputs to adopt an appropriate strategy and to weigh the variables with significant influence on the results.

[Table 14.2 near here]

Finally, as fruits and vegetables are the main contributors to the carotenoid intake in human diet, most of the micellarization data obtained by *in vitro* digestion protocols apply to such food sources, although studies in other natural or formulated foods are also available, as in whole grain and degermed maize meal products,¹⁹³ baked foods,^{194,195} pasta,¹⁹⁶ dairy products,¹⁹⁷ micronutrient-fortified flour,¹⁹⁸ pistachios,¹⁹⁹ and food emulsions.^{23,200,201}

14.2.4.1 The Xanthophyll Esters: A Case Study

Esterification of xanthophylls is becoming a very active research subject in the carotenoid arena, and meaningful advancements are covered in this book. Indeed, the effects of esterification of xanthophylls on the *in vivo* digestion of carotenoid esters-rich food sources, including the participation of unravelled enzymes in the overall efficiency of digestion of xanthophyll esters, and the accessible metabolic routes to re-esterify xanthophylls in mammals²⁰²⁻²⁰⁵ are detailed in Chapter 13 and are issues definitively within the *state of the art*.

The *in vivo* studies²⁰⁶⁻²¹⁰ were not able to predict that xanthophyll esters are not substrates for lipases from human pancreas, a fact observed *in vitro*²¹¹ with an assay that models the intestinal digestion stage. Hence, no activity of human pancreatic lipase on xanthophyll esters was observed, while lipases from different origins showed hydrolytic activity towards carotenoid esters in a limited extent. In addition, the same study²¹¹ demonstrated that carboxyl ester lipase hydrolyses β -cryptoxanthin esters, while the activity on carotenoid esters from paprika and marigold oleoresins was less efficient. This enzyme, denoted as bile-salt dependent lipase, also known as cholesteryl ester hydrolase or cholesterol

esterase, is a nonspecific lipolytic enzyme that shows activity towards a wide range of acyl esters, such as triacylglycerols, diacylglycerols, monoacylglycerols, phospholipids, lysophospholipids, with a complementary hydrolytic activity to that performed by pancreatic lipase. The occurrence of complementary enzymes that contribute to the hydrolytic activity of pancreatic lipase is not singular. Digestion of retinyl esters involves several enzymes, including retinyl ester hydrolases associated with the brush border membrane.²¹² Thus, it is reasonable to suggest that in addition to carboxyl ester lipase other hydrolytic enzymes may also contribute to the hydrolysis of carotenoid esters, a point that has been recently shown,²¹³ although the identity of such enzymes is still unknown.

Thus, it is clear that besides the number of effectors in the micellarization of carotenoids, some others come into the scenario in the case of esters. Carotenoid esters should be released from the food matrix, transferred to lipid droplets and emulsified, but the esterification increases the hydrophobicity so that the physicochemical phenomena required for such process are demanded to a higher level. Moreover, the carotenoid esters distribute within the core of the lipid droplets, not at the surface what hinders the activity of hydrolytic enzymes (whatever they are), because the hydrolytic process of this class of enzymes takes place at the lipid-water interface. Again, the requirements of bile salts and intestinal enzymes are intensified to reach efficient hydrolysis of the hydrophobic bulk while the diffusion and aggregation events to build micelles with the products resulting from the hydrolytic activity could be compromised under certain circumstances as well.⁵⁰

What lessons have we learned from *in vitro* studies? Firstly, the hydrolysis of carotenoid esters is incomplete, under the experimental conditions of *in vitro* digestion protocols, and highly variable with values in the 10-50% range.^{155-157,179,180,214} Secondly, the incomplete hydrolysis of xanthophyll esters does not completely hamper their incorporation into micelles, that is, the hydrolytic products arising from the activity of digestive enzymes but also the partially de-esterified lipids and even those not processed by the enzymes may aggregate within the micelles. Thus, several reports show^{50,155-157} that micelles contain carotenoid esters as well as other lipids that elude the hydrolysis, although the higher the efficiency of hydrolysis the higher micellarization rate.^{174,202,213} This is an important fact because the available numbers of the efficiency of hydrolysis of carotenoid esters exclude the possibility of subsequent hydrolysis once the micellar aggregates diffuse in the cellular membrane of the enterocytes, where they could be substrates for the hydrolytic activity of enzymes at the apical side. Intestinal epithelial cells do have carboxyl ester lipase that promotes the absorption of free and esterified forms of dietary cholesterol,²¹⁵ so that the degree of hydrolysis of lipid esters, including the esterified xanthophylls, might be higher

than noted above. Consequently, it must be emphasised that inputs from experimental models aimed to measure the *cellular absorption* and *trafficking of lipids* are priceless to accurately establish how the digestibility of lipid esters, including the esterified carotenoids, is managed by the physiology of human digestion. Indeed, it has been shown *in vitro* that xanthophyll esters are absorbed¹⁷⁴, and endogenous cellular re-esterification also takes place to a lesser extent.²¹⁶

The effect of *esterification* introduced another uncertainty in the experimental design of *in vitro* digestion protocols: should the protocols include the addition of carboxyl ester lipase in the intestinal phase? It was suggested^{111,174} that to resemble physiological conditions (see Chapter 13) the enzyme should be incorporated, a point that has been generally accepted by most of *in vitro* protocols developed so far. However, the study published by O'Connell et al.¹⁷⁷ did not report any difference in the micellarization of carotenoid esters. Recent comparative studies^{178,181} do not include the carboxyl ester lipase in the *in vitro* digestion protocol following the consensus protocol⁵³, whereas other claims⁵⁵ for the inclusion of this enzyme as one of the necessary modifications to adapt the harmonised protocol of the INFOGEST network to the digestibility of carotenoids and carotenoid esters. The reader should consider that the use of pancreatin extract in the protocols means the addition of an enzymatic cocktail containing lipase and probably some other enzymes that could resemble the activity of the carboxyl ester lipase.^{202,213,217} Indeed, other factors excluding the enzymatic ones, may significantly contribute to the efficiency of hydrolysis of xanthophyll esters. Some of them are related with the fat amount that may enhance the hydrolysis of esters through the stimulating effect on pancreatic secretions, as well as the ability to boost kinetics of lipid digestion, conditions that could be resembled by dynamic *in vitro* protocols.²¹⁸

14.3 Conclusions

The application of *in vitro* digestion protocols to resolve scientific and technical questions related with the nutritional efficiency of food ingredients and bioactive compounds have increased our knowledge of the molecular events that participate in the gastric, intestinal and absorptive stages of food, in addition to a deep understanding of the physiological parameters involved in human digestion. This knowledge has allowed the correlation between several effectors in the micellarization of nutrients and non-nutrients with their fate in health-related issues, reinforcing the concept of health-through-food. However, the *in vitro* digestion protocols are not, by its very nature, an experimental approach that may encompass the whole complexity of human digestion and they show several constraints because the kinetics of digestion, homeostasis and host- and genetic-related factors are not accessible with this

technique. Nevertheless, parallel data obtained with *in vivo* and *in vitro* studies at once show that *in vitro* digestion protocols behave as a predictive tool to ascertain the direction of an *in vivo* effect instead of the magnitude of such outcome. Indeed, *in vitro* digestion protocols are easily implemented in any laboratory, require accessible equipment, and are cost-effective. The future of *in vitro* digestion protocols lies on the refinement of standardisation, the introduction of different digestive conditions of target populations, quality control of the digestive process to obtain reliable data and their coupling with additional experimental tools to increase the predictive value of *in vivo* situations.

Acknowledgements

The financial support of the Ministerio de Economía y Competitividad (MINECO-CICYT, Spanish Government, projects AGL2015-63890-R and AGL2017-87884-R) is gratefully acknowledged. Antonio Pérez-Gálvez is a tenured scientist at the Spanish National Agency for Research (CSIC).

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Table captions

Table 14.1 Correlation or consistency between *in vivo* studies and *in vitro* digestion protocols data in different carotenoid-rich meal, and the approach applied for data comparison.

Table 14.2 Summary of the main effectors in the micellarization of carotenoids, including a brief description of the carotenoid source, the *in vitro* digestion protocol applied, and the lessons learned in each case. Proposed (or already used in the studies) quality controls to reinforce the confidence in the observed effect are shown.

Figure captions

Figure 14.1 A practical characterisation of bioavailability consists on to split into two functions, bioaccessibility and bioactivity.

Figure 14.2 Principal effectors related to food processing and structure features and physiological issues in the micellarization index of food lipids.

Figure 14.3 Summary of the *in vitro* digestion protocols designed to measure the micellarization index of carotenoids and the consensus protocol developed by the INFOGEST network. The main reason to change the conditions applied in the *in vitro* protocol designed by Garrett et al.¹⁵⁰ is shown in each case.

Table 14.1

<i>In vitro</i> protocol	Approach applied for comparison	Carotenoid-rich meals	Carotenoids	Published human study for comparison
152	Numerical data of carotenoid micellarization percentage <i>in vivo</i> and <i>in vitro</i>	Carrot and tomato purees, and spinach	α -carotene, β -carotene, lycopene and lutein	152
152	Numerical data of relative bioaccessibility values reported in published human studies and relative micellarization values measured <i>in vitro</i>	Comparison of different food matrices: (1) lactolycopene vs. tomato puree; (2) tomato juice vs. raw tomatoes; (3) tomato juice vs. lycopene oleoresin; (4) tomato juice vs. lycopene oleoresin; (5) tomato puree vs. raw tomatoes; (6) skin-enriched vs. classic tomato puree; (7) pumpkin vs. spinach; (8) β -carotene supplement vs. carrot juice; (9) cooked vs. raw carrots; (10) lutein supplement vs. spinach	β -carotene, lycopene and lutein	158 (1); 159 (2); 160 (3); 99 (4); 99 (5); 161 (7); 162 (9); 164
154	Microscopy of carrot tissue microstructure and cell swelling from human ileostomy effluents, and from <i>in vitro</i> digestion	Raw grated carrot or carrot puree	-	153
111	Lutein content in the post-prandial period in serum samples and in micellar isolates from <i>in vitro</i> digestion	Lutein-fortified fermented milk at low or high lutein dose content	Lutein esters	155
111	β -cryptoxanthin content in serum samples and in micellar isolates from <i>in vitro</i> digestion	β -cryptoxanthin-enriched drink with or without added phytosterols	β -cryptoxanthin esters	156
111	β -cryptoxanthin content in serum samples and in micellar isolates from <i>in vitro</i> digestion	Fruit juices alone or with modifiers (milk or milk plus iron)	β -cryptoxanthin esters	157
29 ^b	<i>In vitro</i> β -carotene content in the filtrates+ileal efflux+ residues	Mixed diet containing vegetables high in β -carotene and oil diet containing vegetables low in β -carotene	β -carotene	165
167	β -cryptoxanthin content in serum samples and in micellar isolates from <i>in vitro</i> digestion	Fresh oranges or orange juice	β -cryptoxanthin	168
111	β -cryptoxanthin content in serum and faeces samples and in micellar isolates from <i>in vitro</i> digestion	β -cryptoxanthin-enriched drink with added phytosterols in the presence of different lipid emulsions (soy lecithin, whey protein with milk fat globule membrane plus milk fat, soy lecithin plus olive oil)	β -cryptoxanthin	169

^aThe numbers in parenthesis indicate the food matrices used in the published human study. ^bThe *in vitro* data were published in reference 166.

Table 14.2

Tested effect	Carotenoid source	<i>In vitro</i> method	Lessons learned
Food structure and/or food matrix			
- Natural physical barriers	Ripe red-fleshed papaya, mango, tomato fruits and carrot roots: washed, peeled and cut into small cubes, then homogenised with a kitchen blender. Some samples were mixed with 2.5% sunflower oil. Aliquots of the test foods were frozen with liquid nitrogen and stored at -80 °C	Garrett et al. ¹⁵⁰ (excluding Caco-2 cell uptake)	Chromoplast morphology and carotenoid content had significant effects on the micellarization of carotenoids. Lipid addition was a low factor in the micellarization of carotenoids
- Natural physical barriers	Carotenoid-enriched olive oil fraction from red carrots, red tomatoes, orange carrots and orange tomatoes prepared as described in ref. 115. Chromoplast fraction-enriched fraction. Cell cluster fractions	Hedrén et al. ¹¹⁰ with modifications ¹⁸⁵ ; micelles isolated by filtering the digesta (Macherey-Nagel 615 _{1/4})	The increased level of bioencapsulation resulted in a significant decrease in micellarization
- Natural physical barriers	Butternut squash, carrot, red grapefruit, mango, melon, papaya, sweet potato, tomato, watermelon, each one chopped in a blender for 1 min and mixed with full-fat yoghurt and then homogenised at 40 rpm for 1 min	Garrett et al. ¹⁵⁰ (excluding Caco-2 cell uptake)	Correlation between carotenoid micellarization and food matrix interactions were observed. Influence of meal was detected on β -carotene micellarization. Micellarization was affected by cell wall and chromoplast morphology
- Pectin	Carotenoid-rich soybean oil with two concentrations of pectin	Garrett et al. ¹⁵⁰ (excluding Caco-2 cell uptake); micelles isolated by centrifugation of digesta at 15000 g for 20 min and then filtered (0.22 μ m)	At low pectin concentration, binding of carotenoids to bile salts were highly active, favouring micellarization of carotenoids, while at high pectin concentration, the effect was observed at high pectin concentration. Viscosity and degree of methyl esterification were also effectors of micellarization of carotenoids
Mechanical/Thermal/Non-thermal processing			
- High-pressure homogenization (HPH) or HPH followed by thermal processing	Pulp of ripe red tomatoes: washed, dried, quartered, frozen with liquid nitrogen, then thawed, mixed and sieved	Hedrén et al. ¹¹⁰ with modifications ¹⁵¹ ; micelles isolated as described in ref. 150	HPH resulted in increased strength of the network and negative effect on the micellarization.
- Mechanical breakdown and human mastication	Carrots: peeled and cut into pieces, used as such, cooked in boiling water for 3 min or 25 min, then blended and sieved	Hedrén et al. ¹¹⁰ with modifications ¹⁸⁵ ; micelles isolated by filtering the digesta (Macherey-Nagel 615 _{1/4})	<i>In vitro</i> micellarization was highly dependent on particle size for raw and gently cooked carrots. Conditions for mimicking the human mastication were provided
- Thawing conditions	Ultrafrozen orange juices were thawed at room temperature, refrigeration temperature, and microwave defrosting (800 W for 20 s)	Garrett et al. ¹⁵⁰ in combination with Liu et al. ¹⁷⁵ (excluding Caco-2 cell uptake); micelles isolated as described in ref. 110	Microwave thawing had lower micellarization of provitamin A carotenoids than it produced a significant decrease in micellarization levels of the juice
- Effect of HPH in microstructure	Pulp of ripe red tomatoes: washed, dried, quartered, frozen with liquid nitrogen, then thawed, mixed and sieved	Hedrén et al. ¹¹⁰ with modifications ¹⁸⁵ ; micelles isolated as described in ref. 150	Although HPH decreased tomato product consistency increased, so that the micellarization of carotenoids was negatively affected. Particle size was an effector of micellarization of carotenoids
- Mechanical processing and particle size	Carrots- and tomatoes-derived particles and purees. Plant materials were peeled, cut into pieces and vacuum-packed, then heated at 95 °C for 5 min. Blending or HPH was applied, and pulp fractions with different particle sizes were separated by wet sieving	Hedrén et al. ¹¹⁰ with modifications ¹⁸⁵ ; micelles isolated as described in ref. 150	The correlation between particle size and micellarization of carotenoids could not predict the micellarization of carotenoids from carrot- and tomato-derived purees
- Boiling	Yellow-fleshed cassava roots, peeled, cut into cubes and then boiled (120 °C for 45 min) or boiled and then fried in soybean oil at 180 °C for 8 min	Garrett et al. ¹⁵⁰ (excluding Caco-2 cell uptake); micelles isolated by centrifugation of digesta at 5000 g for 45 min	The highest micellarization of β -carotene was reached from fried cassava roots, compared to boiled samples

-	Boiling	Cooked or raw potatoes were sliced, then frozen in liquid nitrogen and finally lyophilised, milled and stored at -70 °C	Garrett et al. ¹⁵⁰ (excluding Caco-2 cell uptake); micelles isolated by centrifugation of digesta at 3000 g for 10 min	Boiled potatoes provided significant lutein and zeaxanthin
Amount and type of fat				
-	Fatty acid composition of dietary oil	Pulp of ripe red tomatoes: washed, dried, quartered, frozen with liquid nitrogen, then thawed, mixed and sieved with an amount (0-10%) of oil	Hedrén et al. ¹¹⁰ with modifications ¹⁵¹ ; micelles isolated as described in ref. 150	All tested oils increased the micellarization of lycopene, but some of them had a high effect on the final result. Quantity of oil is also an important factor for digestibility
-	Fatty acid composition of dietary oil and processing	Pulp of ripe red tomatoes: washed, dried, quartered, frozen with liquid nitrogen, then thawed, mixed and sieved with an amount (5%) of oil, used directly or subjected to HPH, microwave heating (20 min at 70 °C, 90 °C or 120 °C) or HPH+microwave heating	Hedrén et al. ¹¹⁰ with modifications ¹⁵¹ ; micelles isolated as described in ref. 150	Addition of oil before processing improved micellarization of lycopene. Type of oil and processing method were important effectors on micellarization, while in the case of lycopene, processing improved lycopene release from the matrix
-	Transfer to lipid phase	Tomato juice mixed with peanut oil or pre-prepared emulsion in different weight to weight ratios at different pH and temperature	Isolation of the oil phase after centrifugation at 10000 g for 2 min	Limited diffusion of lycopene in the case of high oil to tomato ratios. No effect on lycopene release was observed with increased diffusion was observed at higher temperatures. Emulsions limited the micellarization of lycopene
-	Amount and fatty acid composition of dietary oil	Western-type salad meal with 20% spinach, 35% tomato, 25% carrot, 10% romaine lettuce and 10% orange pepper. The salad was homogenised until pureed and then stored under nitrogen gas at -80 °C	Garrett et al. ¹⁵⁰	Micellarization of carotenes enhanced by the presence of oil and dependent on the fatty acyl chain length, not on the degree of unsaturation. Carotenoid release of β-carotene was independent of the composition of micelles
-	Amount and fatty acid composition of dietary oil	Leaves of washed spinach, qing jin cai and komatsuma were blanched for 2 min and then quickly cooled. Carrot and pumpkin were sliced and blanched. Homogenized samples were stored at -80 °C until needed. Lipids were added during digestion	Garrett et al. ¹⁵⁰ (excluding Caco-2 cell uptake) and Hedrén et al. ¹¹⁰ ; micelles isolated by centrifugation of digesta at 1500 g for 20 min and then filtered (0.2 µm)	Micellarization of β-carotene enhanced by the presence of fats and oils and long-chain triacylglycerols. Hydrolysates released from the triacylglycerols during digestion were more significant than β-carotene than bulk lipids

^aAnalysis carried out by the authors. ^bAnalysis proposed in this Table.

Figure 14.1

Bioavailability	<u>Bioaccessibility</u> <ul style="list-style-type: none">• Events that take place during digestion of food to yield potentially bioaccessible material• Absorption and transport through the intestinal epithelium• First-pass metabolism	<u>Experimental approaches</u> <ul style="list-style-type: none">• Micellarization → <i>in vitro</i> digestion protocols• Absorption and transport through cellular models• First-pass metabolism models
	<u>Bioactivity</u> <ul style="list-style-type: none">• Systemic circulation and deposition in the target tissue• Interaction with biomolecules, metabolism and bioefficacy• Generation of the biomarker and physiological response	<u>Experimental approaches</u> <ul style="list-style-type: none">• <i>In vivo</i> models for pharmacokinetic response and accumulation• System biology and high-throughput screening• <i>In vivo</i> models and omics techniques

Figure 14.2

Figure 14.3

<p>Garrett et al.¹⁵⁰</p> <p>Sampling: Homogenization (30 s) and microwave heating (25 s, twice)</p> <p>Oral phase: -</p> <p>Gastric phase: pH 2, porcine pepsin (40 mg/mL), shaking at 95 rpm for 1 h at 37 °C</p> <p>Intestinal phase: pH 5.3, bile extract and pancreatin (12 mg/mL and 2 mg/mL, respectively); then pH is raised at 7.5, shaking at 95 rpm for 2 h at 37 °C</p> <p>Isolation of micelles: 167000 g at 4 °C for 95 min and filtering through 0.22 µm</p> <p>Caco-2 cell culture model</p>	<p>Hedrén et al.¹¹⁰</p> <p>Sampling: Mechanical ground or cut into pieces and mixing with different amounts of oil stabilized with tocopherol</p> <p>Oral phase: -</p> <p>Gastric phase: pH 2, porcine pepsin (0.5%), shaking at 250 rpm for 1 h at 37 °C</p> <p>Intestinal phase: pH 5, bile extract and pancreatin (25 g/L and 4 g/L, respectively, stabilized with tocopherol); then pH is raised at 7.5, shaking at 250 rpm for 30 min at 37 °C</p> <p>Isolation of micelles: 5000 g for 20 min</p> <p>The protocol was designed to estimate the maximum amount of carotenoids released, not micellarized</p>	<p>Liu et al.¹⁷⁵</p> <p>Sampling: Mechanical chopping and thawing, then cooking at 100 °C for 15 min</p> <p>Oral phase: -</p> <p>Gastric phase: pH 2, porcine pepsin (0.1%), shaking for 1 h at 37 °C</p> <p>Intestinal phase: pH 6.9, bile extract and pancreatin (0.14% and 0.02%, respectively, stabilized with BHT), shaking for 2 h at 37 °C</p> <p>Isolation of micelles: 12000 g for 5 min and dilution</p> <p>Caco-2 cell culture model</p> <p>Direct delivery of diluted micellar fraction to the Caco-2 cell culture</p>	<p>Reboul et al.¹⁵³</p> <p>Sampling: Homogenization at 37 °C for 10 min, stabilized with pyrogallol</p> <p>Oral phase: -</p> <p>Gastric phase: pH 4, porcine pepsin (0.23%), shaking for 1 h at 37 °C</p> <p>Intestinal phase: pH 6, bile extract and pancreatin (1.1% and 0.04%, respectively), shaking for 30 min at 37 °C</p> <p>Isolation of micelles: 20000 rpm at 10 °C for 18 h and filtering through 0.22 µm</p> <p>The amounts of enzymes and bile extract were increased as the protocol is not coupled with Caco-2 cell culture model</p>	<p>C</p> <p>B</p> <p>C</p> <p>P</p> <p>r</p> <p>In</p> <p>ex</p> <p>en</p> <p>I</p> <p>U</p> <p>co</p> <p>es</p>
<p>Minekus et al.⁵³</p> <p>Sampling: Mince meal with electric mincer. Oral phase: Simulated salivary fluid is added to the minced meal in a 50:50 (w/v) ratio, including human salivary α-amylase (100 U/mL) and 10 mM NaHCO₃ (10 min at pH 7. Gastric phase: Simulated gastric fluid (SGF) is added to the oral bolus to obtain a final ratio of food to SGF of 50:50 (v/v). Porcine pepsin (2000 U/mL, pH reduced to 3. The mixture is mixed by shaking at 37 °C for 2 h. Intestinal phase: The gastric chyme is mixed with simulated intestinal fluid (SIF) to obtain a final ratio of chyme to SIF of 50:50 (v/v). pH is raised to 7 and enzyme extract containing pancreatin (100 U/mL, based on trypsin activity) and bile (10 mM) is added. In addition, trypsin, 100 U/mL; bovine chymotrypsin, 25 U/mL; porcine pancreatic α-amylase, 200 U/mL; porcine pancreatic lipase (2000 U/mL) and porcine pancreatic lipase molar excess) are added when the meal contains high amounts of fat or the digestion of food lipids is the aim of the study. The intestinal phase is followed by continuous shaking of the sample. The protocol emphasises the addition of digestive enzymes to achieve specific activity values in the final mixture, instead of enzyme concentrations.</p>				