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In Vitro and *in Vivo* Bioavailability of Carotenoids and Tocopherols from Fruits and Vegetables: A Complementary Approach

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ABSTRACT

Bioavailability is a critical feature in the assessment of the role of micronutrients in human health, and the approaches to this issue include *in vitro* and *in vivo* methods. Food- and host-related factors affect the bioavailability of carotenoids and tocopherols, and major challenges in the study of bioavailability include the release of these compounds from the food matrix, micellization, the measurement of the plasma response and the inter-individual variability. To evaluate bioaccessibility, *in vitro* gastrointestinal models have been used to assess stability, hydrolysis of carotenol esters and transfer efficiency of carotenoids (i.e. β-cryptoxanthin, lutein, β-carotene, lycopene) and tocopherols (i.e. α- and γ-tocopherol) from fruits and vegetables. *In vivo* (human) bioavailability has been studied mostly by assessing the responses in chylomicron fractions and serum produced by different dietary intervention protocols. Available *in vitro* data show that the stability of carotenoids and tocopherols is high, although micellization is a critical determinant of the bioaccessibility. In human studies, upon dietary intervention, changes in serum concentrations may be observed for some compounds (i.e. β-cryptoxanthin, lutein, γ-tocopherol), but not for others (α-tocopherol, β-carotene). Overall, the behaviour of these phytochemicals under *in vitro* gastrointestinal conditions does not fully explain the changes observed in *in vivo* studies. The results indicate that *in vitro* methods are useful for assessing food-related factors affecting bioavailability, although host-related factors, physiological processes and methodological constraints may limit the comparability and the "predictive value" of *in vitro* models. In this respect, the two approaches should be considered complementary, but not necessarily interchangeable.

Keywords: bioaccessibility, carotenoids, human study, vitamin E, xanthophyll esters

CONTENTS

INTRODUCTION

Carotenoids and tocopherols

Carotenoids correspond to a group of lipid pigments present in plants and microorganisms that can not be synthesised by animals. Carotenoids are C-40 compounds that contain numerous conjugated double bonds and can be broadly classified into carotenes (hydrocarbon, apolar) and xanthophylls (oxygen-containing carotenoids, polar). More than 600 carotenoids containing more than 60 functional groups have been described in nature (Straub 1987; Goodwin and Britton 1988). Most carotenoids in plants exist in the *all-trans* configuration, although *cis* isomers may be

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Invited Review

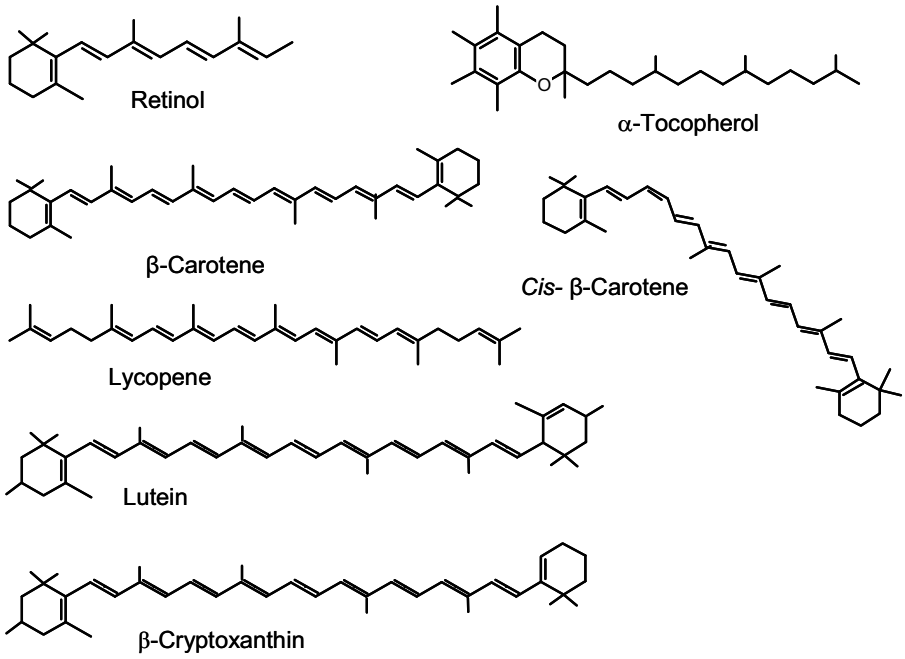


Fig. 1 Chemical structures of common carotenoids, all-*trans*-retinol and α-tocopherol.

formed during food processing (Rodriguez-Amaya 1997) (**Fig. 1**).

In mammals, less than 10% of the carotenoids can be converted into vitamin A (retinal) (Bauerfeind *et al* 1981; Bendich and Olson 1989), this being the only proven function of carotenoids in humans (provitamin A activity), and constituting the basis for their being considered micronutrients (Parker 1997). *Cis* isomers of both dietary carotenoids and retinoids are also found in tissues and may exhibit different biological activities (Ross 1999).

The term *vitamin E* should be used as a generic description of the "tocol" and "tocotrienol" derivatives that show qualitative biological activity of α-tocopherol (**Fig. 1**) (Machlin 1984). The term tocopherol is not synonymous with vitamin E, but refers to all mono, di and tri-methyl tocols, regardless of their biological activity. The "tocol" molecule, a hydroxy-chroman nucleus linked to a saturated phytyl chain (tocol) or one with three double bonds (tocotrienol), represents the basic structure of these compounds, which can be differentiated by the number and position of the methyl groups linked to the nucleus. The group includes α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol.

In developed countries, 70% to 90% of carotenoid intake comes from fruit and vegetable consumption. Although more than 40 carotenoids are present in the plant foods frequently consumed by humans, only a few, including β-carotene, α-carotene, β-cryptoxanthin, lutein, zeaxanthin and lycopene, are present in blood and tissues in significant amounts (Goodwin and Britton 1988).

Carotenoids are processed during digestion in the same manner as retinyl esters and other fat-soluble compounds, such as tocopherols, including their release from the food matrix, intestinal cell uptake and incorporation into chylomicrons for transport to the liver, although their distribution and recycling between tissues and excretion may differ substantially. Nevertheless, humans are considered indiscriminate carotenoid absorbers (Goodwin 1984), meaning that we are capable of absorbing a wide range of carotenes and xanthophylls and, moreover, to partly metabolize them (i.e. conversion into retinol).

While dietary tocopherols are mostly present in the free form (except in fortified foods), dietary carotenoids may be present as protein-bound, ester and free forms. Thus, additional factors, such as the activity of enzymes, the integrity of secretions and other intraluminal conditions are important for the preabsorptive processes (i.e. matrix release and micellization) of carotenoids.

Nutritional relevance

Fruits and vegetables are major sources of biologically active compounds, many of which may have beneficial effects against chronic diseases (WCRF and AICR 1997; WHO 2003). Carotenoids and tocopherols constitute important groups in human diets that display several biological activities, including vitamin activity (Bendich and Olson 1989; Jiang *et al*. 2001; Stahl and Sies 2005), antioxidant capacity (Bendich and Olson 1989; Biesalski 2001; Jiang *et al*. 2001; Stahl and Sies 2005), blue light filtering (Bendich and Olson 1989; Beatty *et al*. 1999; Stahl and Sies 2005), modulation of immune function (Bendich and Olson 1989; Stahl and Sies 2005), modification of inflammatory processes (Biesalski 2001; Stahl and Sies 2005) and regulation of cell differentiation and proliferation (Bertram and Bortkiewicz 1995; Jiang *et al*. 2001; Kris-Etherton *et al*. 2002; Asai *et al*. 2004; Stahl and Sies 2005).

It is widely assumed that serum concentrations of carotenoids reflect, at least to some extent, the consumption of carotenoid-containing foods and, thus, that the serum carotenoid status can be used as an index related to a healthy diet (Brevik *et al*. 2004). Inadequate consumption of fruit and vegetables has been identified as a probable risk factor in relation to hypertension, cardiovascular disease, cancer and age-related eye diseases (WCRF and AICR 1997; WHO 2003; Comisión Europea 2003), and epidemiological studies have consistently shown that a high carotenoid intake and serum concentrations are associated with a lower risk for developing chronic diseases (Bunce 1994; Kohlmeier and Hastings 1995; Steinmetz and Potter 1996).

However, growing evidence, including the failure of supplementation trials with single nutrients to prevent certain chronic diseases (Hennekens 1998; Institute of Medicine 2000) and the apparent advantage of providing mixtures of phytochemicals at levels achievable through dietary intake, supports a holistic view of the diet-health relationship. This would involve considering it in terms of including the variety of foods, processing methods and food habits (Scali *et al*. 2001). Thus, due to insufficient evidence and the lack of nutrient specificity, a food-based rather than a compound-based approach is recommended (WHO 2003). In this respect, to achieve the benefits associated with the consumption of fruits and vegetables, different approaches are considered to increase the content and/or the bioavailability of these components including agricultural practices, biotechnology and food technology (Olmedilla *et al*. 2001a).

Within this framework, the food industry is playing an

increasing role in the development and marketing of new products with added nutritional value, including new approaches to preservation and packaging (i.e. modified atmospheres, electric pulses), to safeguard and stabilize food products. This is having a relevant impact on food quality and the stability of micronutrients in foods (Lindley 1998), as well as on the food supply and the dietary patterns of the population. However, although increasingly used and accepted by consumers, little is known about the effect of these emerging technologies on the retention and bioavailability in humans of the nutrients and phytochemicals contained in these foods and, thus, their potential impact on public health and the nutritional status of the population is still uncertain.

BIOAVAILABILITY

Concepts and determinants

Bioavailability can be defined as the fraction of a dietary nutrient capable of being absorbed and available for use or storage (Bender 1989). The overall process includes food digestion, absorption from the intestinal lumen and postprandial nutrient (vitamin) metabolism. Within this concept, *bioaccessibility* (synonymous with "digestibility", or the amount of a nutrient released from the food and available for absorption) and *bioconversion* (amount transformed in the body into active forms) are two concepts that can be included in a more refined definition, and both are important for some vitamins and precursors (i.e. provitamin A carotenoids).

Interest in vitamin bioavailability has greatly increased for a number of reasons which apply differently to developed and developing countries. They include: 1) the great interest in the proportion of vitamins from foods that are available for utilization in the body, especially regarding the vast proportion of undernourished people worldwide and groups at risk of developing micronutrient deficiencies; 2) the epidemiological evidence that suggests protective effects associated with (high) intakes above the recommended dietary intakes (RDI) and/or high serum levels of several vitamins and related compounds against a number of highly prevalent diseases in developed countries (cancer, cardiovascular diseases, cataracts, neural tube defects); 3) the increasing percentage of elderly people with lower energy intake but the same micronutrient requirements and the concept of "healthy ageing"; and 4) the increasingly enormous role of the food industry in developing new products with "added nutritional values" and their potential impact on public health and nutritional status of the population.

However, while the study of nutrient (vitamin) bioavailability is a growing field, it is highly complex since, in addition to the available methodology, many other issues are involved. Several food and host-related factors are capable of influencing carotenoid bioavailability at different stages (West and Castenmiller 1998). Although these factors, grouped under the mnemonic SLAMENGHI (Species of vitamin; molecular Linking; Amount consumed; food Matrix; Effect of modifiers; Nutrient status; Genetic factors; Host-related factors and Interactions) were primarily related to carotenoids, many of them are applicable to other micronutrients and phytochemicals (i.e. tocopherols) contained in foods (Borel 2003; Granado-Lorencio and Olmedilla-Alonso 2003).

Methodology for assessing vitamin bioavailability in humans

Approaches to the study of the bioavailability of food components can be broadly classified as *in vitro* and *in vivo* methods, each having its pros and cons. Studies of the bioavailability of carotenoids and tocopherols, however, are difficult due to the endogenous presence of these substances in plasma and tissues, and most methods only yield information regarding the *relative bioavailability* (relative to a reference dose or control), not the *absolute bioavailability* of the vitamins.

Studies in human subjects can be divided into those using large, pharmacological doses, which are only partly available due to limitations in the absorption process, and those using more physiological intakes, either as pure substances or contained in different matrices, including foods. Another approach, regardless of the dose, includes short-term, single-dose, pharmacokinetic studies (i.e. area under the curve, peak concentration, steady-state concentration) and long-term, multiple-dose supplementation trials. In this case, the information obtained is somewhat different since the protocol used and samples collected are interpreted in terms of "acute" postprandial metabolism or "chronic" nutritional status, repletion and/or saturation processes (van den Berg 1993; Granado-Lorencio and Olmedilla-Alonso 2003).

In most cases, doses larger than those provided by mixed diets are needed in order to detect variations in plasma. This problem is overcome through the use of stable isotope-labelled carotenoids and tocopherols, both intrinsically (in growing foods) and extrinsically (single compounds), which enables the study of carotenoid and tocopherol bioavailability (i.e. absorption, transport, distribution, storage, excretion) at dietary levels, regardless of the endogenous presence (Traber *et al*. 1993; Parker *et al*. 1999; Lineau *et al*. 2003; Tang *et al*. 2003). These techniques, however, also have limitations (i.e. the perceived health risk and the costs associated with the necessary methodology). Because of these limitations, many studies have involved the use of *in vitro* and animal models. Although animal models may provide relevant information with regard to bioavailability in man, no one animal model completely mimics human absorption and metabolism of carotenoids (Lee *et al*. 1999) and, thus, extrapolation of these results and their relevance to humans should be considered with caution.

In vitro models based on human physiology have been developed as simple, inexpensive, noninvasive and reproducible tools to study digestive stability, micellization, intestinal transport and metabolism and to predict the bioavailability of different food components (i.e. ascorbic acid, carotenoids, chlorophylls, polyphenols) (**Fig. 2**) (Garret *et al*. 1999; Sugawara *et al*. 2001; During *et al*. 2002; Hedren *et al*. 2002; Chitchumroonchokchai *et al*. 2004; Vallejo *et al*. 2004; Yun *et al*. 2004; Failla and Chitchumroonchokchai 2005; Serrano *et al*. 2005; Goñi *et al*. 2006; Reboul *et al*. 2006). However, since there are a number of factors capable of influencing vitamin bioavailability at different stages, *in vitro* methodology for bioavailability assessment and its potential predictive value regarding human absorption of phytochemicals should be validated in different *in vivo* situations (Oomen *et al*. 2002).

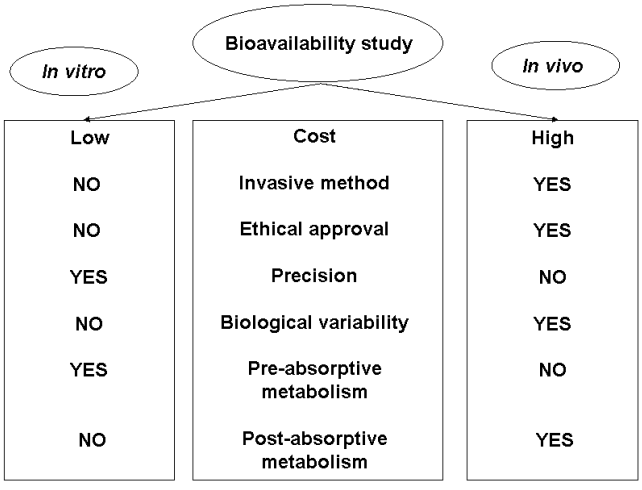


Fig. 2 Some factors to consider in the study of bioavailability of phytochemicals.

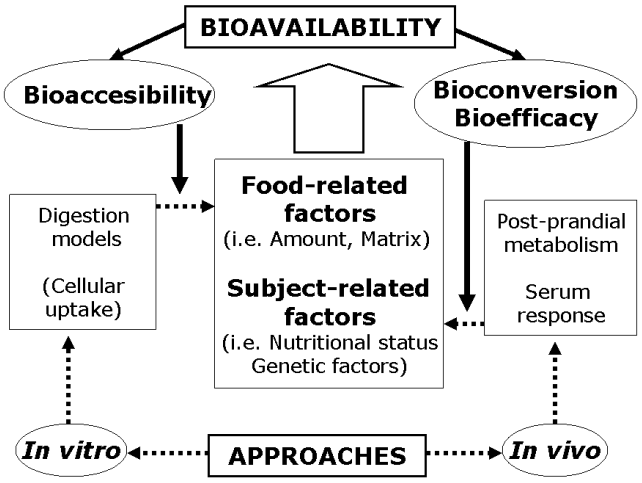


Fig. 3 General approach to the study of carotenoid bioavailability. Although several approaches can be used to study different food- and host-related factors of the nutrient bioavailability, *in vitro* studies constitute a cost-efficient alternative to provide information on the bioaccesibility while *in vivo* approaches may be better suited for assessing bioconversion and bioefficacy.

Finally, although compartmental modelling in nutrition is in its infancy, the use of mathematical models to estimate bioavailability of vitamins as an alternative to other methods (i.e. area under the curve) is of great interest due to their applicability in the understanding of biological control mechanisms, including timescale analysis (fast vs. slow processes) and non-linear kinetic models (Wilson and Dainty 1999).

In vitro and *in vivo* assessment: a complementary approach

The release of carotenoids from the food matrix, the solubility, the measurement and interpretation of the plasma response and the differences in inter-individual response have recently been referred to as the challenges to the understanding and measurement of carotenoid bioavailability (Faulks and Southon 2005). Thus, since both food and host-related factors influence carotenoid and tocopherol bioavailability, a combined approach may be useful in its study (**Fig. 3**). Nevertheless, while *in vitro* methods may be appropriate for studying preabsorptive processes, it will be necessary to determine whether they are valid and applicable in humans regarding carotenoid/tocopherol stability during digestion, solubility and micellization as an index of absorbability and/or bioavailability (Failla and Chitchumroonchokchai 2005).

The rationale implies the assessment of the value of an *in vitro* gastrointestinal model applicable to different food matrices and conditions (including technological processes) in predicting the *in vivo* bioavailability (response). Within this context, two aspects should be differentiated: 1) a qualitative approach, i.e. information on species (i.e. epoxy-carotenoids, ceto-carotenoids), molecular links (free forms *vs*. esters), structural and geometrical isomers (lutein vs. zeaxanthin; *cis*- *vs*. *trans*-β-carotene), etc.; and 2) a (semi) quantitative comparison, i.e. the extent to which *in vitro* data on stability, isomerization, hydrolysis and transfer into an aqueous phase can provide reliable information regarding the expected *in vivo* response.

However, comparisons between *in vitro* and *in vivo* methods are often difficult since both approaches lack standardization parameters or traceability to reference methods. Moreover, comparisons between studies may be misinterpreted because of differences in the design, execution and end-points of the assays. In principle, the validity of an *in vitro* method relies on its consistency with other *in vitro* data and its comparability with *in vivo* results under different physiological and food matrix conditions (Oomen *et al*. 2002; Yun *et al*. 2004). This rationale assumes that there is a relationship between the two approaches; *in vivo* results are considered the reference method ("gold standard") for *in vitro* validation and, thus, consistency between the two approaches is necessary. Obviously, *in vivo* (human) data constitute the reference standard ("true response") as the basis for comparisons and *in vitro* protocols are used as surrogates for prediction, although very few studies have employed this approach (Yun *et al*. 2004; Reboul *et al*. 2006; Granado *et al*. 2006). However, we must be aware of the fact that the *in vivo* (human) model also involves methodological and biological uncertainties of its own and, thus, it should not be considered as the "gold standard". Hence, a lack of agreement between *in vitro* and *in vivo* data does not necessarily mean inadequacy of the *in vitro* protocol, since this inconsistency may be related to both physiological and methodological factors associated with the *in vivo* approach.

FOOD-RELATED FACTORS AFFECTING BIOAVAILABILITY

Stability and micellization

The bioaccessibility of a nutrient depends on the physical properties of the food matrix, which affect the efficiency of the physical, enzymatic and chemical digestion process. Following release from the food matrix, the major factor determining absorption is the solubility of carotenoids in digesta (Faulks and Southon 2005; Failla and Chitchumroonchokchai 2005). Overall, *in vitro* studies are highly consistent with regard to the stability of carotenoids and tocopherols under simulated gastrointestinal conditions (>70%) and, thus, in the amounts available for micellization (Garret *et al*. 1999; Chitchumroonchokchai *et al*. 2004; Serrano *et al*. 2005; Granado *et al*. 2006), a circumstance that is also consistent with *in vivo* human data (Tyssandier *et al*. 2003; Reboul *et al*. 2006).

The transfer from the food matrix into micelles is one of the steps that determine the extent of carotenoid absorption from different foods (Garret *et al*. 1999; Tyssandier *et al*. 2001, 2003). Overall, micellization of lutein and zeaxanthin has been reported to be more efficient than that of β-carotene or lycopene (Garret *et al*. 1999; van het Hoff *et al*. 1999a; Garret *et al*. 2000; Tyssandier *et al*. 2003; Chitchumroonchokchai *et al*. 2004). However, transfer efficiency is also affected physiological factors (Tyssandier *et al*. 2001) as well as by the food matrix and varies according to the type of vegetable (Rich *et al*. 2003; Failla and Chitchumroonchokchai 2005; Serrano *et al*. 2005; Chitchumroonchokchai and Failla 2006). In this respect, the results from our laboratory are in agreement with this finding, since foods containing xanthophyll esters (i.e. fruits) apparently transfer free xanthophylls more efficiently than those containing only free forms (i.e. green and non-green vegetables) (**Fig. 4**), a fact consistent with some observations in humans (i.e. zeaxanthin)(Breithaupt *et al*. 2004). Additionally, these results are somehow consistent with *in vivo* data reporting that, regardless of the hydrophobicity, the formulation in which the carotenoids are supplied (i.e. food, beadlet, gelcap, oleoresin) affect the bioavailability of several carotenoids, i.e. β-carotene, lycopene, astaxanthin (Pateau *et al*. 1999; Fuller *et al*. 2001; Odeberg *et al*. 2003).

However, the extent of micellization may be also related to methodological issues (Rich *et al*. 2003; Granado-Lorencio *et al*. 2007) and the aqueous-micellar phase generated by overnight sedimentation has been reported to render a higher recovery of xanthophylls than low speed centrifugation protocols (**Fig. 5**). This fact is consistent with physiological events such as the estimated transit time (under fed conditions, up to 12-14 hours), the time-dependence of carotenoid uptake by Caco-2 cells (up to 16 hours), the peak maxima obtained in blood after a single ingestion of carotenes and xanthophylls (<12 hours) (Wingerath *et al*. 1995; Garret *et al*. 2000; During *et al*. 2002; Rich *et al*. 2003; Chitchumroonchokchai *et al*. 2004; Failla and Chitchumroonchokchai *et al*. 2005) and observations in humans, in

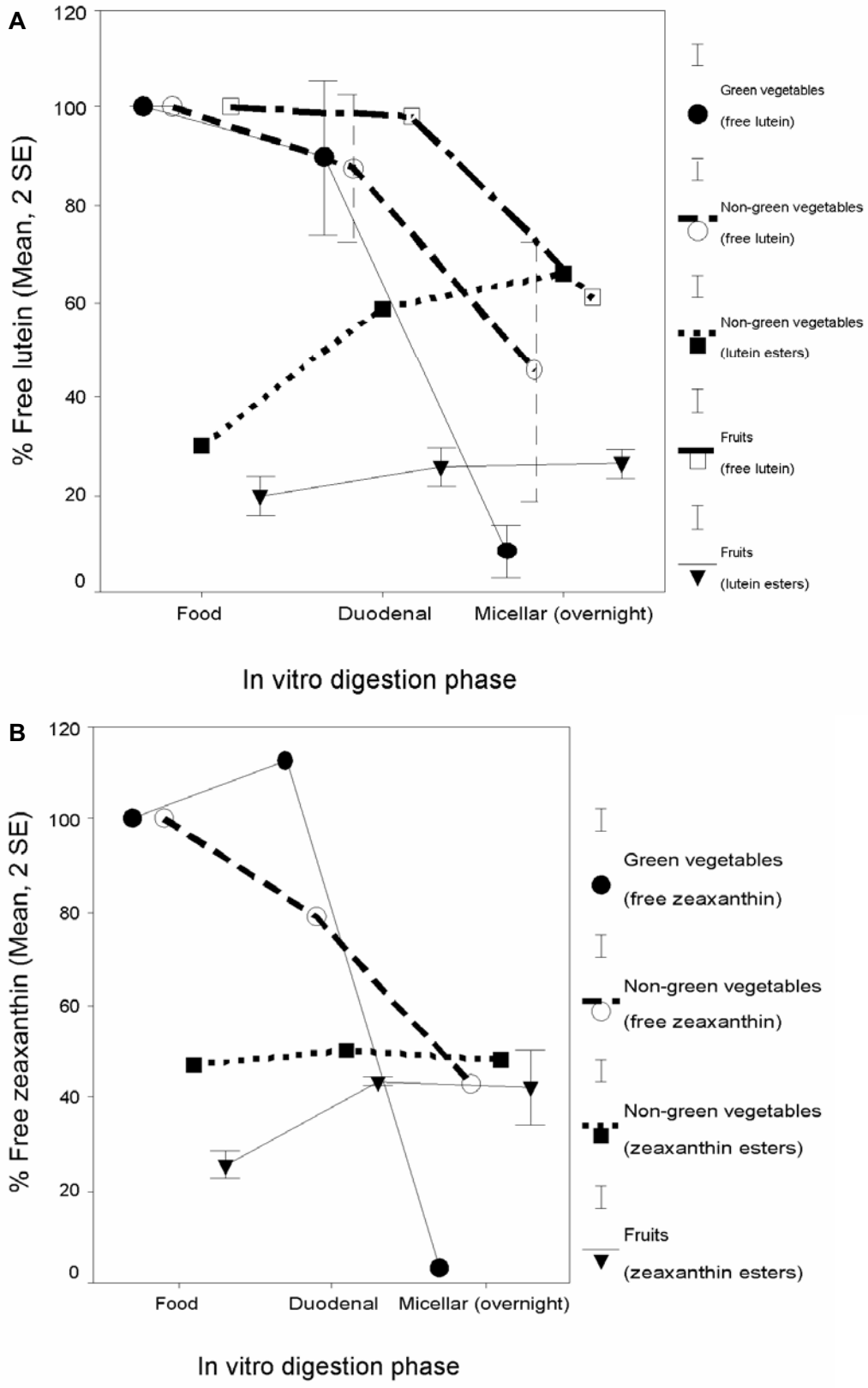


Fig. 4 Percentual transference of free xanthophylls into micellar phase according to the type of vegetable (food matrix) and the major chemical forms present in the food[1]. Overall, non-green vegetables and fruits containing xanthophylls esters showed a greater percentual transference of free forms of lutein (**A**) and zeaxanthin (**B**) compared to that observed in green vegetables containing mostly free xanthophylls. [1]Foods included in the groups were: Spinach, broccoli and lettuce (green vegetables with free xanthophylls); sweet corn, tomato paste and carrot (non-green vegetables with free xanthophylls); red bell pepper (non-green vegetables with xanthophylls esters); kiwi (fruits with free xanthophylls); orange, loquat and pineapple (fruits with xanthophylls esters).

whom a longer food transit time is related to a higher lutein absorption, indicating that time is a much more crucial controlling factor during transfer to lipid phase for lutein than for β-carotene (Faulks *et al*. 2004).

Food matrix and molecular linkage

Fruits containing xanthophyll esters (i.e. loquat, oranges, papaya) are good sources of provitamin A carotenoids and, in many countries, constitute the best source to cover vitamin A requirements (Rodriguez-Amaya 1997; de Pee *et al*. 1998), although, to date, food matrices containing xanthophyll esters have seldom been evaluated under *in vitro* conditions (Breithaupt *et al*. 2002; Pérez-Gálvez and Mínguez-Mosquera 2005; Chitchumroonchokchai and Failla 2006; Granado-Lorencio *et al*. 2007). Interestingly, in humans, the plasma response to a single dose of xanthophyll esters, as compared to free forms, has yielded both similar (for β-cryptoxanthin) and higher bioavailability (for zeaxanthin esters) (Breithaupt *et al*. 2003, 2004).

To our knowledge, only two studies have specifically addressed the bioaccessibility of carotenoids *in vitro* using foods containing xanthophyll esters. Regardless of the food studied, hydrolysis of carotenol esters was incomplete under *in vitro* conditions, with an average estimate of ca. <40% (Chitchumroonchokchai and Failla 2006; Granado-Lorencio

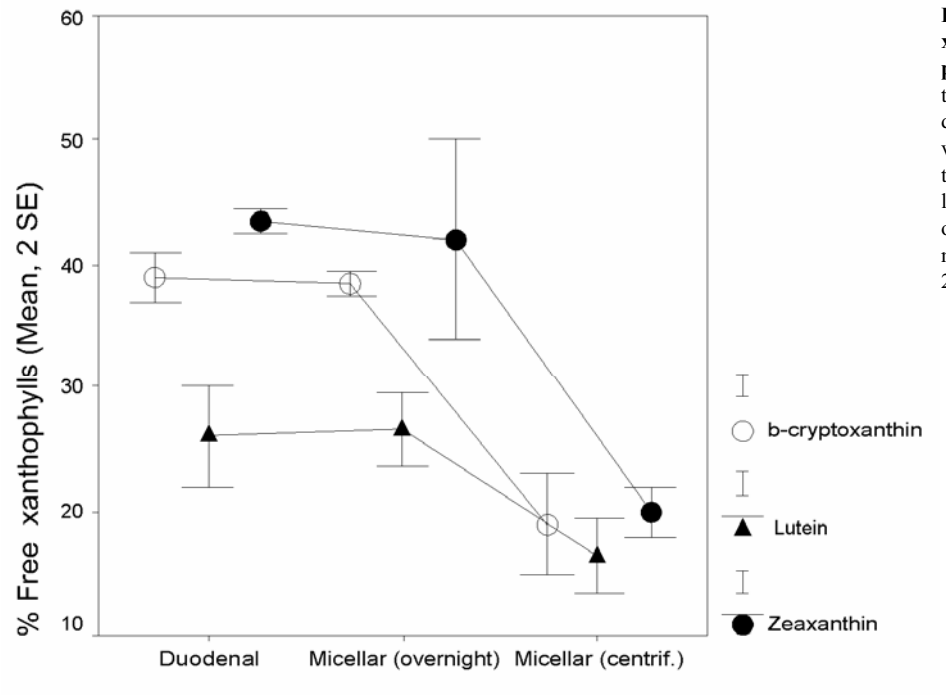


Fig. 5 Percentual transference of free xanthophylls into supernatants (micellar phase) according to the protocol used. For the three xanthophylls assessed, overnight decantation (at room temperature) showed a virtually complete transference of the xanthophylls present in the final digesta while low-speed centrifugation resulted in >50% of losses during transference. (Adapted and modified from Granado-Lorencio *et al.* 2007).

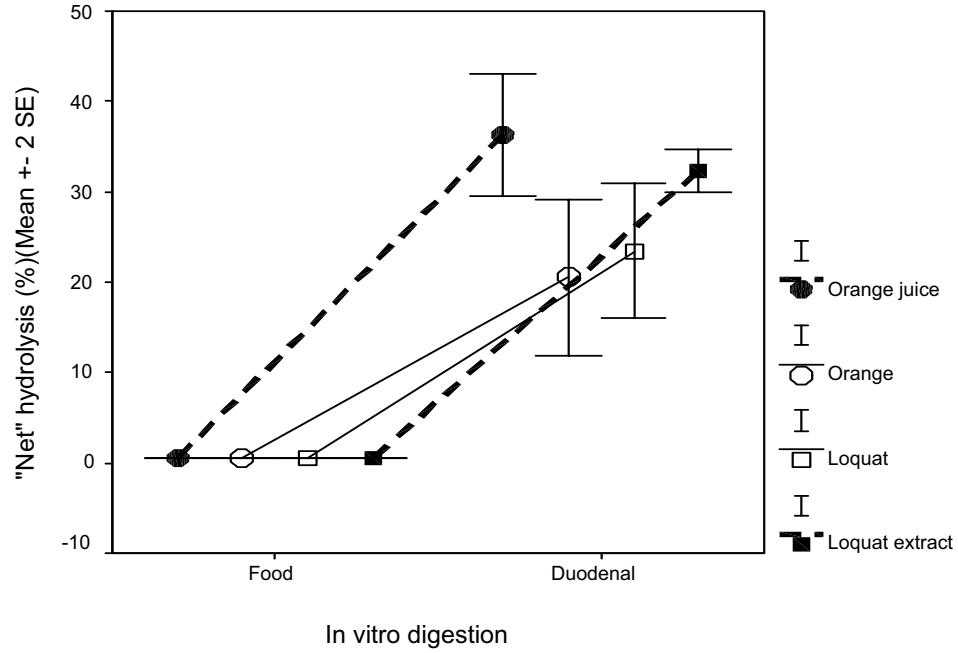


Fig. 6 Effect of matrix disruption on the final degree of hydrolysis of β-cryptoxanthin esters[1]. Regardless of the presence of esters of different xanthophylls (i.e. orange) or acyl moieties of the same xanthophyll (i.e. loquat), accessibility to the matrix such as that in juices and extracts, appears to be a key determinant of the degree of carotenol esters hydrolysis contained in the food and the final amount of free forms available for absorption. (Adapted from Granado-Lorencio *et al.* 2007). [1] "Net" hydrolysis: Final amount of free β-cryptoxanthin corrected for the initial content in the food.

et al. 2007). However, it is worth mentioning that different xanthophyll monoesters and diesters were cleaved, although the extent of hydrolysis was highly variable for different xanthophylls in a given food, as well as for a given xanthophyll in different foods (Granado-Lorencio *et al*. 2007). Interestingly, a determining factor of the degree of ester hydrolysis was the homogenization of the food matrix, regardless of the carotenoid profile of the food (i.e. orange vs. loquat) (**Fig. 6**).

This effect of matrix disruption has been previously observed (van het Hoff *et al*. 1999b; Chitchumroonchokchai and Failla 2006). Thus, based on *in vitro* data, a higher *in vivo* response could be expected (predictive value) when subjects consumed a more homogenized food. However, results from our laboratory suggest that this may not always be observed. We tested this prediction by comparing two human studies in which serum responses (dose-adjusted) were measured after the consumption of multiple doses of oranges (as juice and in segments). Using β-cryptoxanthin as a marker of intake, we found that the *in vivo* (human study) response was inconsistent with the *in vitro* prediction since, after chronic intake, the responses in serum were higher in the volunteers that consumed the less homogenized matrix (orange segments) (**Fig. 7**).

Species and vitamin isomers

Bioaccessibility of carotenoids and vitamin E has been reported to be highly variable, ranging from 0.1% to almost 100%, and depends on several factors, including microconstituent species, food matrix and food processing (Traber *et al*. 1993; Parker 1997; West and Castenmiller 1998; Reboul *et al*. 2006; Granado-Lorencio *et al*. 2007). Carotenes, xanthophylls and tocopherols display different molecular linkages and physicochemical properties and behave differently under *in vitro* conditions (i.e. micellization). Similarly, both carotenoid species and tocopherol vitamin isomers display differential *in vivo* transport and metabolism (Traber and Kayden 1989; Gaertner *et al*. 1996; Traber and Sies 1996; Parker 1997; Granado-Lorencio and Olmedilla-Alonso 2003).

A significant positive relationship between the percen-

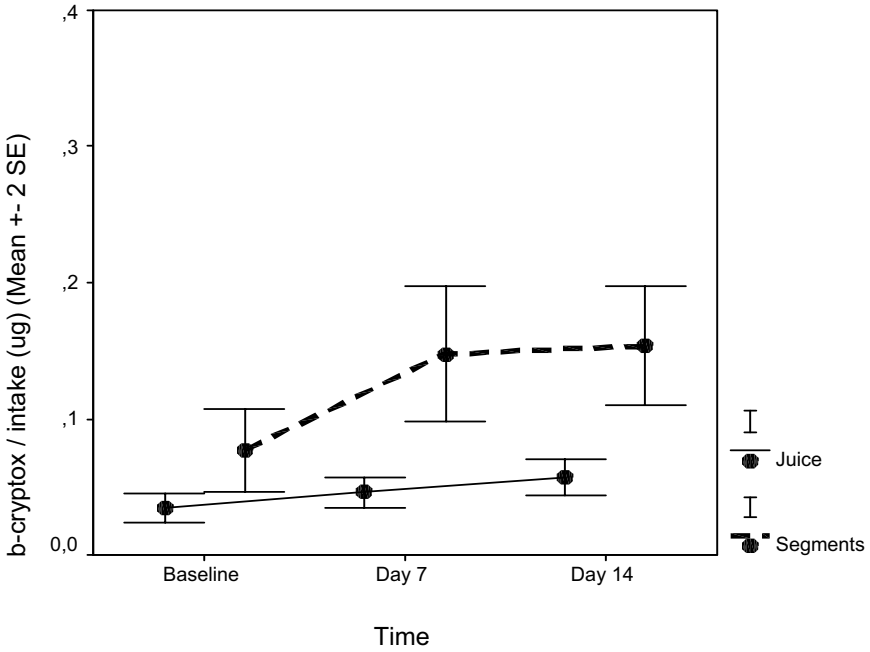


Fig. 7 Dose-adjusted serum response of β-cryptoxanthin in humans upon consumption of orange (juice or segments). In contrast to *in vitro* data, the serum response of β-cryptoxanthin after the intake of orange juice or segments for 14 days was inversely related to the degree of matrix disruption (n=14/group) (unpublished observations).

tage of carotenoids transferred into micelles in the *in vitro* model and those observed *in vivo* (micelles in the gastrointestinal lumen) has recently been reported, with values for lutein higher than those for β- and α-carotene which, in turn, are higher than those for lycopene (Reboul *et al*. 2006). Similarly, α-tocopherol has generally shown a bioaccessibility *in vitro* equal to or higher than that of γ-tocopherol in foods (Granado *et al*. 2006; Reboul *et al*. 2006).

Again, the predictive value of the *in vitro* model has been tested using broccoli as a frequently consumed dietary source of carotenoids and tocopherols (Granado *et al*. 2006). As expected, lutein, β-carotene, γ-tocopherol and α-tocopherol were highly stable (>75%) under the *in vitro* conditions while the incorporation into the supernatants (micellization) accounted for <20% of the initial content (Granado *et al*. 2006). Interestingly, the *in vitro* model showed that α- and γ-tocopherol display a similar rate of incorporation into supernatants, a finding consistent with the lack of biodiscrimination between the two vitamin isomers at the intestinal level observed in humans (Traber and Kayden 1989; Traber and Sies 1996). However, in a human crossover study, after consumption of broccoli for seven days, mean serum levels of lutein and γ-tocopherol increased, although, on the group level, only the change in lutein reached statistical significance, and no significant changes were found for α-tocopherol (**Fig. 8A**) and β-carotene (**Fig. 8B**). Thus, although the *in vitro* results predicted similar *in vivo* responses for lutein (**Fig. 8C**) and β-carotene and for α- and γ-tocopherol (**Fig. 8D**), the results in the human study were only partly consistent with this prediction and the behaviour of these phytochemicals under *in vitro* conditions did not fully explain the changes observed in the *in vivo* study (Granado *et al*. 2006).

Technological processing

Overall, processing may be beneficial as it disrupts the food matrix (cell walls), which facilitates the release of bound carotenoids and solubilization of free and ester forms, increasing the bioavailability (i.e. carotenoids). It is a well known fact that food processing (i.e. heating, canning) can induce conversion of the all-*trans*-form to *cis*-isomers (Rodriguez-Amaya 1997). Under *in vitro* digestion conditions, the *cis-trans* ratio in processed foods (i.e. cooked, canned) is apparently unaffected during the gastric and duodenal phases (Granado-Lorencio *et al*. 2007), coinciding with findings observed in humans (Tyssandier *et al*. 2003), but this fact may be different depending upon the carotenoid or the food matrix (i.e. lycopene) (Re *et al*. 2001) and could explained the reported higher transfer efficiency of *cis*-isomers to the aqueous-micellar phase (Failla and Chitchumroonchokchai 2005; Granado *et al*. 2006; Granado-Lorencio *et al*. 2007). Unfortunately, the consistency of these results with *in vivo* data is difficult to verify since the *cis-trans* ratio of carotenoids, both in chylomicrons and serum, seems to be fairly constant, regardless of their proportion in the diet and the population studied (**Fig. 9**) (Gaziano *et al*. 1995; Stahl *et al*. 1995; Gaertner *et al*. 1996; You *et al*. 1996; Olmedilla *et al*. 2001b, 2002).

As mentioned above, the food industry is increasingly using emerging technologies and approaches in food packaging (i.e. modified atmospheres), although little is known about their effect on the bioavailability of nutrients. The effect of modified atmosphere packaging (MAP) on carotenoid bioavailability has been explored both *in vitro* and *in vivo* (Serafini *et al*. 2002; Granado *et al*. 2005). Under *in vitro* conditions, MAP did not affect the hydrolysis of xanthophyll esters present in orange or the amount of free xanthophylls transferred and made available for absorption (Granado *et al*. 2005). Similarly, the stability of lutein, β-carotene, α- and γ-tocopherol and isomerization of lutein and β-carotene under *in vitro* conditions were not affected in broccoli stored under MAP conditions (Granado *et al*. 2005). In this context, the consumption of MAP-stored lettuce failed to improve the plasma levels of these nutrients and plasma total antioxidant capacity in humans (Serafini *et al*. 2002), although we observed the lack of a significant effect of MAP technology on the bioavailability of carotenoids and tocopherols both *in vitro* (Granado *et al*. 2005) and *in vivo* (unpublished observations). Thus, *in vivo* studies provide conflicting results, although differences in packaging materials and the modified atmospheres developed inside the packaging may affect the food constituents in the vegetables in different ways (Jacobsson *et al*. 2004).

Absorption modifiers

Dietary factors also affect the bioavailability of carotenoids and tocopherols (Hollander *et al*. 1978; West and Castenmiller 1998; Borel 2003). It is a well known fact that dietary fat increases carotenoid bioavailability and in humans; both the amount and type of fat have been reported to affect the bioavailability of some (i.e. lutein), but not all, carotenoids (Borel *et al*. 1998a; Roodenburg *et al*. 2000). On the other hand, plant sterols and water-soluble fibres (i.e. pectin) decrease the absorption of β-carotene, lycopene, lutein and tocopherols in humans (Rock and Swenseid 1992; Riedl *et al*. 1999; Richelle *et al*. 2004).

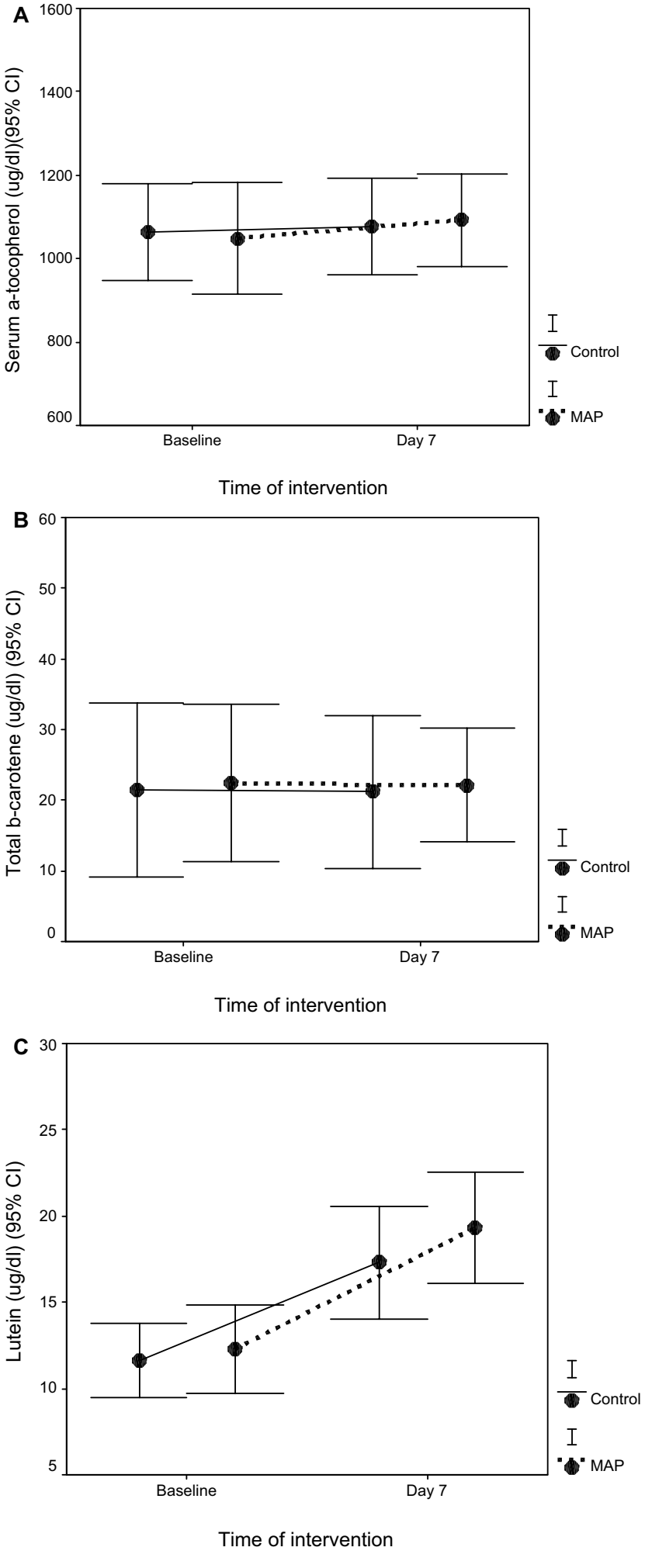
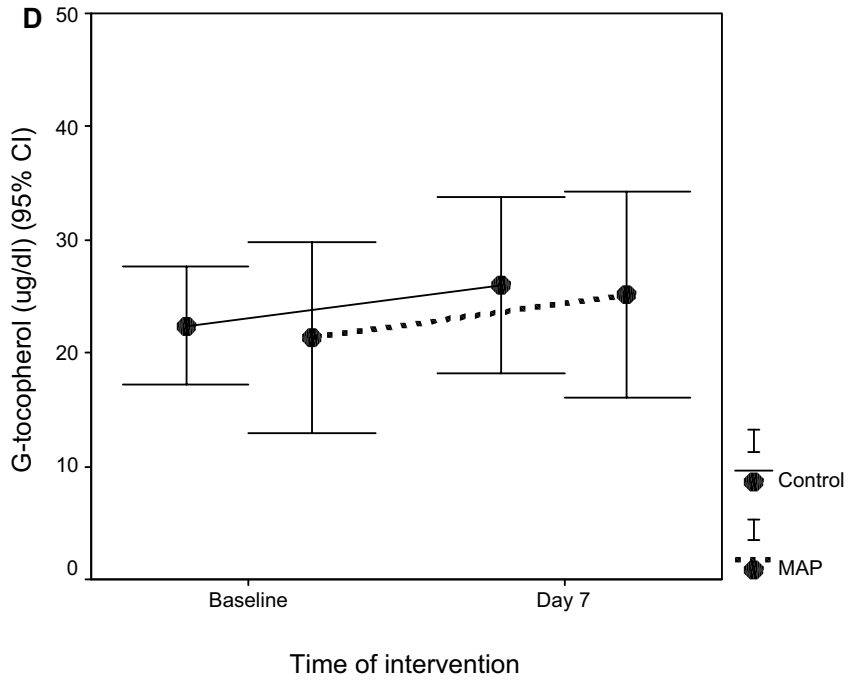


Fig. 8 Effect of modified atmospheres packaging (MAP). In a human, crossover, multiple-dose intervention study, we assessed the changes of carotenoids and tocopherols in serum caused by the daily consumption (n=14; 7 days) of minimally-processed broccoli (MAP) compared to conventional storage. MAP did not affect the bioavailability of carotenoids or tocopherols *in vivo* and changes were only significant for lutein, regardless of the type of broccoli consumed (Adapted and modified from Granado *et al*. 2005).



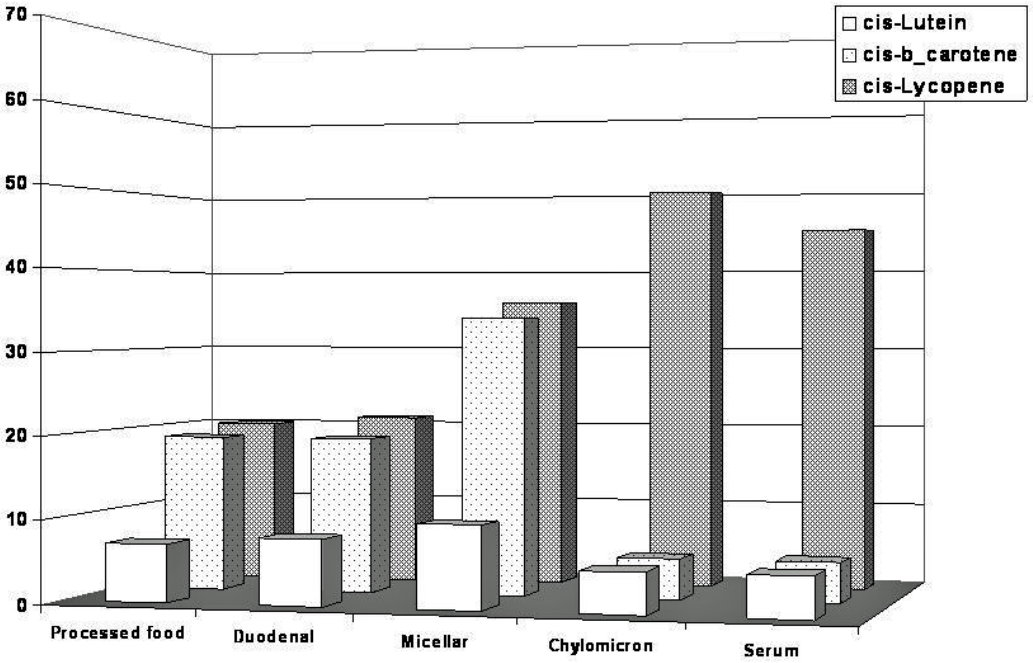


Fig. 9 Isomerization: Comparison between *in vitro* and *in vivo* results. The bars show the average content of *cis*-forms reported under *in vitro* and *in vivo* (serum) conditions. Overall, simulated digestion conditions do not increase significantly the content of *cis*-forms although it appears to be a preferential incorporation into the micellar phase. Although this differential micellarization of *cis*-forms (available for absorption) is somehow concordant with the *cis-trans* pattern of lycopene *in vivo*, it is consistently independent from the profile observed for other carotenoids (i.e. lutein, β-carotene) in *in vivo* studies and populations. X-axis: Percentage of cis-isomer; Y-axis: *In vitro* (processed food, duodenal, micellar) and *in vivo* phase (chylomicron, serum).

In vitro studies have also shown an effect of absorption modifiers (i.e. fat, fibre). The transfer into micelles constitutes a key step that determines the extent of carotenoid absorption from different foods, and influencing factors include soluble and insoluble indigestible fractions, amount of taurocholate, amount and type of lipids and acyl moieties, phospholipids and bile salt content (Jacobs *et al*. 1982; Hedren *et al*. 2002; Rich *et al*. 2003; Tyssandier *et al*. 2003; Failla and Chitchumroonchokchai 2005; Serrano *et al*. 2005; Yonekura *et al*. 2006). For example, a significant correlation between *in vitro* availability of carotenoids (lutein + β-carotene) and the content of Klason lignin and non-starch polysaccharides has been reported in green leafy vegetables (Serrano *et al*. 2005). Similarly, when the major dietary sources of carotenoids were submitted to *in vitro* digestion, negative correlations were found between fibre content and the transfer of lutein, β-carotene and β-cryptoxanthin, while positive correlations were observed between the fat content and the transfer of β-cryptoxanthin, β-carotene and, especially, lutein. However, none of these relationships reached statistical significance nor fully explained the different transfer efficiency observed across the different foods (unpublished observations).

Currently, the food industry is adding several biologically active compounds to food products, although there is little information regarding the impact on their bioavailability. We studied the effect of adding milk (casein phosphopeptides [CPPs]) and iron to fruit juices on the *in vitro* bioaccessibility and *in vivo* serum response of carotenoids (i.e. β-cryptoxanthin). *In vitro* data showed that the degree of ester hydrolysis of β-cryptoxanthin increased in the presence of milk and milk plus iron (**Fig. 10A**), a circumstance maintained in the micellar phase. In this case, as predicted, dose-adjusted concentrations of β-cryptoxanthin in serum were higher, although not significantly, when subjects consumed fruit juices containing milk (and milk plus iron) than when they ingested fruit juices alone (**Fig. 10B**) (unpublished observations).

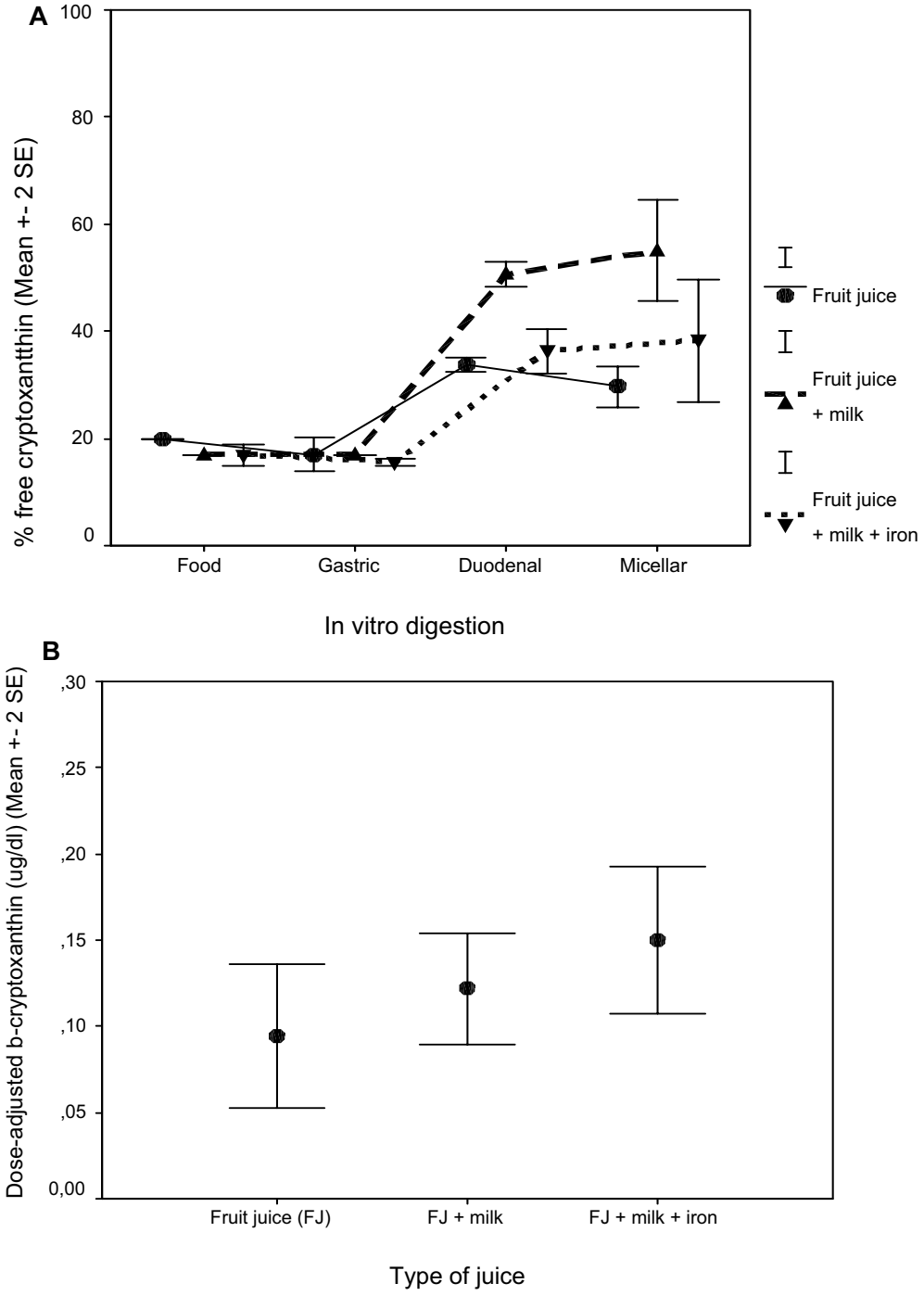


Fig. 10 (**A**) Free β-cryptoxanthin available for absorption under *in vitro* conditions. We assessed the potential effect of different absorption modifiers on the degree of hydrolysis of β-cryptoxanthin and its transference into micellar phase. As shown, the presence of milk (and milk plus iron) increased the amount of free β-cryptoxanthin in the final digesta and available for absorption (unpublished observations). (**B**) Dose-adjusted increments of β-cryptoxanthin in serum upon consumption of fruit juices with and without absorption modifiers. Human crossover study (n=26).

Predictive value of *in vitro* models

As mentioned above, the predictive value of the *in vitro* models relies on the consistency between the results under simulated conditions and those observed *in vivo*. **Table 1** summarizes some qualitative findings at different stages using both approaches. As can be observed, agreement between the two approaches depends on the compound and the stage assessed, while, for some components, information is still lacking.

The absence of xanthophyll esters during the postprandial state in humans (Wingerath *et al*. 1995; Granado *et al*. 1998; Breithaupt *et al*. 2003; Schlatterer *et al.* 2006) has led to the assumption that xanthophyll hydrolysis is a prerequisite for xanthophyll absorption. However, the presence of ester forms in the aqueous-micellar phase after different *in vitro* protocols has been reported (Chitchumroonchokchai and Failla 2006; Granado-Lorencio *et al*. 2007), and minor, although quantifiable, amounts of ester forms have been reported to be absorbed by Caco-2 cells (Chitchumroonchokchai and Failla 2006).

In addition, free epoxy-carotenoids have been found in the supernatants (Granado-Lorencio *et al*. 2007) and, although xanthophyll epoxides have not been detected in humans after single-dose ingestion (Barua and Olson 2001), studies in Caco-2 cells and rats showed that these xanthophylls are absorbed and distributed to specific tissues (Sugawara *et al*. 2001; Asai *et al*. 2004). Similarly, keto and keto-hydroxy-carotenoids (i.e. astaxanthin, capsanthin) have hardly been tested *in vitro*, although they are found in human chylomicrons and plasma upon acute ingestion (Oshima *et al*. 1997; Odeberg *et al*. 2003; Coral-Hinostroza *et al*. 2004). Regarding the occurrence and behaviour of oxidative metabolites and *cis*-isomers of carotenoids, the former does not seem to be detectably formed during *in vitro* digestion, while the *cis-trans* ratio, as mentioned above, seems to be relatively constant *in vivo* (i.e. serum). Nevertheless, as shown in **Table 1**, information regarding the metabolite formation both *in vitro* and *in vivo* is scarce. For example, the occurrence of some "lycopenoids" (lycopene

Table 1 Some comparative aspects between *in vitro* and *in vivo* observations[a].

Food	*In vitro* Duodenal /Micellar	Caco-2 cells	Chylomicron	Plasma-Serum
XANTHOPHYLLS				
Free forms	YES	YES	YES	YES
Ester forms	YES[1, 2]	YES[1]	NO [3,4, 5, 6]	NO [3, 5, 6,7]
Cis-isomers	YES	YES	YES	YES
Xanthophyll epoxides	YES [2, 8]	YES [8]	Not reported	NO [9] /YES [10]
Oxidative metabolites from:				
Lutein/Zeaxanthin	Not formed [2]	Not reported	Not detected [3]	YES [3, 11]
Neoxanthin	YES [2, 10]	YES [10]	Not reported	Not reported
Lycopene	Not reported	Not reported	Not reported	YES [11]
Keto- and keto-hydroxy-carotenoids (i.e. astaxanthin, capsanthin)	YES [8]	YES [8]	YES [12, 13, 14]	YES [12, 13, 14]
CAROTENES				
β-carotene	YES	YES	YES	YES
Lycopene	YES	YES	YES	YES
Others (i.e. phytoene, phytofluene)	YES [3, 8]	YES [8]	Not reported	YES
Cis-isomers	YES	YES	YES	YES
VITAMIN E				
α-tocopherol	YES	YES	YES	YES
γ-tocopherol	YES	YES	YES	YES
Tocopheryl esters (fortified foods)	YES [2]	Not reported	NO	NO

[a] Forms of carotenoids and tocopherols without superscripts are usually present in *in vitro* systems and/or human serum and tissues. [1] Chitchumroonchokchai and Failla (2006); [2] Granado-Lorencio *et al*. 2007; [3] Granado *et al*. 1998. Ester forms detected in human serum upon long-term supplementation with lutein; [4] Wingerath *et al*. 1995; [5] Breithaupt *et al*. 2003; [6] Breithaupt 2004; [7] Schlatterer *et al*. 2006; [8] Sugawara *et al*. 2001; [9] Barua and Olson 2001 (in human); [10] Asai *et al*. 2004 (in mice); [11] Khachick *et al*. 1992; [12] Coral-Hinostroza 2004; [13] Odeberg *et al*. 2003; [14] Oshima *et al*. 1997.

metabolites) (Lindshield *et al*. 2007) have been reported in serum and human milk but, to our knowledge, not tested under *in vitro* digestion models, while other lycopene metabolites formed *in vitro* have not been found *in vivo* (Lindsshield *et al*. 2006).

Concerning consistency, on a (semi) quantitative basis, between the *in vitro* and *in vivo* models, several results have already been mentioned and some are summarized and discussed in more detail elsewhere (During and Harrison 2004; Failla and Chitchumroonchokchai 2005; Reboul *et al*. 2006). They mostly refer to the stability and limited isomerization under gastrointestinal conditions (Garret *et al*. 2000; Re *et al*. 2001; Reboul *et al*. 2006; Granado-Lorencio *et al*. 2007), the greater micellization efficiency of lutein compared to carotenes (van het Hof *et al*. 1999b; Rich *et al*. 2003; Goñi *et al*. 2006), the greater bioaccessibility of carotenoids after food processing (Rodriguez-Amaya 1997; Garret *et al*. 2000; Failla and Chitchumroonchokchai 2005), the impact of matrix disruption on micellization and absorption (van het Hof *et al*. 1999b), the effect on hydrolysis and micellization of absorption modifiers (Riedl *et al*. 1999; Roodemburg *et al*. 2000; Richelle *et al*. 2004; Serrano *et al*. 2005; Granado-Lorencio *et al*. 2007), the linear and saturable uptake by Caco-2 cells (Garret *et al*. 2000; During and Harrison 2004) and the lack of biodiscrimination of tocopherols during gastrointestinal digestion (Traber and Kayden 1989; Traber *et al*. 1993; Granado *et al*. 2006; Reboul *et al*. 2006). However, more accurate quantitative prediction of *in vivo* responses is problematical as the latter also depend on host-related and methodological factors, although mathematical modelling will probably help to evaluate and test its validity.

LIMITATIONS IN THE COMPARABILITY OF *IN VITRO* AND *IN VIVO* STUDIES

In vitro constraints

Overall, *in vitro* studies indicate that carotenoids (and tocopherols) are relatively stable when exposed to gastrointestinal conditions, and that transfer efficiency is critical for their bioaccessibility in foods, although it varies widely depending on the carotenoid structure and the food that contains them. The positive correlation between the percentage of carotenoids and tocopherols in micelles (*in vitro*) and observations *in vivo* suggests that the *in vitro* approach may be suitable for predicting the bioavailability of phytochemicals from foods (Failla and Chitchumroonchokchai 2005; Reboul *et al*. 2006), although exceptions do exist and *in vitro* data are not fully concordant with *in vivo* responses (Granado *et al*. 2006; Reboul *et al*. 2006).

As mentioned above, *in vitro* methodology for bioavailability assessment and its potential predictive value regarding human absorption of phytochemicals should be validated in different *in vivo* situations (Oomen *et al*. 2002). However, due to the lack of standardization of *in vitro* protocols, comparisons between the two approaches are subject to methodological uncertainties. For example, the dose of the carotenoid ingested is known to affect the relative (in percentage terms) bioavailability *in vivo*, but to mimic this factor *in vitro* implies a modification of the model, i.e. the substrate-enzyme relationship, final reaction volume, saturation of partition liquids, or probably to saturate micellar incorporation capacity (Borel 2003). Similarly, as mentioned above, incorporation of phytochemicals into the supernatants (micellization) may differ depending on the protocol used, the components of the final digesta and the food matrix studied (Tyssandier *et al*. 2001; Hedren *et al*. 2002; Borel 2003; Failla and Chitchumroonchokchai 2005; Granado-Lorencio *et al*. 2007). Moreover, most of the *in vitro* protocols do not assess other common carotenoids simultaneously present in the foods (i.e. neoxanthin, violaxanthin, capsanthin, phytoene); thus, their behaviour under *in vitro* conditions and the potential interactions with other food components is unknown, limiting the predictive value of the model.

Caco-2 cell lines have been used as a surrogate for small intestinal enterocytes and human absorption, including chylomicron secretion (Failla and Chitchumroonchokchai 2005). Available data indicate that intestinal absorption of certain carotenoids (i.e. lutein, β-carotene) is, at least in part, an active protein-mediated process in at least certain subsets of cell lines (During and Harrison 2004; Fransen-van Hal *et al*. 2005; During *et al*. 2005; Reboul *et al*. 2005). However, while Caco-2 cell models may provide relevant mechanistic information (rate and mechanisms of transport time-dependence, saturation and interactions between carotenoids), not all human intestinal cell clones metabolise carotenoids in the same way or at the same rate as the human intestinal mucosa (During and Harrison 2004), and each cell line

appears to have a distinct metabolite profile. Thus, it is not possible to establish which one corresponds to the *in vivo* situation (Fransen van Hal *et al*. 2005). Similarly, incubation of Caco-2 cells with micelles have shown that these cells take up a wide range of carotenoids, even those not de-tected in human serum (i.e. neoxanthin, xanthophylls esters) (Sugawara *et al*. 2001; Chitchumroonchokchai and Failla 2006) and, as mentioned before, carotenoid uptake by Caco-2 cells shows a time-dependence (up to 16 h) (Garret *et al*. 2000; During *et al*. 2002; Reboul *et al*. 2005), a fact that does not necessarily mimic the postprandial response mostly observed *in vivo* (bimodal) (Faulks and Southon 2005). Altogether, these facts may compromise its comparability with *in vivo* studies.

In vivo limitations

Serum concentrations of carotenoids reflect, at least to some extent, the consumption of carotenoid-containing foods, although absorption studies are best carried out by measuring chylomicron excursion even when the amount of carotenoids that remain in this fraction or travel to tissues during their clearance by the liver is not clear (Faulks and Southon 2005).

Because of the endogenous presence of carotenoids and tocopherols, large doses have been used to provoke a measurable response. However, large doses may alter the physiological response, and some kinetic studies (after high oral dose) have been questioned due to the lack of linear dose responses (i.e. vitamin E) or because linear, nonsaturable models are assumed. Thus, intakes at achievable dietary levels are used, although they sometimes result in undetectable responses. Nevertheless, it should be noted that the lack of a detectable response does not necessarily mean the absence of absorption, as reported in ileostomy patients (Faulks *et al*. 2004), and probably there is a very small proportion of true non-responders to pharmacological doses in the healthy population (Borel *et al.* 1998b). Another explanation for the lack of response (and the apparent inconsistency with *in vitro* data) may be related to first-pass metabolism at the intestinal level of some compounds (i.e. provitamin A activity of β-carotene) compared to others (i.e. lutein), and the preferential incorporation of vitamers (i.e. α- vs. γ-tocopherol) in VLDL in the liver, although the absorption is nonselective in the gastrointestinal tract (Traber and Sies 1996).

In biological systems, large deviations from linearity occur due, for example, to saturation of carrier systems or the action of homeostatic control mechanisms (Wilson and Dainty 1999). Moreover, the carotenoid response seems to be bimodal, and the assumption of equal plasma clearance, distribution or exchange rate among lipoproteins for the different carotenoids (i.e lutein vs. β-carotene) may lead to the misinterpretation of the "true" degree of absorption (Granado *et al*. 2002; Faulks and Southon 2005).

Due to differences in fat absorption and clearance, a great within- and between-subject variability is usually observed in human studies. However, because of methodological and statistical requirements, *in vivo* studies are performed in groups of subjects of a (small) calculated size. In this respect, it is generally accepted that humans can be divided into "responders" and "non-responders" according to their chylomicron metabolism and plasma response (Borel *et al*. 1998b). However, postprandial behaviour does not predict long-term responses, and acute responses (after a single dose) usually show larger inter-individual variability than chronic (multiple) intakes (Borel *et al*. 1998b; Olmedilla *et al*. 2002; Granado-Lorencio and Olmedilla-Alonso 2003; Faulks and Southon 2005). Moreover, serum responses after supplementation may also be related to the serum levels at entrance (i.e. nutritional status), the dose supplied, the timing of intake (i.e. with or without meals) and duration of the intervention, all of which may determine the serum response or even provoke a "ceiling effect" (Olmedilla *et al*. 2002; Riso *et al*. 2004; Schlatterer *et al.* 2006). Finally, most of the *in vivo* studies rely on the statistical significance of the changes observed which, in turn, depend on the number of subjects involved and the magnitude of the change. Thus, a lack of consistency between *in vivo* and *in vitro* data may also be related to the design and the statistical power of the *in vivo* study rather than to the lack of concordance in the results.

CONCLUDING REMARKS

In vitro protocols have proved to be a rapid and cost-efficient alternative for the initial screening for bioavailability of micronutrients and phytochemicals, and its potential predictive value regarding human absorption of phytochemicals should be further validated in different *in vivo* situations (Oomen *et al*. 2002; Yun *et al*. 2004; Failla and Chitchumroonchokchai 2005; Reboul *et al*. 2006; Granado *et al*. 2006).

The behaviour of several phytochemicals under *in vitro* conditions does not fully explain the responses observed *in vivo*. Thus, the predictive value (*in vivo* response) and the interchangeability of the two approaches (*in vitro* and *in vivo*), especially for quantitative purposes, will be compromised because of the differential metabolism of the phytochemicals (i.e. first-pass metabolism), host-related factors (i.e. nutritional status, biological variability) and methodological aspects (i.e. study design, statistical power). Moreover, since *in vitro* protocols are not completely physiological and do not mimic the host-related factors, the potential predictive value of *in vitro* models includes an uncertainty factor that will limit its applicability. In this respect, the two approaches should be considered complementary, but not necessarily interchangeable.

Nowadays, *in vitro* models are increasingly used and they should be considered as a preliminary step to test novel food ingredients (i.e. microalgae) and newly designed (functional) foods before being tested in human trials. Data from *in vitro* studies will certainly contribute to unravel the underlying mechanisms, interactions and determinants of the bioavailability of phytochemicals from foods. Undoubtedly, the *in vitro* approach will become of help to optimize the design of foods and processing methods, to develop food-based dietary guidelines for health promotion on a community basis and to support health claims of functional foods.

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