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Effect of differently processed fruit and vegetable soups on β-carotene and lycopene bioavailability and oxidative status in humans.

Efecto de sopas vegetales elaboradas con diferente procesado sobre la biodisponibilidad de β-caroteno y licopeno y el estado oxidativo en humanos.

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A los voluntarios

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ABBREVIATIONS

8-OHdG	8-hydroxy-2´-deoxyguanosine
ABC	Adenosine triphosphate (ATP)-binding cassette
AICR	American Institute for Cancer Research
ANOVA	Analysis of variance
apoA-IV	Apolipoprotein A-IV
apoB100	Apolipoprotein B100
As	Ascorbic
ATBC	Alpha-Tocopherol Beta-Carotene
ATCC	American Type Culture Collection
AUC	Area under the curve
BCDO2	β,β-carotene-9',10'-oxygenase
BCMO1	β,β-carotene-15,15'-monooxygenase
BHT	Butylated hydroxytoluene
BMI	Body Mass Index
C	Cholesterol
Caco-2	Human colon carcinoma cell line
CARET	β-carotene and Retinol Efficiency Trial
CD36/FAT	Cluster determinant 36/Fatty acid translocase
CHD	Coronary heart disease
CI	Confidence interval
CM	Chylomicrons
COS-7	African green monkey kidney cell line (Simian Virus 40 transformed)
Cr	Creatinine
CV	Coefficients of variation
CVD	Cardiovascular disease
CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1
DCF	2',7'-Dichlorofluorescein
DCFH	2',7'-Dichlorodihydrofluorescein
DCFH-DA	2',7'-Dichlorodihydrofluorescein diacetate
DHR	Dihydrorhodamine 123
DMSO	Dimethylsulfoxide

DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
Eq	Equivalent
F&V	Fruit and vegetable
FABP	Fatty acid-binding protein
FAO	Food and Agriculture Organization of the United Nations
GC/MS	Gas chromatography/mass spectrometry
GOT	Glutamic-oxaloacetic transaminase
GPT	Glutamic-pyruvic transaminase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
H	Hip
Hb	Haemoglobin
HDL	High density lipoprotein
HepG2	Human hepatocyte carcinoma cell line
HPH	High pressure homogenization
HPLC	High performance liquid chromatography
HPLC/ECD	High performance liquid chromatography/electrochemical detection
ISX	Intestine-specific homeobox
LDL	Low density lipoprotein
LPC	Lysophosphatidylcholine
MDA	Malondialdehyde
MEM	Minimum essential medium with Earle's salts
MeOH	Methanol
MTBE	Methyl tert-butyl ether
MTP	Microsomal triglyceride transfer protein
MTT	3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NPC1L1	Niemann–Pick C1-like 1
Opt	Optimised
PBS	Phosphate-buffered saline
PUFA	Polyunsaturated fatty acid

RCT	Randomized control trial
RDI	Recommended daily intake
Ref	Reference
ROS	Reactive oxygen species
RR	Relative risk
SD	Standard deviation
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SPSS	Statistical Package for Social Science
SR-BI	Scavenger receptor class B type I
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TG	Triglyceride
tHcy	Homocysteine
Total-C	Total-cholesterol
TRL	Triglyceride-rich lipoprotein
VLDL	Very low density lipoprotein
W	Waist
WCRF	World Cancer Research Fund
WHO	World Health Organization
Wk	Week

I. JUSTIFICATION AND OBJECTIVES

I. JUSTIFICATION AND OBJECTIVES

An adequate dietary intake of fruit and vegetables (F&Vs) has been related to a decreased risk of chronic diseases such as cardiovascular disease (CVD) and some types of cancer [1-2]. In the European Union, the low F&V consumption has been estimated to be responsible for over 1 million deaths annually [3]. Consequently, an increase in consumption of F&Vs has been advocated by national and international organizations, which recommends 400-600 g of F&Vs per day [4-6]. Nevertheless, the intake of F&Vs in the European countries remains below the recommendations [7-8]. The observed decrease in the F&V consumption of the population and its consequence on the health are contributing to the interest for the positive aspects of diet. Foods have assumed a new status, in which should be able to provide additional physiological benefits, such as preventing or delaying onset of chronic diseases, as well as meeting basic nutritional requirements [9]. This is also aligned with a change in consumers' life styles, looking for more healthy and natural products, which has influenced the way of food companies to manufacture their products [10].

The combination of phytochemicals from F&Vs was proposed to be responsible of some of the healthy properties attributed to these foods [11]. Carotenoids are phytochemicals with antioxidant activity that have been related to the prevention of carcinogenesis and atherogenesis, avoiding the oxidation of some important biomolecules (DNA, proteins, lipids and LDLs) [12-14]. The biological actions of carotenoids and their potential positive impact on human health are limited by their bioavailability in F&V products [15]. Bioavailability is the fraction of an ingested nutrient that is absorbed in the gut and reaches systemic circulation [15-16]. The bioavailability of carotenoids is influenced by both, absorption and metabolism, but also by food matrix, formulation and food processing techniques [17-18]. These factors may affect serum carotenoid levels and hence the antioxidant effect in humans [9]. The food industry is playing an increasing role in developing novel "healthy" F&V products [19], by designing processes that maximizes the preservation of key nutrients from raw material, such as carotenoids, and enhancing their bioavailability. Overall, these food products are aiming to contribute to achieve the health benefits of a diet rich in F&Vs.

Processing solutions that improve nutrient bioavailability should be evaluated in human studies. Serum and chylomicron response after the consumption of food rich in carotenoids, may provide an estimation of relative bioavailability using simple procedures [20]. Biomarkers of oxidative stress status are commonly used for estimating the antioxidant activity of carotenoids in dietary intervention studies [21]. Nevertheless, human studies are restricted by technical and ethical considerations, they can be expensive, and the number of samples that can be investigated are limited. *In vitro* models have been developed as more simple, inexpensive and reproducible alternative to human studies [15,22]. However, *in vitro* methods evaluating the beneficial role of antioxidant nutrients against degenerative diseases should be supported with *in vivo* studies to draw logical conclusions.

The present thesis is involved in the Healthy Structuring project (European community's sixth programme), which aims to develop F&V food products with improved structural and nutritional qualities by optimizing all steps involved in the manufacturing of such products from growing the raw materials, through processing conditions. The overall aim of the research presented in this thesis was to evaluate the effect of differently processed fruit and vegetable soups on the bioavailability of β-carotene and lycopene as well as their antioxidant effect in humans.

For that purpose the following specific objectives were established:

1. To perform a pilot study evaluating the effect in humans of a fruit and vegetable soup with high *in vitro* carotenoid bioaccessibility. The obtained data would be useful for calculating sample size in a subsequent large randomized control trial. In addition, to discern whether a 4-week washout period is sufficient to allow β-carotene and lycopene levels to return to their baseline values for future crossover studies with similar food products.

2. To develop a new method to study the antioxidant effect of fruit and vegetable food products by stimulating HepG2 cells with human postprandial chylomicrons obtained after the intake of the selected food products.

3. To compare the effect of the consumption of two differently processed fruit and vegetable soups on serum concentrations of β-carotene and lycopene and on markers of oxidative stress and cardiovascular risk in healthy subjects.

4. To quantify the postprandial carotenoid chylomicron response in humans after a single intake of the two differently processed fruit and vegetable soups, and to evaluate the antioxidant effect of the postprandial chylomicrons on HepG2 cells.

This PhD Thesis is based on the studies reported in the following papers:

I. **Martínez-Tomás R**, Larqué E, González-Silvera D, Sánchez-Campillo M, Burgos MI, Wellner A, Parra S, Bialek L, Alminger M, Pérez-Llamas F.
Effect of the consumption of a fruit and vegetable soup with high *in vitro* carotenoid bioaccessibility on serum carotenoid concentrations and markers of oxidative stress in young men.
European Journal of Nutrition (2012), 51(2): 231-239.
(Objective 1)

II. Sánchez-Campillo M, Pérez-Llamas F, González-Silvera D, **Martínez-Tomás R**, Burgos MI, Wellner A, Avilés F, Parra S, Bialek L, Alminger M, Larqué E.
Cell-based assay to quantify the antioxidant effect of food-derived carotenoids enriched in postprandial human chylomicrons.
Journal of Agricultural and Food Chemistry (2010), 58 (20): 10864-10868.
(Objective 2)

III. **Martínez-Tomás R,** Pérez-Llamas F, Sánchez-Campillo M, González-Silvera D, Cascales AI, García-Fernández M, López-Jiménez JA, Zamora Navarro S, Burgos MI, López-Azorín F, Wellner A, Avilés Plaza F, Bialek L, Alminger M, Larqué E.
Daily intake of fruit and vegetable soups processed in different ways increases human serum β-carotene and lycopene concentrations and reduces levels of several oxidative stress markers in healthy subjects.
Food Chemistry (2012), 134 (1): 127-133
(Objective 3)

IV. Sánchez-Campillo M, Larqué E, González-Silvera D, **Martínez-Tomás R**, García-Fernández M, Avilés F, Wellner A, Bialek L, Parra S, Alminger M, Zamora S, Pérez-Llamas F.
Changes in the carotenoid concentration in human postprandial chylomicron and antioxidant effect in HepG2 caused by differently processed fruit and vegetable soups.
Food Chemistry (2012), 133 (1): 38-44.
(Objective 4)

Related publications not included in the thesis:

I. Burgos Alves MI, Avilés Plaza F, **Martínez-Tomás R**, Sánchez-Campillo M, Larqué E, Pérez-Llamas F, Martínez Hernández P, Parra Pallarés S.
Oxidized LDL and its correlation with lipid profile and oxidative stress biomarkers in young healthy Spanish subjects.
Journal of physiology and biochemistry (2010), 66 (3): 221-227.

II. Alminger M, Svelander C, Wellner A, **Martínez-Tomás R**, Bialek L, Larqué E, Pérez-Llamas F.
Applicability of *in vitro* models in predicting the *in vivo* bioavailability of lycopene and β-carotene from differently processed soups.
Food and Nutrition Sciences (2012), 3 (4): 477-489.

III. Van Buggenhout S, Ahrné L, Alminger M, Andrys A, Benjamin M, Bialek L, Cleaver G, Colle I, Langton M, Larqué E, Lemmens L, Löfgren A, Lopez-Sanchez P, Pérez-Llamas F, **Martínez-Tomás R**, Robertson J, Schalow S, Svelander C, Wellner N, Hendrickx M, Waldron K.
Structural design of natural plant-based foods to promote nutritional quality.
Trends in Food Science & Technology (2012), 24 (1): 47-59.

II. BACKGROUND

II. BACKGROUND

1. FRUIT AND VEGETABLE PRODUCTS AND HEALTH IMPLICATIONS

1.1 Low consumption of fruit and vegetables and risk of chronic diseases

An adequate dietary intake of F&Vs has been related to a decreased risk of CVD, some types of cancer [1-2,23-24], but also cataracts, age-related macular degeneration, central neurodegenerative diseases, and diabetes [25].

Evidence that F&V consumption has a protective effect against ***CVD*** comes mainly from observational epidemiological studies [26]. In a meta-analysis of cohort studies, the pooled relative risk (RR) of coronary heart disease (CHD) for subjects consuming 3–5 servings/day was 0.93 ($P = 0.06$), and 0.83 ($P < 0.0001$) for those consuming more than 5 servings/day compared with individuals who had less than 3 servings/day of F&Vs [2]. Nevertheless, controlled nutritional prevention trials show limited protective effect of F&Vs on CHD [26]. In a randomized controlled dietary modification trial, women who achieved the highest level of F&V consumption (≥6.5 servings per day), tended to a lower risk of CHD than the control group, but this difference was not statistically significant (adjusted hazard ratio, 0.89; $P = 0.11$) [27]. In contrast, blood pressure, which is an important cardiovascular risk factor, was lower in individuals encouraged to consume 5 portions of F&Vs per day for 6 months than in the control group (differences between groups for the change in systolic and diastolic blood pressure were 4 mmHg and 1.5 mmHg, respectively) [28]. The effects of F&V consumption on others established CVD risk factors have not been thoroughly explored.

As regards ***cancer***, epidemiological studies have observed that people with relatively high intake of F&Vs have a moderately reduced risk of the upper gastrointestinal tract cancer [29-30]. A prospective cohort study in the United States of cancers of the oral cavity, pharynx and larynx, reported that, people with intake of ≈5.8 servings/day per 1000 calories of F&Vs (assessed by food frequency questionnaire), had a reduced risk of cancer compared with people who consumed ≈1.5 s/d/1000 calories (0.71, $P = 0.018$) [30]. In an European study on squamous cell cancers of the upper aero-digestive tract, people with high intake of F&Vs (> 480 g/d) had a RR = 0.60 ($P = 0.035$) compared with those with a relatively low intake (≤ 240 g/d) [29]. Nevertheless, findings in these studies may be influenced by the low number of cases (787 and 352 cases, respectively)

[31]. Besides, although the statistical analyses of epidemiological studies adjusted their data by smoking and alcohol consumption, some authors suggest these factors may still influence the results [31]. For lung cancer, an inverse association with the consumption of F&Vs has been described [32] while for others types of cancer (colorectal, breast, prostate, etc.) a little or no association have been proposed [33,31]. In cells and animal models antitumorigenic effects of vegetables have been found [34-37]. Thus, it is still possible that there are benefits related to the F&V consumption to be confirmed.

The World Health Organization (WHO), on the Global Burden of Disease study (2005) [38], suggested that the total worldwide mortality attributable to inadequate consumption of F&Vs was up to 2.635 million deaths per year. Increasing individual F&V consumption to up to 600 g per day could reduce the total worldwide burden of disease by 1.8%, and reduce the burden of ischaemic heart disease and ischaemic stroke by 31% and 19%, respectively. For stomach, oesophageal, lung and colorectal cancer, the potential reductions were 19%, 20%, 12% and 2%, respectively. In the European Union, in which inadequate F&V consumption has been estimated to be responsible for over 1 million deaths annually, the decrease in the total disease burden could even reach the 3.6% [3].

Consequently, an increase in consumption of F&Vs has been advocated by national and international organizations, on the assumption that such a change would reduce the incidence both of CVD and cancer [4-5,39]. A published WHO/Food and Agriculture Organization of the United Nations (FAO) report (2003) recommends a minimum of 400 g of F&Vs per day [4], while a later report from the World Cancer Research Fund/American Institute for Cancer Research (2007) established as public health goals at least 600 g/d of F&V consumption [5].

1.2 Challenges of novel fruit and vegetable food products

Despite these recommendations, intake of F&Vs remains low in adults, but also in children [7-8]. Data from the last European Nutrition and Health Report (2009) [8] reported that only four countries (Poland, Germany, Italy, and Austria) met the recommendation of 400 g/day, while the populations of northern European countries (Denmark, Estonia, Finland, Latvia, Lithuania, Norway, and Sweden) consumed an

average of 269 g/day of F&Vs. In a dietary survey of schoolchildren in nine European countries (Austria, Belgium, Denmark, Iceland, The Netherlands, Norway, Portugal, Spain, and Sweden), none met F&V intake guidelines [7]. The traditional Mediterranean diet is characterized by an increased intake of F&Vs, but also bread, cereals, legumes, nuts and olive oil, some of which are important sources of dietary antioxidants [40]. This dietary pattern may protects against myocardial infarction, some tumours (e.g., breast, colorectal, etc.), and other diseases associated with oxidative stress [41-45]. In Spain, nevertheless, the adherence to the Mediterranean diet during the past two decades tended to decrease likely as a result of socio-economic changes [46-47].

The observed decrease in the F&V consumption of the population and its consequence on the health are contributing to the interest for the positive aspects of diet. Foods have assumed a new status, in which should be able to provide additional physiological benefits, as well as meeting basic nutritional requirements [9]. The benefit of a diet rich in F&Vs is attributed to the complex mixture of phytochemicals present in these foods [25]. Thus, the food industry is playing an increasing role in developing "healthy" F&V products [19], by designing processes that maximizes the preservation of key nutrients from raw material, such as antioxidants, and improving their bioavailability.

2. FRUITS AND VEGETABLES AS A SOURCE OF ANTIOXIDANTS

Fruit and vegetable are a rich source of potentially beneficial components including folate, vitamins, minerals, dietary fiber and bioactive phytochemicals including carotenoids and phenolic compounds responsible for their antioxidant and anticancer activities [48,25,11,49]. These compounds may act independently or in combination as anticancer or cardio-protective agents by a variety of mechanisms [9]. The benefits of theses phytochemicals in F&Vs may be even greater than is currently understood, considering that some of them possess antioxidant properties, and that the oxidative stress induced by free radicals is involved in the etiology of a wide range of chronic diseases [25].

2.1 Oxidative stress in the pathogenesis of chronic diseases

The oxidative stress has been defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage" [50]. The energetic benefit of aerobic metabolism is associated with the generation of ***reactive oxygen species (ROS)*** [51], a collective term that includes both free radicals and nonradicals (**Table 1**) [52]. A *free radical* is any species that contains one or more unpaired electrons, that is, electrons singly occupying an atomic or molecular orbital [52]. N*onradicals* are oxidizing agents and/or are easily converted into radicals. Free radicals react quickly with other compounds, trying to capture the electrons needed to gain stability. Generally these chemicals species attack the nearest stable molecules, "stealing" its electrons. When the molecule attacked loses its electron, it becomes a free radical itself, beginning a chain reaction. Once the process is started, it can cascade, initiating lipid peroxidation which results in destabilization and disintegration of the cell membranes or oxidation of other cellular components like proteins and DNA, finally resulting in the disruption of cells [53]. The oxidation caused by free radicals sets reduced abilities to combat chronic diseases such as cancer and CVD [54-55] (**Figure 1**).

Table 1. Nomenclature of reactive oxygen species (ROS)

Free radicals	*Nonradicals*
Superoxide, $O_2^{\bullet -}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Hypobromous acid, HOBr
Hydroperoxyl, $HO_2^{\bullet}$	Hypochlorous acid, HOCl
Peroxyl, $RO_2^{\bullet}$	Ozone O_3
Alkoxyl, $RO^{\bullet}$	Singlet oxygen, $^1O_2$ (or O_2)
Nitric oxide, $NO^{\bullet}$	Organic peroxides, ROOH
Nitrogen dioxide, $NO_2^{\bullet}$	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH

Adapted from Halliwell & Whiteman [52].

Some free radicals arise normally during metabolism. For example, sometimes the body's immune system's cells create them to neutralize viruses and bacteria. However environmental factors such as pollution, radiation, cigarette smoke and herbicides can also generate free radicals. Thus, free radicals on one hand can produce beneficial effects but can also induce harmful oxidation and cause serious cellular damages, if generated in excess [9].

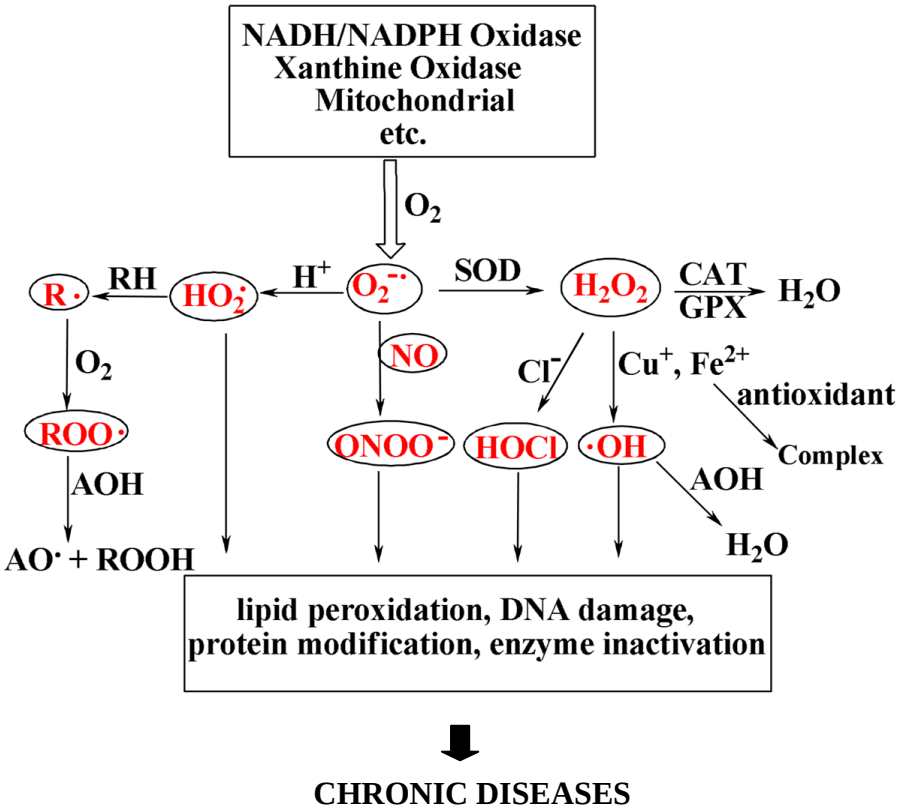


Figure 1. Summary of ROS types and sources, and action point of antioxidants (Adapted from Lü *et al.* [55]). $O_2^{-\bullet}$, superoxide anion; $HO_2^{\bullet}$, perhydroxyl radical; $^{\bullet}OH$, hydroxyl radical; H_2O_2, hydrogen peroxide; NO, nitric oxide; HOCl, hypochlorous acid; $ONOO^-$, peroxynitrite; $R^{\bullet}$, lipid alkyl radical; RH, lipid; $ROO^{\bullet}$, lipid peroxyl radical; ROOH, lipid hydroperoxide; SOD, superoxide dismutase; CAT, catalase; and GPX, glutathione peroxidase.

The detoxification of ROS is a prerequisite of aerobic life which involves a veritable ***antioxidant defence system*** [56]. Antioxidants have been defined as "any substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate" [57]. Antioxidants quench or stabilized free radicals by donating one of their own electrons, ending the electron-stealing reaction. They do not themselves become free radicals by donating electrons because they are stable in either form [9]. Moreover, antioxidants can inhibit the activity or expression of free radical generating enzymes or modifying the activity or expression of intracellular antioxidant enzymes [55]. The repertoire to counteract reactions initiated by oxygen metabolites covers all levels of protection: prevention, interception, and repair [58] and includes compounds of *enzymic* nature as well as *nonenzymic* [56].

The main *antioxidant enzymes* are the superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx or GSH peroxidase) [58] (**Figure 2**). SOD catalyzes the

dismutation of superoxide anion radicals and catalase catalyzes the reduction of hydrogen peroxide to water. GPx catalyzes the degradation of peroxides with concomitant oxidation of glutathione [59]. From a kinetic point of view, catalase and GPx are both able to destroy hydrogen peroxide, but GPx has a much higher affinity for hydrogen peroxide than does catalase, suggesting that hydrogen peroxide is degraded mainly by GPx under normal conditions [60]. Antioxidant enzymes detoxify oxidant compounds, but they also possess indirect antioxidant functions such as the replenishment of glutathione from glutathione disulfide by the flavoprotein glutathione reductase (GR or GSH reductase) [58]. Dietary non-enzymatic antioxidants (e.g., carotenoids, vitamins E and C, phenolic compounds, minerals, etc.) play a key role in the antioxidant system [9,61]; endogenous nonenzymatic antioxidants (e.g., eurate, bilirubin, etc.) are also involved in this antioxidant system (**Figure 3**) [62].

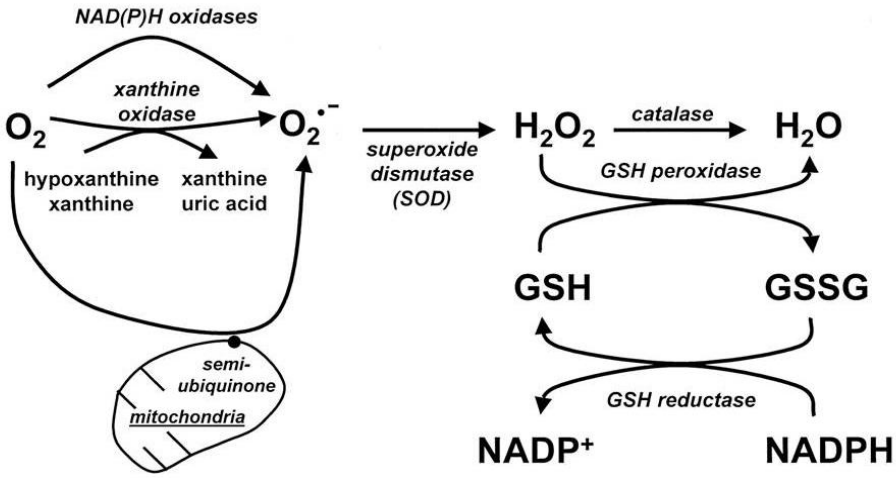


Figure 2. Pathways of reactive oxygen species (ROS) production and clearance [63]. $O_2^{\bullet-}$, superoxide anion; H_2O_2, hydrogen peroxide; GSH, glutathione; GSSG, glutathione disulfide; NADPH; Nicotinamide adenine dinucleotide phosphate.

The ***oxidative stress*** can result from: I) diminished levels of antioxidants, such as mutations or toxins that deplete antioxidant defences or deficiencies in dietary antioxidants; II) increased production of reactive species, for example by exposure to elevated levels of O_2 or to toxins that are themselves reactive species or excessive activation of immune system's cell neutralizing viruses and bacteria [52].

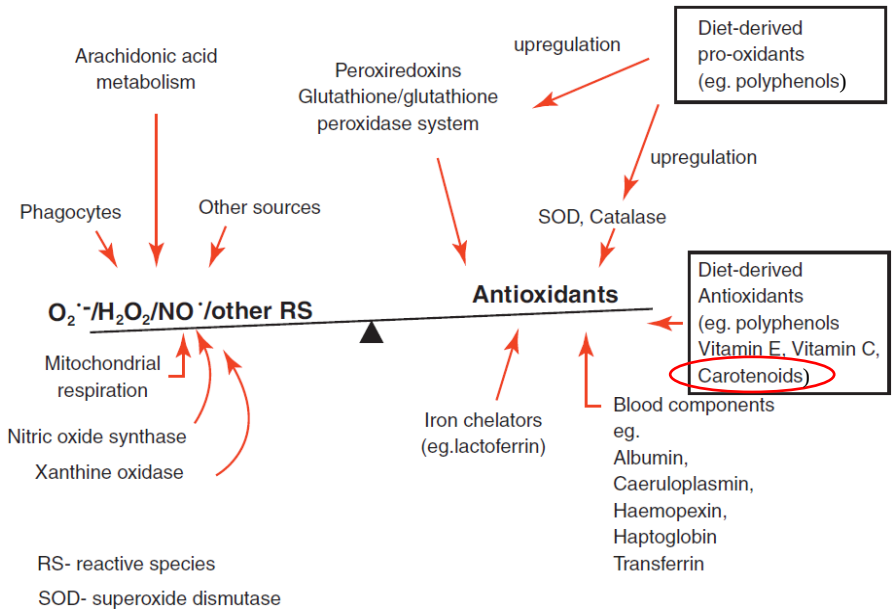


Figure 3. The approximate balance of antioxidants and reactive species *in vivo* (Adapted from Halliwell [62]). $O_2^{\bullet-}$, superoxide anion; H_2O_2, hydrogen peroxide; $NO^{\bullet}$, Nitric oxide; SOD, superoxide dismutase; RS, reactive species.

2.2 Role of antioxidants in the prevention of cardiovascular disease and cancer

Oxidative stress is believed to play a significant role in the initiation and progression of atherosclerotic ***cardiovascular disease*** [64]. The oxidation of low density lipoproteins (LDL) and other lipoproteins, creates proinflammatory lipid mediators that drive a chronic inflammatory state. This chronic inflammatory state leads to complex plaque formation, rupture, and vessel occlusion [65]. Oxidized LDL have enhanced uptake by macrophages, which leads to foam cell formation [66,64,67]. Recognition of oxidized LDL by scavenger was postulated to result from oxidative modification of apolipoprotein B100 (apoB100). Peroxidation of the polyunsaturated fatty acids (PUFAs) esterified in the phospholipids, triglycerides, and cholesterols of lipoproteins generates lipid oxidation products (e.g., hydroxy, hydroperoxy, and epoxy fatty acids; hydroxyalkenals; etc.), some of them possess biological activities that could also contribute to atherogenesis [65]. Dietary antioxidants that are incorporated in LDL are themselves oxidized when these LDL are exposed to prooxidative conditions before any extensive oxidation can occur in the lipoprotein, sterol or PUFAs [68]. For example, the consumption of food products containing moderate amounts of antioxidant nutrients (vitamin E plus carotenoids) have shown to decrease plasma concentrations of F_2-isoprostanes, a biomarker of lipid peroxidation [69]. Nevertheless, other studies supplementing with carotenoids have showed contradictory results [70-71,49], therefore

further investigation about the protective effects of dietary antioxidant against CVD would be necessary.

Other phytochemicals exert their antioxidant activity in the prevention of CVD by other mechanisms. For example, although natural folates cannot be really considered as food antioxidants, they may act as effective antioxidants *in vivo* [72]. There is a strong evidence for a relationship between inadequate folate status, high plasma homocysteine (tHcy) concentrations and the risk of CVD [73]. Plasma tHcy is associated with increased cardiovascular risk, by increased vascular oxidative burden. It has been observed that homocysteinaemia, but also the self-oxidation of tHcy, are associated with increased superoxide radicals and ROS production in the vascular endothelium. The superoxide radicals react with NO to form peroxynitrite radicals, leading to an endothelial dysfunction. In addition, the alteration of the function of intracellular antioxidant enzymes such as SOD and GPx has been observed in the homocysteinaemia [74]. Folic acid supplementation can lower plasma tHcy concentrations safely. Moreover, i*n vitro* evidence demonstrates that 5-methyltetrahydrofolate, the main circulating metabolite of folate, can increase nitric oxide production and can directly scavenge superoxide radicals [75]. Thus, the dietary supplementation with food rich in phytochemicals as folate could prevent oxidative events involved in cardiovascular disorders.

Carcinogenesis is a multistep process in which oxidative damage is linked to formation of tumors through several mechanisms [76-78]. Oxidative stresses induced by free radicals cause DNA damage, which, when left unrepaired, can lead to base mutation, single and double strand breaks, DNA cross-linking, and chromosomal breakage and rearrangement [76]. This potentially cancer-inducing oxidative damage might be prevented or limited by dietary antioxidants found in F&Vs [11]. For example, reduction of urinary 8-hydroxy deoxoguanosine (8-OHdG) concentrations, a biomarker of DNA damage, was observed in subjects supplemented with 30 mg purified lycopene/day [70]. Phytochemicals' action against diseases has been attributed to their antioxidant activity, but also to other mechanisms of action that may or may not be related to antioxidant function such as the regulation of gene expression in cell proliferation, cell differentiation, etc., [48,79,11,22] (**Table 2**). Some authors, although

not all [80-82], have observed a decreased prostate cancer risk/progression by lycopene/tomato consumption [83-89], while in postmenopausal women at high risk for breast cancer the observed effect was low [90]. Additional investigation about the effect of phytochemicals on cancer and its mechanism of action is required.

Table 2. Proposed mechanisms by which dietary phytochemicals may prevent cancer

• Antioxidant activity	-Superoxide dismutase
-Scavenge free radicals and reduce oxidative stress	• Enzyme inhibition
• Inhibition of cell proliferation	-Phase I enzyme
• Induction of cell differentiation	-Cyclooxygenase-2
• Inhibition of oncogene expression	-Inducible nitric oxide synthase
• Induction of tumor suppress gene expression	-Xanthine oxide
• Induction of cell-cycle arrest	• Enhancement of immune functions
• Induction of apoptosis	• Antiangiogenesis
• Inhibition of signal transduction pathways	• Inhibition of cell adhesion and invasion
• Enzyme induction and enhancing detoxification	• Inhibition of nitrosation and nitration
-Phase II enzyme	• Prevention of DNA binding
-Glutathione peroxidase	• Regulation of steroid hormone metabolism
-Catalase	• Regulation of estrogen metabolism
	• Antibacterial and antiviral effects

Adapted from Liu [11].

It has been suggested that the actions of the antioxidant nutrients alone do not explain the observed health benefits of diets rich in F&Vs [11]. The individual antioxidants studied in clinical trials do not appear to have consistent preventive effects [39,11]. The isolated pure compound may lose its bioactivity or may not behave similarly as the compound in the F&Vs [25]. For example, human studies have supported the ***pro-oxidant properties of β-carotene***. Supplementation of β-carotene at pharmacological levels increased lung cancer incidences in smokers in the Alpha-Tocopherol Beta-Carotene (ATBC) trial [91]. Increased mortality from CVD in a group of smokers, former smokers and asbestos exposed individuals has also been reported in the β-carotene and Retinol Efficiency Trial (CARET) [92]. These observations have been interpreted as a possible biphasic response of β-carotene, that promotes health when taken at dietary levels, but may have adverse effects when taken in higher amounts [93], at least for some subgroups of the population [39,92]. Phytochemical extracts from F&Vs have shown to have potent antioxidant and antiproliferative effects.

Thus, the combination of phytochemicals from F&Vs was proposed to be responsible of some of the healthy properties attributed to these foods [11].

3. ANTIOXIDANT PROPERTIES OF CAROTENOIDS

Carotenoids are a family of pigmented compounds that are synthesized by plants and microorganisms but not animals [93]. In developed countries, 80–90% of the carotenoid intake comes from F&V consumption. Of the more than 700 naturally occurring carotenoids identified thus far, as many as 50 are present in the human diet and can be absorbed and metabolized by the human body [16]. However, only six (β-carotene, lycopene, β-cryptoxanthin, α-carotene, lutein and zeaxanthin), representing more than 95% of total blood carotenoids, are present in the blood of people from different countries, and have been studied and associated with some health benefits (**Figure 4**). β-carotene and lycopene, together with lutein are the carotenoids that present higher relative contribution to the total amount of carotenoid intake in Europe [16,94].

Carotenoids possess a potent antioxidant activity and have been related to the prevention of carcinogenesis and atherogenesis, avoiding the oxidation of some important biomolecules such as DNA, proteins, lipids and LDLs [12-14]. The Kuopio Ischemic Heart Disease Risk Factor Study, observed that a low serum lycopene concentration was significantly associated with a higher mean and maximal intima-media thickness of the common carotid artery, which is related to *coronary events* [95]. Case-control and cohort studies have shown that serum lycopene (RR = 0.74; 95% confidence interval [CI] 0.59-0.92), lycopene intake (RR = 0.89; 95% CI 0.81-0.98) and cooked tomato intake (RR = 0.81; 95% CI 0.71-0.92) were associated with a significant decrease in prostate cancer risk [96]. Nevertheless, other case-control or supplemental studies suggested a weak or no benefit of carotenoid on the incidence of cancer and CVD [97-100]. For example, a randomized factorial trial reported no overall effects of the daily intake of β-carotene in combination with other antioxidants on cardiovascular events among women at high risk for CVD [97]. In a case-control study no associations between plasma concentrations of carotenoids and overall prostate cancer risk were observed, although an inverse association with the risk of advanced disease was reported [98]. In addition, the molecular relationship between consumption of antioxidants and the elevation of antioxidant ability is not clear [55].

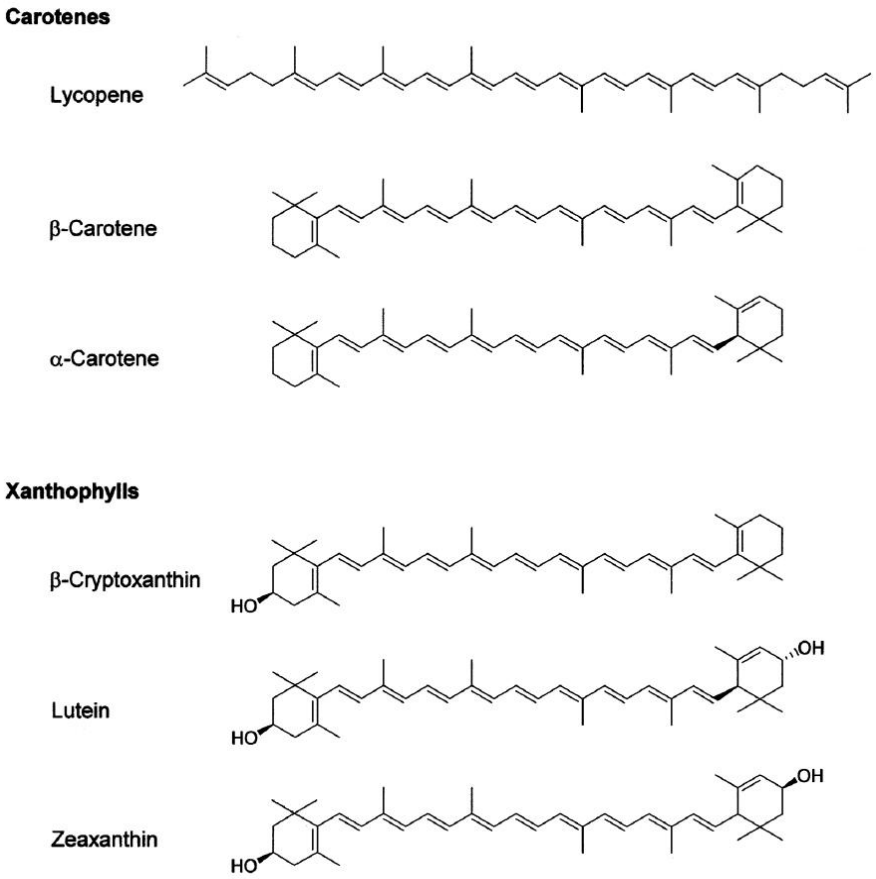


Figure 4. Structures of major carotenoids found in human plasma [101].

3.1 Carrots, tomatoes and broccoli as sources of β-carotene and lycopene

Although carotenoids are present in many common human foods, deeply pigmented F&Vs constitute the major dietary sources. Thus, yellow-orange vegetables (e.g., carrots) and red fruits (e.g., tomatoes) providing most of the β-carotene and lycopene, respectively [16,102-103]. In fact, in five European countries (France, Republic of Ireland, United Kingdom, The Netherlands and Spain) carrots and tomato/tomato-products have shown to be the major foods contributing to β-carotene and lycopene intake, respectively [16].

The *tomato*, it is one of the most versatile and widely-used food plants, being consumed both raw, and as a constituent of other products and recipes [104]. The worldwide and European production of tomato was estimated at over 153 and 23 million tonnes in 2009, respectively making it one of the most important agricultural commodities in the world [105]. The largest producers of tomatoes are China, USA,

India and Turkey [105]. *Carrot* has gained popularity in recent decades due to increased awareness of its nutritional value [106]. About 34 million tonnes of carrots and turnips were produced worldwide in 2009 and about 8 million tonnes in Europe in the same period [105]. China, Russia, and the United States are the top 3 producers of carrots globally, contributing almost 50% of the world carrot crop [106]. Broccoli it is a frequently consumed dietary source of carotenoid [107] and posses other nutrients with exceptional heath benefits such as folate [108]. Moreover, broccoli is a valuable raw material for food emerging technologies [107].

3.2 Structure of β-carotene and lycopene and antioxidant activity

The properties, and therefore functions, of a carotenoid molecule are primarily dependent upon its structure and hence its chemistry [109]. All carotenoids posses a polyisoprenoid structure, a long conjugated chain of double bond and a near bilateral symmetry around the central double bond, as common chemical features [110]. Different carotenoids are derived essentially by modifications in the base structure by cyclization of the end groups and by introduction of oxygen functions giving them their characteristic colours and antioxidant properties [93]. Carotenoid hydrocarbons are known as *carotenes* (carotenoids composed only of carbon and hydrogen atoms) [111] and contain specific end groups. Thus, β-carotene has two cyclohexene type end groups and lycopene have two acyclic end groups [112] (**Figure 4**).

The antioxidant actions of carotenoids are based on their ***singlet oxygen ($^1O_2$) quenching*** properties [109], but also their ability to trap peroxyl radicals [112,22]. Two different pathways are operative with respect to the deactivation of $^1O_2$: physical and chemical quenching [113]. *Physical quenching* implies the deactivation of $^1O_2$ by energy transfer from the excited oxygen species to the carotenoid to yield a triplet excited carotenoid. The energy of the excited carotenoid is dissipated through of rotational and vibrational interactions with the solvent to recover the ground state carotenoid. The carotenoid remains intact in this process and might undergo further cycles of deactivation [112-113]. This physical quenching is directly related to the number of conjugated double bonds of the molecule [112,109], but also it is influenced to a lesser extent by carotenoid end groups (cyclic or acyclic). Lycopene (eleven conjugated and two nonconjugated double bonds) is among the most efficient singlet

oxygen quenchers of the natural carotenoids [112]. *Chemical quenching* contributes less than 0.05% to total $^1O_2$ quenching by carotenoids but is responsible for the eventual destruction of the carotenoid molecule [113].

It has been speculated that ***scavenging of peroxyl radicals*** is a major task of carotenoids *in vivo* [112-113]. β-Carotene is a scavenger of peroxyl radicals, although this activity may be also exhibited by others carotenoids [112]. The addition of a peroxyl radical on a suitable double bond of the carotenoids was postulated as a first reaction step. This would form a resonance-stabilised carbon-centred radical intermediate, e.g., ROO-Car$^{\cdot}$. In a further reaction, another peroxyl radical could add to the species to form the neutral adduct ROO-Car-OOR. This process is thought to take place at low oxygen tension and would consume peroxyl radicals. This antioxidant activity of carotenoids may contribute to the protection of membranes from lipid peroxidation [112-113]. Carotenoids act as antioxidants by reacting more rapidly with peroxyl radicals than do unsatured acyl chains. In this process, carotenoids are destroyed [112].

At higher oxygen levels, however, a carotenoid intermediate radical might add oxygen to form a carotenoid peroxyl radical such as Car-OO$^{\cdot}$or ROOCar-OO$^{\cdot}$. Such an intermediate species could act as a ***pro-oxidant***, initiating for example the process of lipid peroxidation [112-113]. It has been observed that β-carotene at a concentration of 10 μM increased the production of ROS and the levels of cellular oxidized glutathione in leukaemia and colon adenocarcinoma cell lines [114]. Possible pro-oxidant effects of supplementation with carotenoids in some subgroups of the population, have been discussed previously.

Carotenoids may ***interact with free radicals*** in three main ways, namely electron transfer, hydrogen abstraction, and addition of a radical species (Equation 1-3, respectively, **Figure 5**). The mechanisms and rate of scavenging of free radicals by carotenoids in solution is strongly dependent upon the nature of the ROS itself. For example, β-carotene is very reactive to peroxyl radicals but less so to OH and $^1O_2$ [109].

$$ROO^{\bullet} + CAR \rightarrow ROO^{-} + CAR^{\bullet +} \quad [1]$$

$$ROO^{\bullet} + CAR \rightarrow ROOH + CAR^{\bullet} \quad [2]$$

$$ROO^{\bullet} + CAR \rightarrow (ROO - CAR)^{\bullet} \quad [3]$$

Figure 5. Carotenoids interaction with free radicals [109]. $ROO^{\bullet}$, peroxyl radical; CAR, carotenoid; ROO^{-}, peroxide anion; ROOH, hydroperoxide; $CAR^{\bullet +}$, carotenoid radical cation; $CAR^{\bullet}$, carotenoid radical; $(ROO\text{-}CAR)^{\bullet}$, carotenoid peroxyl radical.

3.3 Other considerations of structure and function of carotenoids

Because of the presence of double bonds in the structure of carotenoids, they can exist in both the ***cis and trans isomeric forms*** [93]. In general, the all-*trans* form is thermodynamically most stable and predominant in nature but several *cis* isomers of carotenoids are present in blood and tissues [111]. For example, all-*trans*, representing about 80% - 97% of total lycopene in tomatoes and related products [115-116], while human blood and tissues contain mainly *cis*-isomers [117-118]. Several authors have proposed that *cis*-lycopene isomers are more bioavailable than *trans*-lycopene [119-120]. In contrast, human studies have consistently reported a preferential accumulation of all-*trans* β-carotene in total plasma and chylomicrons, compared to its 9-*cis* isomer [121-124]. The authors suggested a selective intestinal transport of all-*trans* β-carotene *vs*. its 9-*cis* isomer. In addition, an intestinal isomerization during absorption in human has been proposed for both, lycopene and β-carotene [125-126]. Carotenoids can also undergo mono- or poly- isomerisation by light, heat treatment and chemical reactions to its *cis*-isomeric forms [93]. Nevertheless, very little is known about the biological significance of carotenoid isomerization in human health.

The lack of polar groups make carotenes virtually insoluble in water, and they often form stable crystals owing to their elongated, symmetric chain structure [110,127]. The ***crystalline form*** significantly increases the stability of carotenes; nevertheless, due to the unsaturated nature of the carotenoids, they are sensitive to degradation (and isomerisation), especially in the presence of oxygen or heat [128,93]. Other factors such as light and pH can also produce alterations [93].

Besides of its antioxidant activity, β-carotene is the most potent vitamin A precursor of all ***provitamin A carotenoids*** (α-carotene and β-cryptoxanthin). Two pathways have

been described for the cleavage of β-carotene to retinoids (vitamin A): central and eccentric [125]. Per molecule of β-carotene, central cleavage would result in the formation of two molecules of retinal that could be reduced to retinol and then esterified. Eccentric cleavage would produce one molecule of a β-apo-carotenal that could be converted not only to retinal but also to β-apocarotenoic acids and subsequently to retinoic acid [17]. Under normal physiological conditions (when antioxidant levels are adequate), central cleavage would be the predominant pathway, whereas eccentric cleavage could occur preferentially under oxidative conditions [125].

The two major sites of β-carotene conversion to vitamin A in humans are the liver and the gut with a greater extent in the former. In the human gut, it has been reported that about half the dietary provitamin A carotenoids are converted to retinol and about half are absorbed intact [125], although β-carotene conversion can be lower (≈10%) [129]. Moreover, the extent of conversion varies widely among individuals [125]. At high doses of β-carotene, it is possible to saturate the capacity of the gut to convert β-carotene to retinyl esters, and a greater proportion of absorbed material is secreted as intact β-carotene [130], thus avoiding vitamin A toxicity [113]. With respect to the influence of retinol `status' on provitamin A carotenoid absorption, vitamin A has shown to modulate the expression of certain proteins involved in the carotenoid absorption [131] (see section 4.2); nevertheless, there are no strong evidences to suggest that retinol-replete individuals absorb β-carotene or the other carotenoids less efficiently [132,113]. Vitamin A deficiency, however, seems to increase carotenoid bioavailability [22].

4. BIOAVAILABILITY AND BIOACCESSIBILITY OF CAROTENOIDS

The development of novel F&V foods aiming to contribute to achieve the health benefits of a diet rich in F&Vs should tackle new challenges. Absorption and metabolism of carotenoids, and factors affecting its bioavailability/bioaccessibility are key aspects to consider in industrial processing designed to preserve endogenous carotenoid with an increased bioavailability.

4.1 Bioavailability and bioaccessibility concepts

The observed association between high consumption of F&Vs and the decreased risk for chronic diseases depends, among other factors, on the carotenoid bioavailability

[17]. *Bioavailability* is defined as the fraction of carotenoids ingested capable of being absorbed and available for using in physiological functions or to be stored in the human body [15-16]. Bioavailability is a key concept for nutritional effectiveness, since only certain amounts of all carotenoids in food will be used effectively by the organism [15]. Another frequently used term related to the fate of carotenoids in the gastrointestinal tract is *bioaccessibility,* which is generally defined as the fraction of carotenoids that is released from its matrix to mixed micelles and thereby made available for subsequent uptake by the intestinal mucosa [133,18]. Consequently, the bioavailability term can be considered to include the concept of bioaccessibility [134]. The nutritional quality of food is currently approached, by considering the stability of key nutrients of the food, but also their bioaccessibility. Thus, the biological actions of carotenoids, their potential positive impact on human health, and the interest of the food industry in its preservation in F&V products, have promoted the attention on bioaccessibility/bioavailability of carotenoids [15].

4.2 Absorption and metabolism of carotenoids

The *in vivo* intestinal absorption of carotenoids involves several crucial steps: 1) release of carotenoids from the food matrix, 2) solubilization of carotenoids into mixed lipid micelles in the lumen, 3) cellular uptake of carotenoids by intestinal mucosal cells, 4) incorporation of carotenoids into chylomicrons and 5) secretion of carotenoids and their metabolites in chylomicrons into the lymph. This overall process may affect the bioavailability of dietary carotenoids [94].

Carotenoids are highly hydrophobic compounds, and their absorption is similar to that of other lipids. Hence, dispersion of dietary carotenoids into the digestive fluids greatly affects their bioavailability [94]. A decrease in carotenoid bioavailability may be produced by interaction in the gastrointestinal tract with drugs or food constituents. Gastric pH also plays a role, for example it has been observed that a single dose of β-carotene (120 mg) increased plasma concentrations of β-carotene at normal gastric pH to a level twice as high as that at a gastric pH of 6.4 [17]. Carotenoids released from the food matrix are dispersed in bulk lipid droplets in the gastrointestinal tract with the aids of dietary lipids, bile salts, and bile-derived phospholipids [94,135].

Then, consequent to the action of pancreatic lipases and bile salts, carotenoides are solubilised in the mixed micelles consisting of phospholipids, cholesterol, lipid digestion products (free fatty acids, monoacylglycerols, lysophospholipids) and bile salts [94,135] (**Figure 6**). The presence of protein in the small intestine helps stabilize fat emulsions and enhances micelle formation and carotenoid uptake [17]. In general, nonpolar carotenoids (carotenes) as lycopene and β-carotene are not solubilized as readily as polar carotenoids (e.g., xanthophylls) [136,131]. The solubilisation steps determine how much carotenoid becomes accessible to uptake by the intestinal cells [94].

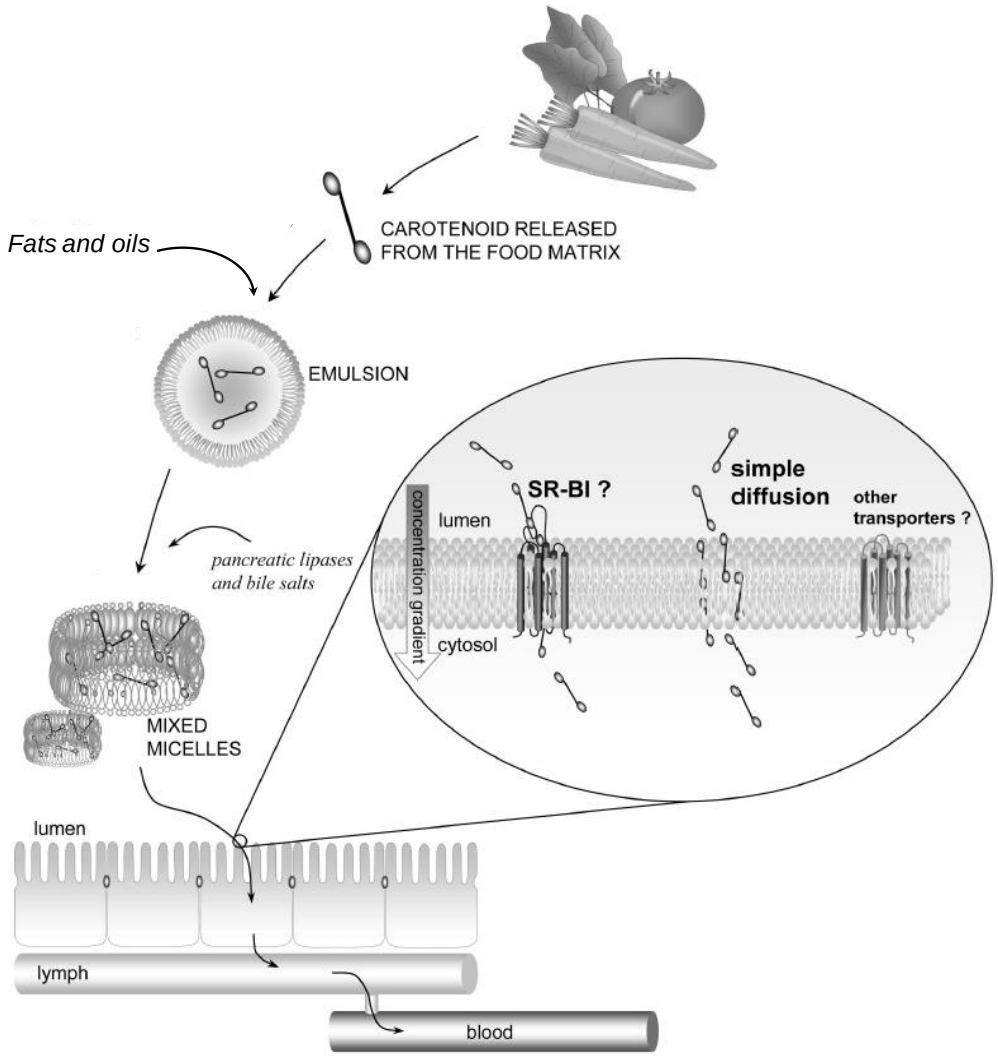


Figure 6. Scheme of dietary carotenoid absorption. SR-BI, receptor class B type I (Adapted from Yonekura *et al.* [101]).

In addition, it is possible that proteins present in the gut and originating either from diet or pancreatic/biliary secretions, can bind a fraction of carotenoids and transport them to the enterocyte [131]. Indeed, β-lactoglobulin, recovered in cow milk, is able to bind retinol and β-carotene [137-138]. It has been suggested that mixed micelles may interact with some membrane proteins of the enterocyte, while proteins may have distinct specific transport pathways. Thus, the mechanisms of absorption may depend on the vehicles with which the carotenoids are associated [131].

After solubilization in the mixed micelles, carotenoids are taken up by the duodenal mucosa. A simple diffusion across the plasma membrane of the epithelial cells has been thought to mediate the cellular uptake [125,135]. It has been reported that the lipophilicity of carotenoids and that the components of the mixed micelles remarkably affected the uptake [94]. In particular, lysophosphatidylcholine (LPC) in the mixed micelles enhanced the carotenoid uptake by human colonic carcinoma cells (Caco-2), suggesting that the physical perturbation of membrane integrity by LPC facilitates the transfer of carotenoids [139-140]. These results are consistent with the simple diffusion mechanism, because more lipophilic compounds are known to penetrate readily into the lipid bilayer of the cells by diffusion [94].

Nevertheless, it is difficult to explain intestinal absorption of individual carotenoids based on a simple diffusion mechanism alone [94]. Data using Caco-2 cells suggested that the intestinal transport of carotenoids might be facilitated by the participation of a specific epithelial transporter [141]. Borel *et al.* reported that carotenoids may be captured from mixed micelles by SR-BI, CD36, and NPC1L1, which are apical membrane transporters from the intestinal cells (**Figure 7**) [132]. The scavenger receptor class B type I (SR-BI), mediates the selective uptake of cholesterol and cholesteryl ester by the liver and other steroidogenic tissues from high density lipoproteins (HDL) particles [101]. In addition, is involved in the uptake of dietary β-carotene and other carotenoids [125,94] controlled by retinoid signaling. Retinoic acid induces the expression of the intestinal transcription factor ISX (intestine-specific homeobox), which represses the expression of SR-BI [142]. Another scavenger receptor, cluster determinant 36 (CD36 or FAT), has the ability to translocate fatty acids across the membranes and have shown to be involved in β-carotene uptake using SV40-

transformed African Green Monkey kidney cell line (COS-7) and mouse brush border membrane vesicles [143]. This involvement in carotenoid uptake by cells was confirmed in mouse 3T3-L1 adipocytes and in mouse adipose tissue cultures [144].

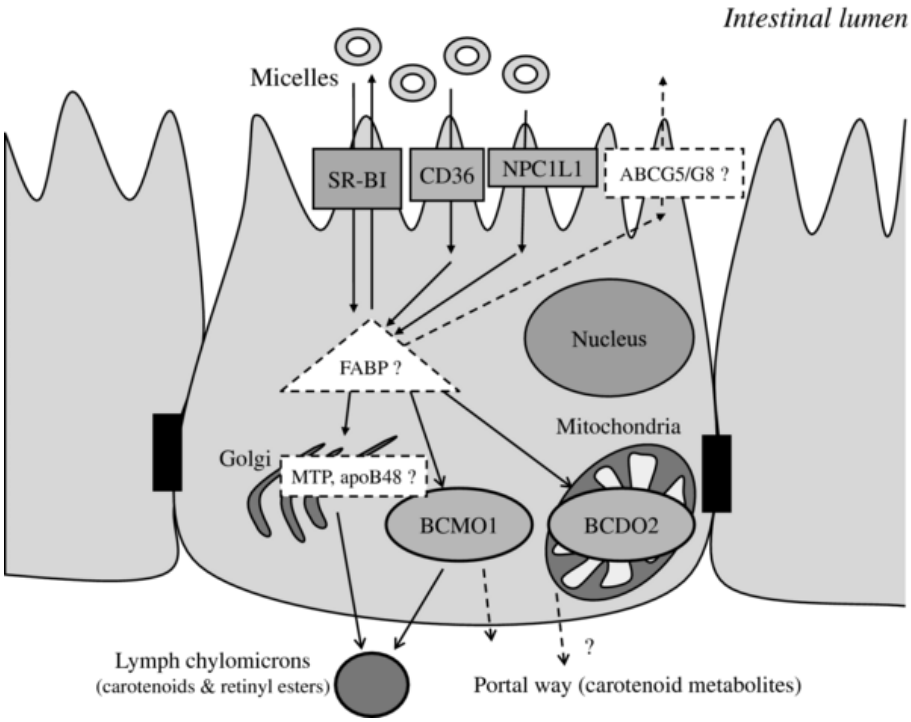


Figure 7. Proteins involved in carotenoid metabolism in the human enterocyte [132]. SR-BI, scavenger receptor class B type I; CD36, cluster determinant 36; NPC1L1, Niemann Pick C1-like 1; ABCG5**,** ATP-binding cassette subfamily G member; FABPs, fatty acid-binding proteins; MTP, microsomal triglyceride transfer protein; apoB48, apolipoprotein B-48; BCMO1, β,β-carotene-15,15'-monooxygenase; BCDO2, β,β-carotene-9',10'-oxygenase .

NPC1L1 (Niemann–Pick C1-like 1) has been described as the main cholesterol and phytosterol transporter in the gut [145]. Carotenoid uptake may be partially inhibited in Caco 2-cells by ezetimibe [146], an inhibitor of cholesterol absorption which binds specifically to NPC1L1 [147], although conflicting results have been also reported [148]. The ABCG5/G8 heterodimer is member of the ATP-binding cassette (ABC) transporter superfamily that is critical in the sterol homeostasis [132]. Besides, ABCG5 polymorphism may play a role in the plasma response to dietary carotenoids [149]. Thus, the difference of intestinal absorption and metabolism among humans and selectivity for individual carotenoids could be due to the properties of the receptors or transporters involved in lipid absorption [132,131]. In addition, protein-mediated transport has been proposed occurring at dietary concentrations while passive diffusion

taking over at pharmacological doses [131]. In summary, the solubilization process in the digestive tracts, as well as the uptake of carotenoids from the mixed micelles by the intestinal cells, determines the bioavailability of dietary carotenoids.

Once in the enterocyte, one or more proteins might be involved in intracellular transport of carotenoids, although they have not yet been identified. Good candidates are the fatty acid-binding proteins (FABPs). Provitamin A carotenoids (e.g., β-carotene) are centrally cleaved into retinal by β,β-carotene-15,15'-monooxygenase (BCMO1). Nonprovitamin A carotenoids (e.g., lycopene), are possibly transported to mitochondria, where they are eccentrically cleaved into apocarotenals by β,β-carotene-9',10'-oxygenase (BCDO2). Nonmetabolized carotenoids and retinyl esters (originated from vitamin A metabolism), are incorporated in chylomicrons. It has been suggested that proteins involved in chylomicron assembly (e.g., microsomal triglyceride transfer protein [MTP] and apoB48), could indirectly participate in carotenoid metabolism in the enterocyte [132]. Furthermore, proteins involved in the secretion of intestinal-HDL (e.g., ABCA1) [150-151], possibly are also implicated in the carotenoids presence within these lipoparticles [132]. Finally, it is hypothesized that polar carotenoid metabolites are secreted in the portal route [132].

Chylomicrons containing newly absorbed carotenoids and retinyl esters are then secreted into the lymph [125]. Enterocytes containing carotenoids not incorporated into chylomicrons that may be sloughed into the lumen during normal turnover of the mucosa [130]. Moreover, it is possible that carotenoids can be not only incorporated into the chylomicrons secreted in the first postprandial period after a meal with lipids, but also secreted after subsequent meals [152]. Both carotenoids and retinyl esters incorporated into chylomicrons, and secreted into the lymph, are released into the blood stream, where the chylomicrons are rapidly hydrolyzed by the endothelial lipoprotein lipase (**Figure 8**). The resulting chylomicron remnants containing carotenoids are rapidly taken up by the liver. The liver secretes carotenoids associated with hepatic very low density lipoprotein (VLDL) [20], but in the fasting state most plasma carotenoids are associated with LDLs and HDLs [130,20]. Postprandial chylomicrons would show a peak in blood at around 3-8h postingestion [153,113,20], and would be clear at about 12h [113].

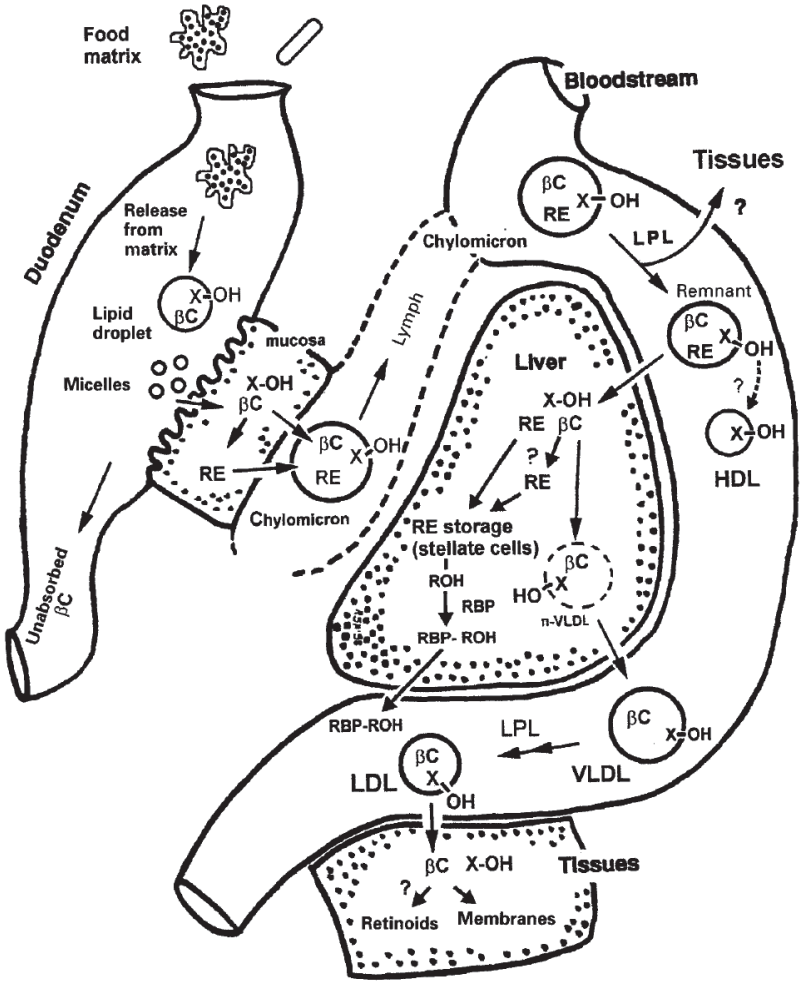


Figure 8. Scheme for uptake, metabolism and transport of carotenoids [130]. βC, β-carotene; X-OH, xanthophylls; RE, retinyl esters; RBP, retinol-binding protein; ROH, retinol; n-VLDL, nascent VLDL; LPL, lipoprotein lipase; HDL, high density lipoprotein; LDL, Low density lipoprotein.

Carotenoids are absorbed differentially by different tissues. Little is known about the mechanisms of tissue absorption of carotenoids. At steady state, plasma carotenoids amount to approximately 1% of the total body content of carotenoids, whereas the highest concentration can be found in the liver [17,93]. In addition, the observed presence of carotenoids in human bile, has suggested a secretion of carotenoids from hepatocytes and a potential excretion from intestinal epithelia into the intestinal lumen [94]. Indeed, it has been proposed that a fraction of carotenoids might be effluxed back to the intestinal lumen via apical membrane transporters (SR-BI and possibly ABCG5/G8) [132], which may be relevant to understand carotenoid metabolism.

4.3 Factors affecting carotenoid bioavailability/bioaccessibility

The bioavailability of carotenoids is influenced by both, absorption and metabolism, but also by various aspects including the food matrix, formulation and food processing techniques [17-18]. These factors may affect serum carotenoid levels and hence the antioxidant effect in humans [9]. Emerging processing operations that reduce the particle size of the food material (e.g., high pressure homogenization) or the incorporation of an oil-phase in food formulations may enhance carotenoid bioaccessibility [133,154-155].

Food matrix

Disruption of the food matrix and release of carotenoids constitute the first step in carotenoid absorption [156] (**Figure 9**). Carotenoids have to be transferred or dissolved in the lipid phase before they are absorbed [17]. Generally carotenoids are present in complexes with proteins as in green leafy vegetables or in semicrystalline structure as in carrots and tomatoes [16]. Although carotenoids are soluble in the intestinal contents, their transit time are probably insufficient for extensive solubilisation to take place during this passage through the intestinal tract [17].

The gastric hydrolysis of dietary lipids and proteins increases the release of carotenoids from the food matrix, and begins the process of solubilization of carotenoids into mixed lipid micelles in the gut lumen. The transfer of carotenoids from the predominantly aqueous environment to lipid micelles requires very close proximity between both of them, and in this process the roles of bile salts and pancreatic secretion are critical [16]. Serrano *et al*. [157], showed a significant inverse correlation between small intestine availability of carotenoids (lutein + β-carotene) and content of klason lignin and resistant protein in green leafy vegetables. These compounds may have affected the intestinal availability of carotenoids acting as a barrier to the action of digestive enzymes and to the release of carotenoids from the food matrix [157].

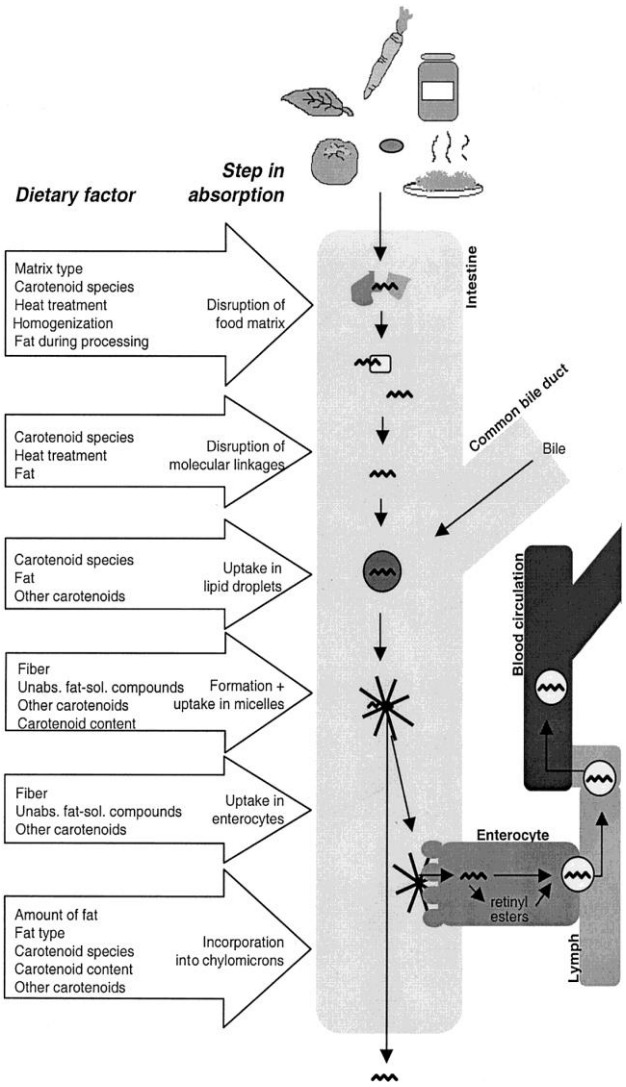


Figure 9. Steps of carotenoid absorption and dietary factors that affect carotenoid absorption [156].

Bioaccessibility of carotenoids in vegetables is low and they are characterized by a slow rate of absorption because of their chemical structure strongly interacts with macromolecules within the plant food matrix [16]. As an example, an *in vitro* digestion model system reported that only 1–3% of the β-carotene in raw carrots is accessible for absorption; and the accessibility of lycopene in canned and fresh tomatoes was <1% [158-159]. Nevertheless, there is a high variability in literature data on carotenoid bioaccessibility from raw or cooked single F&Vs [18]. Further studies indicated that more than 70% of the carotenoids remained in the final digesta [160]. Thus, the modification of matrix may enhance bioaccessibility of carotenoids in the final formulations, so that knowledge of this effect would increase nutritional value of food products [15].

Food processing

Food processing involves changes in structural integrity of the matrix, and this produces both negative (e.g., loss of carotenoids due to oxidation) and positive (e.g., increased bioavailability) effects [113]. Porrini *et al.* demonstrated that plasma total lycopene levels were higher after the intake of a commercial tomato puree that had undergone a process of heating and homogenization than after raw tomato consumption, thus demonstrating a significant effect of food processing on food matrix and on absorption [161].

The effects of *thermal processing* on stability and bioavailability of carotenoids depend mainly on the severity of the thermal treatments applied. Although it is difficult to assess a general effect of food processing [16], carotenoid content of foods is not altered to a great extent by common household cooking methods such as microwave cooking, steaming and boiling, but extreme heat can result in oxidative destruction of carotenoids [93]. *Blanching* (70–105ºC) is a mild heat treatment for short time period used to inactivate enzymes and vegetative microorganisms [16]. Although at these temperatures, may decrease the carotenoid content, most of them are stable and isomerization is negligible. Moreover, the disruption of the matrix of plant tissues and the destruction of the integrity of cell walls, membranes and carotenoid-protein complexes produced, often increase carotenoid liberation and solubilization and therefore its bioavailability [16,22]. In addition, the inactivation of oxidative enzymes prevents further losses during slow processing and storage [113].

Processing reducing the particle size of food material such as mechanical *homogenization*, has the potential to enhance the bioavailability of carotenoids from vegetables [156]. It has been observed in tomato products that homogenization increases the susceptibility of the structured suspensions to disrupt into smaller aggregates under shear [162]. Food technologies such as *high pressure homogenization (HPH)* has been used as a more intense way to disrupt plant material, as it uses pressure to force a coarse plant dispersion through a small constriction [10]. HPH may modify carotenoid bioavailability of food [155], and moreover, it has shown to decrease the viscosity of carrot and broccoli dispersions, while it increase the viscosity of tomato [163]. However, studies on the use of HPH processing on plant-based products are

scarce [162-163,10,164]. Further investigations about processing effects on specific carotenoids from particular vegetables/fruits could be applied in the development of foods with enhanced carotenoid bioavailability.

Fat

The incorporation of released carotenoids into mixed micelles is another critical step in the absorption of carotenoids that may affect their bioavailability. Among other factors, the formation of these micelles is dependent on the presence of fat in the gut. A low amount of fat may limit the solubilisation of carotenoids in the fat phase and the release and activity of esterases and lipase enzymes that hydrolyze carotenoids esters [156]. The co-ingestion of dietary fat has shown to enhance the intestinal absorption of carotenoids [125]. For example, Brown *et al.* observed a greater absorption of carotenoids when salads were consumed with full-fat than with reduced-fat salad dressing [133]. The amount of dietary fat suggested to ensure carotenoid absorption seems to be the threshold of 3–5 g per meal, although it depends on the physicochemical characteristics of the carotenoids ingested [16,156]. On the other hand, the ATP-Binding Cassette proteins ABCA1, ABCG5 and ABCG8 are downregulated by a cholesterol-free high-fat diet [165]. In addition, it has been shown that heart CD36 and hepatic SR-BI expressions are regulated by dietary fat [166-167]. Thus, the fat and cholesterol content in the diet may affect the absorption efficiency of carotenoids by modulating the expression of lipid transporters involved in their absorption [131].

Fiber

The presence of dietary fiber in vegetables and fruits may explain in part the low bioavailability of carotenoids in these foods. It is a known fact that fiber decreases the absorption of carotenoids by entrapping them [16]. In addition, fiber interferes in the micelle formation by partitioning bile salts and fat into the gel phase of dietary fiber [16,156]. This leads to an increase of faecal excretion of bile acids, fats and fat soluble substances such as carotenoids, and hence, decreasing its absorption [17,16].

Interactions between carotenoids and others factors

The inter-relationship of different carotenoids present in the food matrix also affects carotenoid absorption. It has been proposed that a high-dose intake of carotenoids may

antagonize the bioavailability and absorption of other carotenoids [16]. This competitive inhibition may occur at the level of micellar incorporation, intestinal uptake, or lymphatic transport or at one or more of the later steps [16,156,20]. Studies on simultaneous ingestion of carotenoids indicate that β-carotene [168] or the combination of β-carotene plus lycopene [169] may interfere with absorption of lutein, whereas lutein have shown to have the ability to reduce but also enhance the plasma area under the curve (AUC) for β-carotene [168]. Lipid transporters involved in the uptake of carotenoids and other fat-soluble nutrients, likely explains the competition between these molecules for apical uptake [131]. Nevertheless, Hoppe *et al.* observed no interaction of lycopene with β-carotene and others carotenoids [170]. Simultaneous ingestion of various carotenoids may induce an antioxidant-sparing effect in the intestinal tract and thus result in increased levels of uptake of the protected carotenoids [156]. Further research is required to clarify the existence and mechanisms behind these interactions.

Other nutrients apart from the dietary fat can likely modulate the expression of proteins involved in absorption of carotenoids [131]. For example, as has been stated earlier, the expression of SR-BI is modulated by both vitamin A [142], but also by vitamin E [171] [172]; besides, vitamin E downregulates CD36 [173-175]. Finally, other factors that may influence the bioavailability of carotenoids are: species of carotenoids, food storage, amount of carotenoids consumed in a meal, and host-related factors (nutritional status, genetic factors, adiposity, smoking status, ethanol consumption, plasma cholesterol concentrations, etc.) [176,17,22].

4.4 Methodology for estimating carotenoid bioavailability/bioaccessibility

Different methods can be applied to study carotenoid bioavailability from foods, including human studies and *in vitro* models. Human studies can be broadly categorized into metabolic balance techniques [129,177], isotope methods [178-180] and serum/plasma [181,49,182] or chylomicron responses [133,183]. Metabolic balance techniques are non-invasive, but oxidative degradation and excretion of endogenously produced carotenoids are not accounted in this method [20]. Studies of bioavailability of carotenoids are difficult for the endogenous presence in plasma and tissues of carotenoids. In some cases, larger doses than those provided by mixed diets need to be

supplied in order to observe variations in plasma [16]. To overcome this problem stable isotope-labelled carotenoids are being increasingly used to assess nutrient bioavailability [180]. Nevertheless, the perceived health risk and the costs associated with this methodology have limited the number of these studies [16]. Thus, human studies are labor-intensive, time-consuming, and expensive, limiting their use to a few food samples [18,22]. Consequently, the *in vitro* models are increasingly being used to estimate bioaccessibility of carotenoids [18]. However, the ability of these models to predict bioavailability in a healthy human population is not clearly established [16].

4.4.1 *In vitro* studies and animal models

Because of the limitations of the human studies above-mentioned, many studies have been performed using animal models and *in vitro* studies. Animal models may provide relevant information with regard to bioavailability in humans [184]; the main advantages of animal models for nutritional investigations include the ability to induce dietary deficiencies, administer radioisotopes and collect tissues of interest [185]. Nevertheless, no one animal model completely mimics human absorption and metabolism of carotenoids [184]. Extrapolation of these results and their relevance to humans should, therefore, be considered with caution [16].

In vitro models are simple compared with the *in vivo* situation, nevertheless, these systems are inexpensive, rapid and reproducible, allowing the screening of a large number of food samples; besides, do not have the ethical considerations associated with *in vivo* methods [15,22]. *In vitro* methods simulating gastric and small intestine digestive processes, coupled with highly differentiated cultures of Caco-2 cell, have been shown to be valid tools for the initial assessment of the relative bioavailability of carotenoids [22]. The carotenoid bioaccessibility from main dietary sources assessed using a modified *in vitro* digestion model have shown to correlate well with human derived bioaccessibility values ($r = 0.90$, $p < 0.05$), and with published relative mean bioavailability values in humans ($r = 0.98$, $p < 0.001$) [18]. In a study comparing carotenoid bioavailability from broccoli using *in vivo* and *in vitro* assessment, a significant increase in serum level of the subjects was observed for lutein, not for β-carotene. Nevertheless, these two carotenoids had similar behavior *in vitro*, ≤20% of the initial contents in broccoli being micellarized. The authors concluded that food-related

factors affecting carotenoid bioavailability might be addressed by *in vitro* methods, but not host-related factors (health and nutritional status, metabolism, intracellular regulation and homoeostatic control) [107]. Consequently, findings from *in vitro* bioaccessibility trials should be complemented with validation studies using *in vivo* procedures [15-16].

4.4.2 Serum/plasma response after carotenoid ingestion

Plasma or serum carotenoid responses may provide an estimation of relative bioavailability using simple procedures. This method relates amounts of ingested carotenoids and changes in serum carotenoid concentration that can be measured at various time intervals after ingestion [20]. Comparison of relative bioavailability can be estimated for different carotenoids, formulations (e.g., purified vs. food), food preparations (e.g., processed vs. unprocessed food), or individuals. Serum response curves are carried out using either single or multiple doses [16,20]. A rise in serum concentration followed by a fall is generally measured after acute doses. However, daily consumption produces serum carotenoid concentrations that achieve a constant elevated plateau level of various magnitudes [20]. The change from baseline plasma carotenoid concentration can be useful to estimate the relative bioavailability of carotenoids in human subjects [130].

Serum response curves to determine bioavailability are, however, limited by several factors: (a) the serum response to a single oral dose of carotenoid is highly variable; (b) the concentration of carotenoid in serum represents a balance between intestinal absorption, breakdown, tissue uptake, and release from body stores; (c) human serum contains substantial endogenous concentrations of carotenoids such as, β-carotene, lycopene, lutein, etc.; and (d) provitamin A carotenoids can be metabolized to retinyl esters during intestinal absorption. All these factors influence carotenoid serum response curves making difficult the interpretation of results [20].

To reduce the influence of human variability in trials, some authors have used crossover designs, in which subjects consume different plant foods with a washout period between feeding periods [186,49]. Nevertheless, large differences in the length of washout periods have been reported (1-10 weeks) [186,49]. In addition, some studies

did not verify that the selected washout period successfully returned serum carotenoid levels to baseline values [187]. β-carotene and lycopene concentrations have shown to return to basal values after 4-week washout in some studies [182,188], but not in all [189-190]. The half-life for β-carotene in serum has been estimated to be about 7–14 days, while for lycopene about 11–14 days or 12–33 days [190-191]. However, some studies have reported that serum lycopene decreases more rapidly than serum β-carotene [192-193]. Differences in the estimated half-life of each carotenoid may be related to differences in the chemical structure (*trans-cis* isomerization), or in the function (e.g., provitamin A and/or antioxidant activity), etc. [193]; but also related to methodological variability in the studies (length of washout period, carotenoid initial concentrations, diet followed during the washout period, etc.). Due to the many factors that influence the half-life of carotenoids in serum, crossover trials should assess if the selected washout period successfully return carotenoid concentrations to baseline values.

4.4.3 Chylomicron response after carotenoid ingestion

Carotenoid concentrations in triglyceride-rich lipoprotein (TRL) fractions (mixtures of chylomicrons and VLDLs) have also been used to estimate carotenoid absorption. Compared to the serum response curve, this method distinguishes newly absorbed carotenoids from endogenous pools [20]. Newly absorbed carotenoids are initially present in plasma chylomicrons before they are sequestered by body tissues and re-exported in, or transferred to, other lipoprotein fractions [113]. However, because of the rapid rate of chylomicron catabolism and hepatic uptake of chylomicron remnants (half-life 10-15 min) even in the postprandial state, chylomicron-associated carotenoids represent only a small proportion of total plasma carotenoids [130]. The measurement of carotenoids in this fraction, allow the calculation of the absorption based on serum AUC measurements [113].

A potential limitation of this approach is that food matrices that are slowly digested result in slow rates of carotenoid absorption and thus yields little or no rise of carotenoids in the TRL fraction. In addition, as observed with serum response curves, TRL response curves are highly variable among subjects, even when treatment conditions are highly standardized. This may be due to variability in carotenoid absorption as well as in the kinetics of chylomicron secretion and clearance [20]. Based

on plasma and lipoprotein concentrations observed after carotenoid administration, there is evidence to suggest that there are, "low and high responders" (**Figure 10**) [132,153,16]. This fact is frequently observed in single-dose kinetic studies whereas in long term studies most of the subjects show significant, though highly variable responses [153,16].

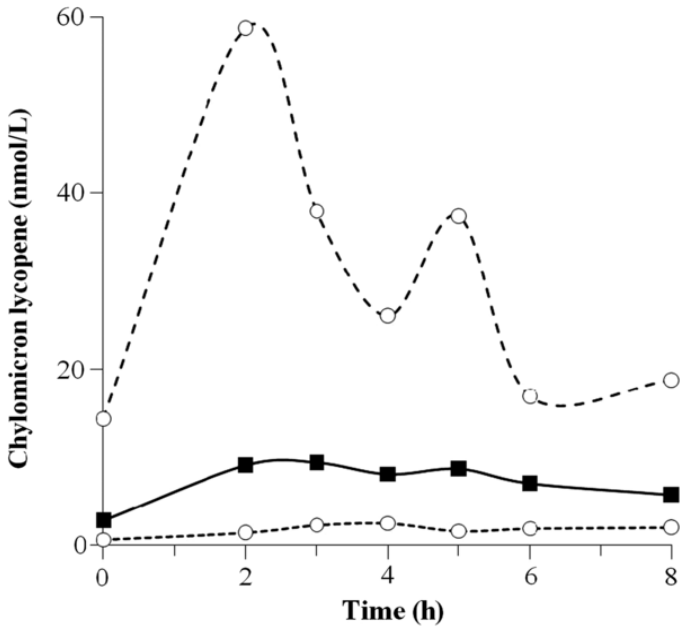


Figure 10. Interindividual variability in absorption efficiency of carotenoids [132]. Postprandial plasma chylomicron lycopene concentrations after intake of 100 g tomato puree as a source of lycopene (n = 39). Bold curve: mean plasma chylomicron lycopene concentrations of the 39 subjects. Dashed curves: curves of the lowest responder (lowest area under the curve [AUC]) and the highest responder (highest AUC). The ratio between the AUC of the highest responder and that of the lowest responder was about 20.

The inter-individual variability in carotenoid assimilation may be related to the presence of genetic variants in genes encoding proteins involved in carotenoid absorption and metabolism [132,131]. Data on associations between genetic variants in SR-BI and CD36 and blood concentrations of carotenoids may support the proposal [194-195]. When most proteins involved in the metabolism of a carotenoid are efficient (**Figure 11**), subjects uptake the carotenoid efficiently (''high responders''). When most of the proteins involved in the metabolism of a carotenoid are inefficient, subjects uptake the carotenoid very poorly (''low responders''). When some proteins are efficient and some are inefficient, the subjects uptake carotenoids with an intermediate efficiency (''medium responders'') [132]. This hypothesis explains the Gaussian

response to β-carotene described in a clinical study [152]. In addition, these receptors are expressed in several tissues; hence, the presence of genetic variants could also affect the uptake or efflux of carotenoids in other tissues [131].

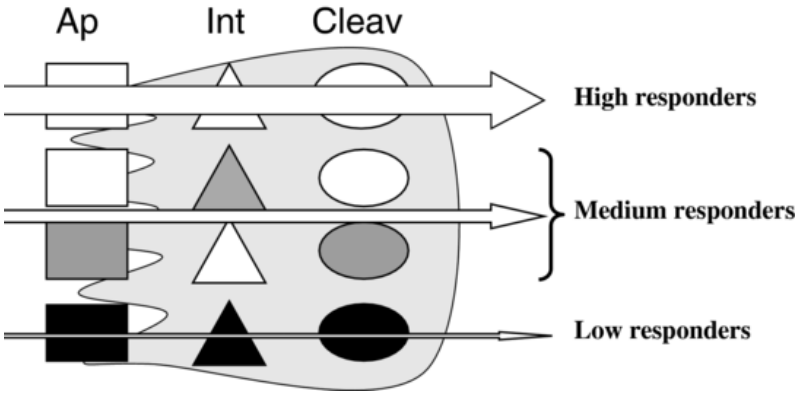


Figure 11. Interindividual variability of absorption efficiency of carotenoids as explained by genetic variation in proteins involved in transport and metabolism of carotenoids in the enterocyte [132]. Ap: apical membrane transporter of carotenoids (e.g., SR-BI). Int: intracellular transporter of carotenoids (e.g., FABP). Cleav: enzyme involved in cleavage of carotenoids (e.g., BCMO1). White symbol: protein working with maximal efficiency. Black and grey symbols: a genetic variation (e.g., an SNP) that leads to an impaired and intermediate expression/activity of the protein, respectively.

5. ASSESSMENT OF ANTIOXIDANT ACTIVITY OF CAROTENOIDS

Biomarkers of oxidative stress status are useful tools for estimating the antioxidant activity of carotenoids in dietary intervention studies in humans. Cell cultures models may also allow the prediction of antioxidant activity of carotenoids from foods, although is critical evaluating how well they correspond with *in vivo* results.

5.1 Biomarkers of oxidative stress status in humans

Biomarkers of oxidative stress status integrate the effect of exposure to oxidants coupled with the full range of antioxidant protective mechanisms *in vivo*. Such biomarkers include various measures of lipid, DNA and protein oxidation [21], and also enzymatic antioxidant defense [196].

5.1.1 Oxidative metabolites

Concerning lipid oxidative metabolites, peroxidation of various PUFAs esterified in the phospholipids, triglycerides, and cholesterols of lipoproteins generates ***lipid***

peroxidation products that possess biological activities that could contribute to atherogenesis. *F_2-isoprostanes* are considered one of the most reliable biomarker to assess oxidative stress *in vivo* [65,197]. These compounds are produced by free radical-induced peroxidation of arachidonic acid. F_2-isoprostanes are formed in phospholipids and then cleaved and released into the circulation before excretion in the urine as free isoprostanes [65] (**Figure 12**). Urine is generally considered a better matrix than serum to quantify isoprostane status [21]. The typical laboratory analyses available for F_2-isoprostanes use gas chromatography/mass spectrometry (GC/MS) or immunoassays methods.

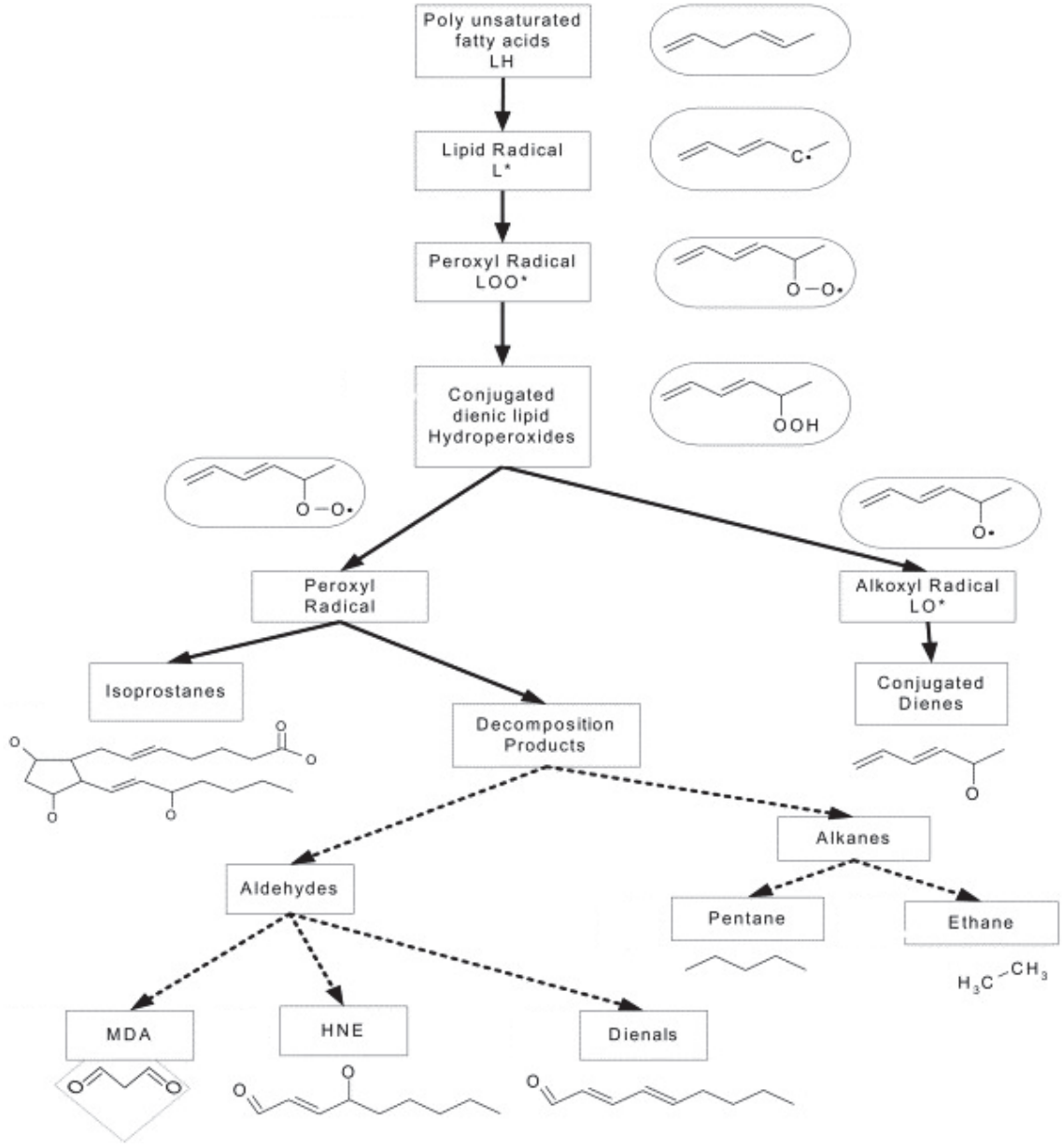


Figure 12. Products and pathways relating to lipid peroxidation [198]. MDA, Malondialdehyde; HNE, 4–hydroxynonenal.

Oxidative modification of apoB100 from LDL, could also contribute to atherogenesis [65]. The techniques used to quantify *oxidized LDL* includes measures the immunogenic response (autoantibodies) to oxidized LDL, or the uses of murine monoclonal antibodies that correspond to different epitopes on the oxidized LDL molecule [64]. In addition, the assessment of the LDL resistance to *ex vivo* induced oxidation is commonly used [21].

Unstable carbon radicals from fatty acids can rearrange to short alkanes and conjugated dienes which are exhaled or react with oxygen further to peroxyl radicals and finally by hydrogen abstraction to result in lipid *hydroperoxides* [196]. Hydroperoxides are quantified by spectrophotometric assay that measure a chromogen produced by peroxide-mediated oxidation [199]. *Thiobarbituric acid reactive substances (TBARS)* is a spectrophotometric assay that measures a chromogen that is produced by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), which is an end product of lipid peroxidation. TBARS can be measured in tissues but is generally measured in plasma. TBARS assay is easy to use, but the specificity is low [21].

Protein carbonyls is the biomarker generally used to estimate ***protein oxidation***. The conventional assay is a colorimetric procedure that involves dinitrophenylhydrazine [200], but also enzyme-linked immunosorbent assay (ELISA) method is used [201]. Nucleic acids can be oxidized to produce many ***DNA oxidative products***; oxidation of the C-8 of guanine is one of the more common oxidative events resulting *8-Hydroxy-29-deoxyguanosine (8-OHdG)* that produces predominantly G-to-T transversion mutations. Several methods for quantitating this biomarker are available (High performance liquid chromatography/electrochemical detection [HPLC/ECD], GC/MS, and ELISA) [21].

5.1.2 Antioxidant enzymes

Several enzymes with antioxidative properties maintain the redox balance after oxidative stress, such as GPx, SOD and GR. Changes in the amount or activity of these enzymes can serve as biomarkers for oxidative stress. The quantification of the enzymes can either be done by the measurement of the enzymatic activity, by immunochemical

detection of the proteins or by analyzing the expression profiles of the corresponding RNAs [196].

5.2 Evaluation of antioxidant activity in cell cultures

Biological systems such as cell cultures, may provide critical information of the bioavailability, metabolism, but also the activity of the antioxidants from plant foods [55]. The liver plays an important role in the lipid and carotenoids metabolism, hence the ***human hepatocyte carcinoma cell line (HepG2)*** has been widely used in nutritional studies [202-203]. This cell line is considered one of the experimental models that more closely resembles the human hepatocyte in culture [203]; besides, in HepG2 cells is easy to detect changes on the antioxidant status in response to the bioactive compounds of different vegetables [202]. Nevertheless, methods that stimulate liver cells or other cells directly with the tested food do not reflect the physiological situation. Thus, incubating HepG2 cells with postprandial human chylomicron serum fraction from subjects consuming tested products may result a suitable approach.

The evaluation of the antioxidant effect of carotenoids from plant foods in cell-based system**,** employs probes usually fluorescence based to detect cellular production of free radicals and other reactive species, for example: 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), Dihydrorhodamine 123 (DHR), etc., or probes of lipid peroxidation/membrane [52,204]. ***2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA)*** is the most popular of these probes, frequently being used to detect cellular peroxides. DCFH-DA enters cells and accumulates mostly in the cytosol. DCFH-DA is deacetylated by esterases to 2',7'-Dichlorodihydrofluorescein (DCFH). This nonfluorescent product is converted by RS into 2',7'-Dichlorofluorescein (DCF), which can easily be visualized by fluorescence [52]. Antioxidants such as carotenoids may prevent oxidation of DCFH and decrease the fluorescence intensity. Cell culture models can be used for initial screening and research of antioxidants prior to animal or human trials [55]. Nevertheless, research evaluating the beneficial role of antioxidant nutrients against degenerative diseases using *in vitro* methods, should be extended as well to *in vivo* studies to draw logical conclusions.

III. METHODS AND EXPERIMENTAL CONSIDERATIONS

III. METHODS AND EXPERIMENTAL CONSIDERATIONS

1. STUDY OVERVIEW

Two consumer panels, a pilot study, a large randomized control trial (large RCT), and a postprandial assay followed by stimulation of HepG2 cells with the postprandial chylomicrons were performed in this research project by our group (**Figure 13**).

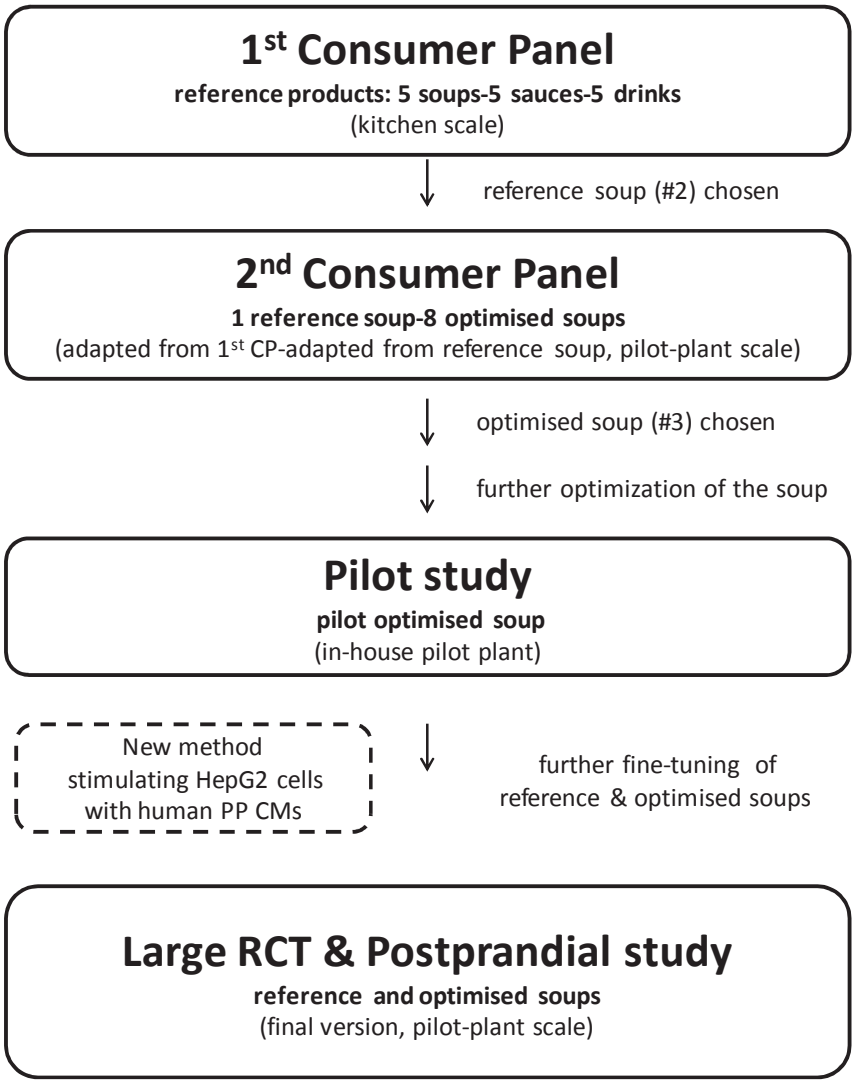


Figure 13. Study overview. CP: consumer panel; HepG2 Cells: human hepatocyte carcinoma cell line; PP: postprandial, CMs: chylomicrons; RCT: randomized control trial.

2. CONSUMER PANEL

Two consumer panel studies were performed previously to the human trials, to identify food products considered acceptable for the consumers. Details of how we performed the consumer panel is beyond the scope of this thesis. The information obtained was considered for decisions on the prototype development. All the F&V products evaluated in this research project were produced by Unilever Discover R&D.

In the ***first consumer panel***, 5 soups, 5 sauces and 5 drinks containing a mixture of carrot, tomato and broccoli, were evaluated by 100 untrained tasters of both genders aged from 20 to 69 years, distributed uniformly regarding gender and age, in Murcia (October-December 2007) (**Table 3**). The prototype products for the consumer panel varied in terms of formulation (varying proportions of carrot, tomato and broccoli; amount of added water/orange juice; presence of oil; other added ingredients related to flavour and appearance), structure (blending, non/partial-blending; amount and size of added pieces; length of cooking time) and processing steps (number, type and order). As a result of the consumer panel, a "soup" format was selected instead of a "sauce" or "drink". In fact, the soup #2 was chosen as the most liked product over all 15 types. This reference soup was selected as the basis for the preparation of optimised products.

Table 3. Details for the reference product set included in the first consumer panel

Sample	Product format		
	Soup	Sauce	Drink
Reference #1	Grainy all-in-one	Smooth all-in-one	Gazpacho A (low tomato)
Reference #2	Creamy all-in-one	All-in-one + pieces	Gazpacho B (high tomato)
Reference #3	Creamy carrot	Creamy carrot	Juice A (40% carrot)
Reference #4	Creamy tomato	Tomato + carrot	Juice B (60% carrot)
Reference #5	Tomato broth	Simple pulpy tomato	Juice C (20% carrot)

In the ***second consumer panel*** (October-December 2008, Murcia), 100 volunteers tested 8 prototypes of optimised soups and one reference soup (**Table 4**). The *reference soup* was adapted from the reference soup #2 chosen in the first consumer panel. The *8 optimised soups* were designed from the adapted reference soup, using a single formulation (20% each F&V) whilst varying some key processing and formulation parameters: olive oil used (2.5% or 5%), the use of HPH at different pressures (none, 100 bar and 600 bar), and the amount of pieces present in the product (none, 10% and 20%). The aim was to produce a range of textures, improving nutrient retention and enhancing nutrient bioavailability. The *optimised soup #3* was chosen since obtained the highest liking score in the second consumer panel, but also because it showed: a high retention of micronutrients; a good bioaccessibility of lycopene and folate; the highest bioaccessibility of β-carotene (60%); and ease of processing (without pieces).

Table 4. Details for the product set included in the second consumer panel

Samples (60% F&V)	Oil (%)	Pretreated	HPH	Pieces
Reference soup	2.5	none	none	none
Optimised #1	"	BB	"	"
Optimised #2	5	"	"	"
Optimised #3	"	"	100 bar	"
Optimised #4	"	"	600 bar	"
Optimised #5	"	"	"	10% C
Optimised #6	"	"	"	20% C
Optimised #7	"	"	none	10% B
Optimised #8	"	"	600 bar	"

F&V: fruit and vegetable; BB: broccoli blanched (added later); HPH: high pressure homogenisation; B: broccoli, C: carrot.

3. CHARACTERISTICS OF THE SOUPS SELECTED FOR THE HUMAN ASSAYS

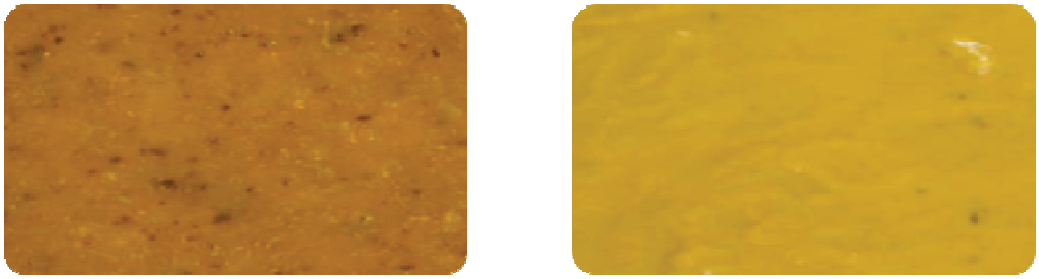
The optimised soup #3 selected in the second consumer panel, was further improved to obtain a pilot optimised soup; its nutrient composition is shown in **Table 5**.

Table 5. Nutrient content of the soups tested in the human assays

	Pilot optimised soup	Reference soup	Optimised soup
β-Carotene		*mg/300mL*	
Total amount	3.9 ± 0.08	2.9 ± 0.2	4.1 ± 0.04
In vitro bioaccessible	2.1 ± 0.09	1.66 ± 0.16	2.34 ± 0.07
Lycopene			
Total amount	4.0 ± 0.14	2.7 ± 0.2	3.9 ± 0.1
Cis-lycopene	2.04 ± 0.09	1.0 ± 0.13	0.78 ± 0.02
All-*trans*-lycopene	1.96 ± 0.12	1.7 ± 0.2	3.12 ± 0.08
In vitro bioaccessible	1.71 ± 0.11	1.31 ± 0.15	1.4 ± 0.08
Folate		*µg/300mL*	
Total amount	52.2 ± 1.2	17.9 ± 5.8	13.3 ± 3.4
In vitro bioaccessible	21.9 ± 1.7	below DL	below DL

Values are means ± SEM. DL: detection limit.

The reference and the optimised soup used in the large RCT and the postprandial study contained the same proportion of carrot, tomato and broccoli (20/20/20) than the pilot optimised soup. The main differences between the reference and the optimised soups are reported in **Table 6**. The reference and the optimised soup contained 2.5% and 5% olive oil, respectively. In the reference soup, tomatoes were pre-treated by blanching, while in the optimised product, all the fresh raw materials were blanched. Tomatoes in the reference soup were par-blanched and the skins and seeds were removed, then flesh and juice were separated and blanched; while in the optimised soup, tomatoes were blanched, blended and then, sieved to remove seeds and skin. The **Figure 14** shows the appearance of both reference and optimised soup.



Reference soup **Optimised soup**

Figure 14. Appearance of the reference and optimised soups.

The reference soup was traditionally made ('all-in-one' process), heating carrots tomatoes, and broccoli, together in one vessel with water, cooked until *al dente* and then blended with oil. The optimised soup was produced using the split-stream approach, that is, by processing each type of vegetable in a different way depending on the type of nutrient to be retained or the ease with which the nutrient becomes bio-accessible. Carrot stream was blended warm with hot oil while broccoli stream was blended with lemon juice and cold water. Tomato was already a finished stream, arriving as cool blenched tomato puree from the pre-processing. Finally, in the slurry stream, dry ingredients were blended in water and boiled. The streams were mixing and pureeing before HPH at 100 bar. The different processing conditions created two very different soups with different characteristics. The reference was pulpy, rough and heterogeneous, containing some small, slightly hard particles of carrot and broccoli, while the optimised soup was smooth, glossy and homogenous in texture.

Table 6. Differences between reference and optimised soups tested in the large RCT and in the postprandial study

	Reference soup	Optimised soup
Formulation		
Carrot/Tomato/Broccoli	20/20/20	20/20/20
Oil	2.5%	5%
Pre-processing		
Carrot & Broccoli	Non-blanched	Blanched
Tomato	Par-blanching, skin and seeds removed, juice & flesh separated and blanched.	Blanched, blended, sieving to remove skin & seeds.
Processing	"All-in-one" approach (essentially made in one pot)	Split-stream approach (4 separate streams – carrot, tomato, broccoli and slurry)
	Non-homogenised	Homogenised at 100 bar (HPH)
Appearance/texture	Grainy, slightly rough and pulpy appearance, thick but flowable, speckled with tiny bits of broccoli & herbs, particles 1-5 mm, clearly heterogeneous	Smooth, creamy, glossy appearance, slightly thinner than reference, speckled with herbs only, fairly homogeneous

HPH: high pressure homogenization.

The content of β-carotene, lycopene and folate in the soups and the *in vitro* bioaccessibility were determined using HPLC [205] and a static *in vitro* model [206], respectively by the team of Chalmers University of Technology (Sweden) (**Table 5**). The *in vitro* bioaccessibility of the carotenoids and folate was estimated by measuring the fraction of the nutrients transferred from the food matrix to the micellar phase. We indicated to the participants how preparing the soup before consumption, that is, pouring the soup in a saucepan and heat it in a stove until boiling. We confirmed that carotenoid losses after been stored (ambient temperature) and heating according to

instructions given to the participants were low [207]. A low content and *in* vitro bioaccesibility of folate was observed, especially in the soups tested in the large RCT and the postprandial study. From these findings, our studies were focused mainly in the assessment of the bioavailability and antioxidant activity of β-carotene and lycopene.

4. PILOT STUDY

Fourteen healthy young men, 24 ± 1 years old (mean ± SD), took part in the study in Murcia (Spain). The study was conducted during May–July 2009. The subjects recruited were normoweight, normolipidemic, non-smoking and non consumers of supplements or drugs. In addition, the participants consumed ≤ 4 portions/day of F&Vs. Volunteers received 300 mL/day of the *pilot optimised soup* for 4 weeks followed by a 4-week washout period. The serum carotenoid concentrations and oxidative markers levels were analyzed at baseline (days -1 and 0), after 3 and 4 weeks of soup consumption and after a 4-week washout period. The consumption of F&Vs of the participants was evaluated by a validated food frequency questionnaire referring to the last year. Anthropometrics and biochemical parameters were analyzed prior to the supplementation period. Subjects were recommended not to change their dietary habits during the study, especially as regards their consumption of a provided list of foods with high β-carotene, lycopene and folate content. To mimic more closely the dietary behaviour of consumers, soup consumption was included in the normal daily diet, and no specific time of consumption or accompanying meal was established. During the first week of the supplementation period, subjects completed a 7-day dietary record to evaluate energy and nutrient intake.

5. LARGE RANDOMIZED CONTROL TRIAL

Sixty-nine healthy volunteers, 35 men and 34 women (27 ± 1 years old), with a BMI of 22.5 ± 0.4 kg/m^2, and normolipidemic, completed the study in Murcia between October and December 2009. The subjects were randomly assigned into two groups to receive either a reference or an optimised soup (300 mL/d) over a 4-week period. The groups were matched by gender. The study was a randomised control trial in which the reference group was considered the control. The serum carotenoid concentrations and oxidative markers levels were analyzed. Blood and urine samples were collected twice before the intervention period (wk 0) (day -1, 0) and after 4 weeks of soup consumption (wk 4) (day 27, 28). The mean values of the samples collected at wk 0 and at wk 4 were

used for the calculation of the change in serum concentrations. At the beginning and the end of the study an anthropometric evaluation of the participants was performed. The impact of the supplementation with the F&V soup was evaluated by a 7-day dietary record completed before the supplementation period and 2 weeks after consuming the soups.

6. POSTPRANDIAL STUDY

Ten healthy men aged 24.8 ± 3.4 years old, with a BMI of 25.3 ± 4.4 kg/m^2, and normolipidemic were recruited. The subjects followed a diet free of carotenoids 24 h before the postprandial assay. They were divided into two groups of five subjects each: Group 1 consumed 600 mL of the reference soup and Group 2 consumed 600 mL of the optimised soup as part of their breakfast accompanied by 125 g of white bread, 20 g olive oil, and water *ad libitum*. Five hours after the breakfast, all subjects consumed a second meal without carotenoids (fish, French fried potatoes, white bread, a pear, and water). Blood samples were collected from the subjects at basal time (fasting) and every hour for 9 h (total 10 blood samples/subject) using an indwelling venous line. The chylomicrons from serum samples were isolated and the carotenoid content quantified. After a 28 day washout period, the two groups repeated the same protocol but with Group 1 consuming the optimised soup and Group 2 the reference soup. HepG2 cells were stimulated with the isolated chylomicrons and the evaluation of the oxidative status was performed treating the cells with DCFH-DA. This method was previously developed and published by our group [208].

7. STATISTICAL ANALYSES

Data were analysed by SPSS Version 15.0 for Windows (SPSS Inc., Chicago, IL). The results are reported as mean ± SEM, unless otherwise stated. In the large RCT, the sample size was chosen to have an α error of 0.05 and a β error of 0.2 for an estimated difference of 20% between groups for the mean value of β-carotene, according to the variance in HPLC serum β-carotene analyses in adult men from the pilot study [209]. The distribution of the variables was examined by Kolmogorov–Smirnov test, and those not following a normal distribution were logarithmically transformed before analysis. Differences were considered statistically significant when $P < 0.05$. The statistical tests used are specified in their corresponding section of each paper.

IV. RESULTS

IV. RESULTS

-PAPER I. Effect of the consumption of a fruit and vegetable soup with high in vitro carotenoid bioaccessibility on serum carotenoid concentrations and markers of oxidative stress in young men

Rebeca Martínez-Tomás[a], *Elvira Larqué*[a], *Daniel González-Silvera*[a], *María Sánchez-Campillo*[a], *María Isabel Burgos*[b], *Anna Wellner*[c], *Soledad Parra*[b], *Lucy Bialek*[d], *Marie Alminger*[c], *Francisca Pérez-Llamas*[a].

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Impact Factor **2.750**

Q2 Nutrition & Dietetics

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Rebeca Martínez-Tomás, *Elvira Larqué, Daniel González-Silvera, María Sánchez-Campillo, María Isabel Burgos, Anna Wellner, Soledad Parra, Lucy Bialek, Marie Alminger, Francisca Pérez-Llamas.*

European Journal of Nutrition 2012; 51: 231–239.

ABSTRACT

Aim To evaluate the effect of the daily intake of a fruit & vegetable soup with high in vitro bioaccessibility of carotenoids on β-carotene and lycopene serum concentrations.
Methods Fourteen healthy young men (24 ± 1 years) received 300 mL/day of a carrot, tomato, and broccoli soup, containing 3.9 mg β-carotene and 4 mg lycopene, for 4 weeks followed by a 4-week washout period. The serum carotenoid response and oxidative markers were analyzed after 3 and 4 weeks of soup consumption and after a 4-week washout.
Results The in vitro bioaccessibility of β-carotene and lycopene was 55 and 43%, respectively, in the soup. Serum β-carotene concentrations were significantly higher than baseline (0.33 ± 0.05 µmol/L) after 3 weeks (0.69 ± 0.06 µmol/L) and 4 weeks (0.78 ± 0.10 µmol/L) of soup consumption ($P < 0.001$). Serum lycopene was also significantly higher compared with baseline levels (0.26 ± 0.08–0.56 ± 0.04 µmol/L and 0.60 ± 0.04 µmol/L, after 3 and 4 weeks, respectively) ($P < 0.001$). Although the highest concentration of both carotenoids was found after 4 weeks, the levels were not statistically different from the levels at 3 weeks. A 4-week washout significantly decreased serum carotenoid concentrations, although only β-carotene returned to baseline. Glutathione peroxidase (GPx) increased significantly after soup supplementation compared with baseline, while superoxide dismutase was significantly lower only after 3 weeks. Glutathione reductase, lipid, protein, and DNA oxidative markers remained unchanged.
Conclusions The soup contributed to increasing the concentration of each carotenoid by more than 100% after 3 and 4 weeks of consumption, the maximum increase being observed after 4 weeks. Oxidative markers did not show any variation except for GPx. Serum lycopene half-life was longer than that of β-carotene, which may be important for studies evaluating both carotenoids.

URL: http://link.springer.com/article/10.1007%2Fs00394-011-0211-6

-PAPER II. Cell-based assay to quantify the antioxidant effect of food-derived carotenoids enriched in postprandial human chylomicrons

María Sánchez-Campillo[a], Francisca Pérez-Llamas[a], Daniel González-Silvera[a], ***Rebeca Martínez-Tomás****[a], M. Isabel Burgos[b], Anna Wellner[c], Francisco Avilés[b], Soledad Parra[b], Lucy Bialek[d], Marie Alminger[c], Elvira Larqué[a].*

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Journal of Agricultural and Food Chemistry 2010; 58, 10864–10868.

Impact Factor **2.823**

Q1 Food Science & Technology

-PAPER II. Cell-based assay to quantify the antioxidant effect of food-derived carotenoids enriched in postprandial human chylomicrons

María Sánchez-Campillo, Francisca Pérez-Llamas, Daniel González-Silvera, ***Rebeca Martínez-Tomás****, M. Isabel Burgos, Anna Wellner, Francisco Avilés, Soledad Parra, Lucy Bialek, Marie Alminger, Elvira Larqué.*

Journal of Agricultural and Food Chemistry 2010; 58, 10864–10868.

ABSTRACT

We developed a new method to evaluate the antioxidant effect of food products in a biological system. The antioxidant status of HepG2 cells was quantified after incubation with postprandial human chylomicrons after the intake of vegetable products. Three subjects consumed in a meal a vegetable soup containing 8.4 mg of β-carotene and 9 mg of lycopene. After 5 h, the subjects consumed a second meal without carotenoids. Blood samples were collected at basal time and every hour for 9 h. Chylomicrons were isolated from serum samples and used for both carotenoid quantification and HepG2 stimulation. Carotenoid in chylomicrons followed an inter-individual and bimodal carotenoid response. We demonstrated the antioxidant effect of postprandial chylomicrons in HepG2 at the time of maximum carotenoid concentration of chylomicrons with respect to basal time. This cell-based assay seems to be a useful method to evaluate the antioxidant effect of fruit and vegetable products in a biological system.

URL: http://pubs.acs.org/doi/abs/10.1021/jf102627g

-PAPER III. Daily intake of fruit and vegetable soups processed in different ways increases human serum β-carotene and lycopene concentrations and reduces levels of several oxidative stress markers in healthy subject

Rebeca Martínez-Tomás[a], *Francisca Pérez-Llamas*[a], *María Sánchez-Campillo*[a], *Daniel González-Silvera*[a], *Ana I. Cascales*[a], *Manuel García-Fernández*[a], *José Á. López-Jiménez*[a], *Salvador Zamora Navarro*[a], *María I. Burgos*[b], *Fernando López-Azorín*[b], *Anna Wellner*[c], *Francisco Avilés Plaza*[b], *Lucy Bialek*[d], *Marie Alminger*[c], *Elvira Larqué*[a]

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Impact Factor **3.655**

Q1 Nutrition & Dietetics

Q1 Food Science & Technology

-PAPER III. Daily intake of fruit and vegetable soups processed in different ways increases human serum β-carotene and lycopene concentrations and reduces levels of several oxidative stress markers in healthy subject

Rebeca Martínez-Tomás, *Francisca Pérez-Llamas , María Sánchez-Campillo, Daniel González-Silvera, Ana I. Cascales, Manuel García-Fernández, José Á. López-Jiménez, Salvador Zamora Navarro, María I. Burgos, Fernando López-Azorín, Anna Wellner, Francisco Avilés Plaza, Lucy Bialek, Marie Alminger, Elvira Larqué.*

Food Chemistry 134 (2012) 127–133.

ABSTRACT

The effect of daily intakes of two differently processed fruit and vegetable soups on β-carotene and lycopene bioavailability, oxidative stress and cardiovascular risk biomarkers was investigated. An optimised soup produced using heat treatments and high pressure homogenisation for high nutrient retention, and a traditionally produced reference soup were tested. Serum β-carotene concentration was significantly higher with the optimised than with the reference soup after the supplementation (0.41 ± 0.05 vs. 0.24 ± 0.03 μM, respectively), whereas the serum lycopene concentration was higher in subjects consuming the reference (0.06 ± 0.02 vs. 0.16 ± 0.02 μM). The change in serum homocysteine levels tended to be greater in the optimised group (−1.67 ± 0.63 vs. 0.02 ± 0.17 μM, $p = 0.06$). Serum antioxidant enzyme activity decreased significantly with consumption of both soups, but to a greater extent with the optimised soup. The consumption of the fruit and vegetable soups increased serum β-carotene and lycopene concentrations and reduced the levels of several oxidative stress makers, particularly in subjects consuming the optimised soup.

URL: http://www.sciencedirect.com/science/article/pii/S030881461200266X

-PAPER IV. Changes in the carotenoid concentration in human postprandial chylomicron and antioxidant effect in HepG2 caused by differently processed fruit and vegetable soups

*María Sánchez-Campillo[a], Elvira Larqué[a], Daniel González-Silvera[a], **Rebeca Martínez-Tomás**[a], Manuel García-Fernández[a], Francisco Avilés[b], Anna Wellner[c], Lucy Bialek[d], Soledad Parra[b], Marie Alminger[c], Salvador Zamora[a], Francisca Pérez-Llamas[a].*

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Impact Factor **3.655**

Q1 Nutrition & Dietetics

Q1 Food Science & Technology

-PAPER IV. Changes in the carotenoid concentration in human postprandial chylomicron and antioxidant effect in HepG2 caused by differently processed fruit and vegetable soups

María Sánchez-Campillo, Elvira Larqué, Daniel González-Silvera, ***Rebeca Martínez-Tomás****, Manuel García-Fernández, Francisco Avilés, Anna Wellner, Lucy Bialek, Soledad Parra, Marie Alminger, Salvador Zamora, Francisca Pérez-Llamas.*

Food Chemistry 133 (2012) 38–44.

ABSTRACT

Ten subjects consumed one serving of an optimised or a reference soup produced using modified or traditional processing methods, respectively. Both soups contained the same proportions of carrot, tomato and broccoli, but with 5% olive oil in the optimised soup and 2.5% in the reference soup. The β-carotene content in 600 mL of the optimised/reference soups was 4.10/2.90 mg, and the lycopene content was 3.90/2.71 mg. The β-carotene and lycopene concentrations in chylomicrons isolated from blood serum samples were similar for both groups. Only 50% of subjects could be considered as carotenoid responders and, in agreement with in vitro accessibility data, the β-carotene concentration in the chylomicrons of these subjects was significantly higher in the group consuming the optimised soup, while no changes were found for lycopene. Postprandial chylomicrons from the optimised soup group exhibited significantly higher antioxidant activity in HepG2 cells than the other group. The stimulation of HepG2 cells by human postprandial chylomicrons seems useful for evaluating the antioxidant effect of different food matrices.

URL: http://www.sciencedirect.com/science/article/pii/S0308814611018498

V. GENERAL DISCUSSION

V. GENERAL DISCUSSION

1. PILOT STUDY

The bioavailability of carotenoids is influenced by the food matrix, formulation and food processing techniques [17-18]. These factors may affect serum carotenoid levels and hence the antioxidant effect in humans [9]. We performed a pilot study in 14 young men to evaluate the effect of the daily intake of the pilot optimised soup, with high *in vitro* bioaccessibility of carotenoids, on β-carotene and lycopene serum concentrations and levels of markers of oxidative stress in young men. The data obtained were used for the calculation of sample size in a subsequent large RCT. We also evaluated whether a 4-week washout period was sufficient time to allow carotenoid levels to return to their baseline values for future crossover studies with similar food products.

The *in vitro* carotenoid bioaccessibility of the pilot optimised soup containing carrot, broccoli, and tomato was 55% for β-carotene and 43% for lycopene, which was markedly higher than the levels reported in other studies (0.1–20%) using raw or cooked single F&Vs [18,210]. Granado-Lorencio reported high levels (about 80%) of β-carotene and lycopene bioaccessibility in a tomato paste, and approximately 75% β-carotene bioaccessibility in carrots, both of which are higher than those obtained in our study, but only about 17% β-carotene bioaccessibility was found for broccoli [211].

The consumption of this soup enhanced significantly serum levels of both carotenoids by 114% at 3 weeks, while after 4 weeks of supplementation, this increase was 141% for β-carotene and 132% for lycopene. Although the highest concentration of both carotenoids was found after 4 weeks, the levels were not statistically different from the levels at 3 weeks. Dragsted *et al.* [212] observed that daily dietary intake of 600 g of F&Vs for similar time periods (16 or 24 days) significantly increased both serum β-carotene and lycopene concentrations compared with the responses observed in a control group, with higher values observed on day 24 than on day 16 (about 50 and 60% change with respect to the basal values for β-carotene and lycopene, respectively, on day 16, and about 65 and 80% on day 24). In our pilot study, we obtained a higher increment in serum β-carotene and lycopene concentrations, suggesting that the supplementation with the F&V soup may contribute to enhance the serum levels of some of the compounds associated with the health benefits of 600 g/day F&Vs proposed

as a public health goal. In other studies, 3 weeks of supplementation with 9.3 mg of β-carotene from spinach products increased serum concentrations by 57% [59], while 8 mg lycopene consumption from tomato products increased serum lycopene by about 70% [213]. Although the carotenoid content of the soups in the present study was lower (4 mg of each carotenoid) than in the previously reported studies, we obtained higher serum levels of both carotenoids, which seems to be in agreement with the estimated high bioaccessibility of carotenoids in this food matrix.

In this pilot study, the serum carotenoid levels reached with the soup consumption, significantly decreased after a 4-week washout period, confirming that the carotenoid levels recorded during the study were mainly the result of soup consumption. The β-carotene concentrations returned to basal values after 4 weeks in our experiment which agrees with the observations made by Rock *et al.* [188]. In a crossover study, plasma β-carotene remained significantly higher than baseline after the subsequent period of placebo treatment for 26 days; however, these authors used a high dose of β-carotene (15 mg/day) provided in capsules [189]. The half-life for β-carotene in serum has been estimated to be about 7–14 days [190-191], thus, a washout period of 4 weeks seems quite reasonable.

The 4-week washout returned serum β-carotene concentrations to basal values but not serum lycopene levels. Differences in the half-life in serum of the two carotenoids (11–14 or 12–33 days for lycopene) [190-191], their chemical structure or function [193] may explain our findings. Nevertheless, other factors such as the diet followed during the washout could be involved [193,190]. Porrini *et al.* reported that a 4-week washout period was sufficient for the serum lycopene concentrations to return to basal values after 4 weeks of 6 mg lycopene supplementation [182]. Nevertheless, as reported in another study, a 4-week washout period tended to increase serum lycopene compared with the values recorded in a 6-week intervention period of tomato juice consumption (12 mg lycopene) plus a controlled diet; the findings were explained by the fact that the self-selected diet contained more lycopene than the controlled diet [190]. The normal diet of the participants in our study contained almost the same amount of lycopene as provided by our soup, while the β-carotene content of the diet was low (0.7 mg/day). These findings also suggest that in populations consuming a diet containing higher

levels of lycopene than other carotenoids, such as β-carotene [214], it is more difficult to control lycopene intake during the washout period in dietary intervention studies, underlining the need for longer washout period during crossover studies considering human lycopene responses.

We evaluated the effect of the soup consumption on the levels of markers of oxidative stress in the subjects, although the number of participants was low (n=14). Thus, we prefer to discuss the effect the soup on the antioxidant status of subjects subsequently in the large RCT.

In summary, we observed in the pilot study that the addition of a F&V food product with enhanced carotenoid bioaccessibility to the diet may contribute to enhanced levels of bioavailable β-carotene and lycopene close to those achieved from a diet containing 600 g/day of F&Vs. Both 3 and 4 weeks of soup consumption resulted in increases in serum carotenoid levels of more than 100%, the maximum increase being observed at 4 weeks. A washout period of 4 weeks was sufficient to return serum β-carotene to basal values, but the serum lycopene concentrations still remained above the basal values after this period.

2. CAROTENOID BIOAVAILABILITY IN THE LARGE RCT

From data from the pilot study, a large RCT including 69 participants (35 men/34 women) was designed. The number of subjects considered for an estimated difference on serum carotenoids of 20% between groups at α error = 0.05 and β error = 0.2, was not too high, and hence a crossover design was not necessary. In the large RCT, two differently processed F&V soups (reference and optimised soups), with different β-carotene and lycopene bioavailability were assayed.

The optimised soup was produced using heat and mechanical treatments aimed at high nutrient retention, followed by HPH, while the reference soup was made using traditional methods without HPH. The fact that the β-carotene and lycopene content of the optimised soup was higher (about 40%) than that of the reference may have been due to the ‘‘split-stream’’ process used. Industrial processes such as homogenisation have been reported to improve β-carotene and lycopene availability, probably due to the

enhanced release of carotenoids from the food matrix [161,155]. Using tomatoes processed in different ways (not, mildly or thoroughly homogenised), it has been observed that mechanical homogenisation of the food matrix enhances both β-carotene and lycopene bioavailability in humans [155]. Microstructural studies [10] demonstrated that intact cells and cell clusters were still present in emulsions of thermally and mechanically processed tomato and carrot, although a complete disruption of the cells could be obtained by subjecting the emulsions to HPH under certain conditions. Nevertheless, recently, an inverse relationship between the homogenisation processing pressure of tomato pulp and *in vitro* lycopene bioaccessibility has been reported [215]. These authors hypothesised that the homogenisation process improved the strength of the fibre network and entrapped the lycopene in the tomato pulp, making it less accessible for digestive enzymes and bile salts. Similar observations were made by other authors, who reported no increases in the *in vitro* bioaccessibility of tomato emulsions containing different carotene/oil ratios after HPH-treatments [216].

In our study, after the consumption of the optimised soup, it was observed a significantly higher serum β-carotene concentration compared with the reference soup. The change of serum β-carotene was significantly higher in the group consuming the optimised soup compared with the reference soup (139% *vs.* 67%, respectively). However, the change of serum lycopene in the optimised group was moderate, and was significantly lower than that observed for the reference soup (24% *vs*. 54%, respectively). Thus, our results with the optimised soup showed that the joint effect of a higher β-carotene content, split-stream approach and an HPH treatment had a positive effect on β-carotene bioavailability, although a similar effect was not observed for lycopene.

We observed an enhanced bioavailability of β-carotene in the presence of the higher amount of fat (5% *vs*. 2.5%) in the optimised soup. In contrast, β-carotene absorption was reported to be enhanced after adding 75 and 150 g avocado to salad (containing 12 and 24 g fat, respectively), but the effect was similar for both low and high fat doses [217]. Lycopene has been shown to be better absorbed from a salad containing 28 g canola oil than 6 g canola oil [133]. Nevertheless, previous studies with diets rich in

lycopene, in which the fat intake consumption was tested at two feasible extremes (16% and 36% of total dietary energy), showed that fat did not appear to influence the serum lycopene levels [218-219]. In our large RCT study, although both the total amount of lycopene and added amount of lipids were higher in the optimised soup, we observed a higher lycopene bioavailability in the reference soup. *In vitro* studies have suggested that the bioaccessibility of lycopene compared with β-carotene is limited by a combination of low solubility in dietary lipids and the low efficiency of micellar incorporation of the solubilised lycopene [119,140]. This fact seems to have influenced the differences observed between serum lycopene and β-carotene responses in the optimised and the reference products. The inter-relationship of different carotenoids present in the food matrix also affects carotenoid absorption [16]. For example, lutein has been shown to have the ability to reduce but also enhance the plasma AUC for β-carotene [168]; thus, it is possible that the presence of other carotenoids may have influenced the β-carotene and lycopene increase observed in serum. However, the content of other carotenoids in our soups was not determined.

Our results *in vivo* in the large RCT for β-carotene, fitted with those obtained by *in vitro* digestion of both soups. The *in vitro* bioaccessibility of β-carotene was higher in the optimised soup than in the reference soup (2.34 ± 0.07 *vs.* 1.66 ± 0.16 mg/300 mL, respectively). Nevertheless, the *in vitro* bioaccessibility of lycopene was similar in both soups (1.4 ± 0.08 *vs.* 1.31 ± 0.15 mg/300 mL), which contrast with the higher lycopene bioavailability from the reference soup observed in serum.

In summary, after the consumption of the optimised soup, produced by processing aiming to maximize the retention and bioavailability of nutrients, it was observed a significantly higher serum β-carotene concentration compared with the reference soup, which was traditionally made. Nevertheless, serum lycopene concentration was higher with the reference soup. These results fitted with β-carotene bioaccessibility data obtained by *in vitro* digestion of the soups, but not with lycopene bioaccessibility.

3. CAROTENOID BIOAVAILABILITY IN THE POSTPRANDIAL ASSAY

The human postprandial carotenoid response after a single intake of the reference and the optimised soup was also studied in a postprandial assay carried out in 10 healthy

men. Comparison of the AUC for total β-carotene and lycopene after intake of the optimised and the reference soup did not show any significant difference between soups considering data from all the participants. Nevertheless, only 50% of subjects could be considered as carotenoid responders. Calculations based just on "responder" subjects (n=5), showed that the AUC for β-carotene was significantly higher after the intake of the optimised compared with the reference soup (AUC = 0.36 ± 0.04 μmol/L * h *vs.* 0.10 ± 0.05 μmol/L * h, respectively, P = 0.007), although no difference was found for lycopene.

The higher oil content in the optimised soup did not enhance the incorporation of lycopene in postprandial chylomicrons, which agree with findings from our large RCT. Furthermore, due to the relatively large contribution of fat in the breakfast meals (20 g of olive oil) in both the reference and optimised soups, and considering the differences in total fat content in the whole meal (22.5 *vs.* 25 mL), factors other than the oil content, might have influenced lycopene bioavailability. *Cis*-lycopene is more bioavailable than all-*trans*-lycopene, due to its higher solubility in bile acid micelles [220]. The absolute content of *cis*-lycopene in the reference and optimised soups was similar (2.0 ± 0.26 and 1.56 ± 0.04 mg/600 mL, reactively), which may explain the lack of difference in lycopene concentrations in human chylomicrons observed in this study. The β-carotene concentration was significantly higher in postprandial chylomicrons from subjects consuming the optimised soup compared with the reference soup. This result agrees with those from the large RCT. Thus, the processing used in the optimised soup showed a positive effect of on β-carotene bioavailability, also when a single meal was provided.

The carotenoid concentration in postprandial chylomicrons showed a bimodal response with two peaks; for the optimised soup at 3 h and at 6 h, and for the reference soup at 4 and 7 h. It seems that carotenoid absorption from the optimised soup was faster than the corresponding uptake from the reference soup, which might have been due to the higher amount of olive oil in the optimised soup.

In the present study, a wide inter-individual variation in the carotenoid response was observed, as reported by other authors [152-153], which probably reflects differences in human absorption, carotenoid clearance rates for the carotenoids in the chylomicrons

and genetic polymorphisms [132,195,129]. To identify carotenoid responders and non-responders among the participants and thus avoid misinterpretation of the results, we followed the ‘‘exclusion criteria’’ reported by Johnson and Russell (1992) [153]. Accordingly, only 50% of the subjects in our study were seen to be carotenoid responders, which agreed with the 40–60% of subjects estimated in some studies as non-responders to dietary carotenoids [152-153].

The *in vitro* bioaccesibility data, agree with the *in vivo* results for chylomicrons from “responder” subjects, whereby subjects consuming the optimised *vs.* reference soup showed significantly higher β-carotene concentrations, while lycopene concentration showed no differences between groups. Reboul *et al.* found a good correlation between an *in vitro* bioaccessibility model and serum bioavailability in healthy humans [18]. Nevertheless, Granado-Lorencio *et al.* reported that the behaviour of carotenoids under *in vitro* gastrointestinal conditions did not fully explain the changes observed in 14 subjects *in vivo* [211]. It was suggested that *in vitro* bioaccessibility methodology may not be sufficient to predict the *in vivo* bioavailability of carotenoids, but our results from the postprandial study confirmed the *in vitro* situation in a reasonable way.

In summary, the β-carotene concentration in postprandial chylomicrons was significantly higher in subjects consuming the optimised soup compared with those consuming the reference soup, while for lycopene no differences were found. Thus, a positive effect of the processing used in the optimised soup was observed on β-carotene bioavailability when a single meal was provided, which agree with findings from the large RCT.

4. EFFECT OF THE SOUP CONSUMPTION ON CARDIOVASCULAR RISK AND OXIDATIVE STRESS MARKERS IN SERUM

In the large RCT, the daily consumption of both F&V soups for 4 weeks decreased serum LDL and increased serum HDL levels. In a previous study [219], the combination of olive oil and lycopene, added to a basic diet of healthy individuals, was reported to significantly decrease the ratio of total cholesterol to HDL, through the increase of HDL-cholesterol, compared with a high-carbohydrate, low fat diet. We also observed a significantly lower intake of meat during the intervention period (-0.551 ±

0.124 serving/d and -0.257 ± 0.118 serving/d change in groups consuming optimised and reference soups, respectively). The reduced intake of meat during the intervention period with the F&V soups might also have contributed to the improved lipid profile of the subjects.

Despite the folate dietary intake was significantly higher in the optimised than in the reference group before the intervention period, no differences in serum folate baseline concentrations were observed between groups. The serum folate concentration did not change during the intervention period, which was to be expected, given the low content in the soups. However, an inverse correlation was found between serum folate concentrations and serum tHcy, which agrees with other studies [221]. The consumption of the optimised soup significantly decreased serum tHcy levels, but this result could have been affected by the high baseline tHcy concentration observed in the optimised group. Nevertheless, the change in serum tHcy levels tended to be higher in the optimised group, even adjusting for baseline values (P = 0.06). The homozygous TT genotype of the C677T MTHFR polymorphism is associated with increased tHcy levels, and subjects with this genotype are more susceptible to a reduction in tHcy concentrations as a result of folate supplementation [222]. However, in the present study no genotyping of the subjects was made to control this effect.

The oxidative status of the participants depends on both the level of oxidised metabolites and the activity of antioxidant enzymes. Several studies have found a significant but not consistent antioxidant effect of carotenoid supplementation [223,79,49]. In our large RCT, both soups decreased significantly the levels of serum oxidised LDL. Nevertheless, the changes in the oxidised LDL concentration between baseline and 4 weeks of supplementation were not significantly different between groups. The change in urinary isoprostanes, which are considered one of the best lipid oxidation biomarkers [197], pointed to a lower lipid oxidation in the optimised group, although the other lipid oxidative biomarkers (hydroperoxides and TBARS) were unaffected. The change of 8-OHdG and carbonyl groups was not significant between soups, which was not unexpected, since these measurements evaluate oxidation in water soluble compounds (DNA and proteins) and the antioxidant effect of carotenoids should be mainly related to lipid-soluble compounds as isoprostanes.

The activity of all the enzymes analysed (GPx, GR and SOD) clearly decreased after the consumption of both types of soup, especially after the intake of the optimised soup. We previously observed in the pilot study that 4 weeks of pilot optimised soup consumption decreased and increased the activity of SOD and GPx enzymes, respectively, while GR remained unchanged. The low number of participants (n = 14) was thought to be related to the variability observed; however, other authors also reported no alteration [223] or increased [59,212] activity of these enzymes after supplementing the diet with carotenoid-rich food.

The effect of carotenoid supplementation decreasing the activity of antioxidant enzymes has also been described in the leukocyte SOD [224]. These authors proposed that supplemental β-carotene may act as a direct scavenger of ROS, decreasing the body's need for certain antioxidant enzymes. Moreover, some of the observed effects may be related to the presence of other carotenoids, such as lutein, zeaxanthin, etc., or phenolic compounds in the olive oil, which also possesses antioxidant properties [16,225]. Subjects under oxidative stress, e.g., during prolonged/high intensity physical activity, show a higher activity of antioxidant enzymes, such as SOD or GPx, which compensate for the excess of radical oxygen species [226]. In our study, the observed decrease in activity of all the antioxidant enzymes analysed suggests a lower oxidative status in subjects consuming the soups.

In summary, the oxidative status of the subjects was modulated mainly through a reduction in the activity of antioxidant enzymes after the consumption of both, the reference and optimised soups; a slight lower lipid oxidation was also observed. The effect was higher in subjects consuming the optimised soup, suggesting that food processing used may play a large role in enhancing the preservation and bioavailability of bioactive phytochemicals.

5. ANTIOXIDANT EFFECT OF POSTPRANDIAL CHYLOMICRONS IN HEPG2

We developed a method to study the antioxidant effect of vegetable food products, stimulating HepG2 cells with human postprandial chylomicrons produced after the intake of the selected food products. In previous *in vitro* studies evaluating the

antioxidant activity of bioactive compounds from vegetable products, the HepG2 cells were incubated directly with the food [202,204]; nevertheless this does not reflect the physiological situation because liver cells are not in direct contact with vegetable products but with chylomicrons.

During the development of the method, we demonstrated the antioxidant effect of postprandial chylomicrons in HepG2 at the time of maximum carotenoid concentration of chylomicrons with respect to basal time (at 3 h). These results indicated that the highest antioxidant effect in the cells seems to be related to the carotenoid content instead of the triglyceride content. The protective effect of carotenoids is ascribed to their ability to act as antioxidants, thereby inhibiting the negative effects of ROS [14] in the form of oxidative damage [49]. This result supports the concept that dietary carotenoids scavenge ROS directly [227]. In contrast, at 7 h, when the concentration of triglycerides was highest, we found an increase in fluorescence intensity and, hence, ROS in the cells. A possible explanation is that the increase in oxidative stress is produced by the postprandial hyperlipidemia [227] with chylomicrons poor in carotenoids. Thus, the proposed cell-based assay, stimulating HepG2 cells with human postprandial chylomicrons, seems to be a useful method to evaluate the antioxidant potential of F&V products.

HepG2 cells incubated with human chylomicrons obtained after the consumption of the optimised produced significantly higher antioxidant effect (lower fluorescence) *vs.* reference soup in the cells (AUC = 897.73 ± 25.53 *vs.* 979.83 ± 25.6% of fluorescence/basal time ∗ h, respectively). Considering the effect of the chylomicrons from carotenoid responders alone in HepG2 cells, the AUC was quite similar to those obtained with all the subjects (893.14 ± 41.54 *vs.* 979.47 ± 47.03% of fluorescence/basal time ∗ h, optimised *vs.* reference, respectively). Nevertheless, the antioxidant effect was not significant (P = 0.07), which could be due to the lower number of subjects in the carotenoid responder group (5 subjects), which could reduce the statistical power. As we discussed before in the large RCT, the antioxidant effect of the soup observed in HepG2 cells, may be related to the carotenoids content, but also to the presence of other compounds with antioxidant properties. For example, hydroxytyrosol from olive oil has been found to have a beneficial effect on the

antioxidant defence system in HepG2 [228]. Thus, other compounds from the food formulation could have contributed to the healthy effect of the optimised soup.

In summary, postprandial chylomicrons from the optimised soup group exhibited significantly higher antioxidant activity in HepG2 cells than those from the reference group. These findings agreed with those from the large RCT, confirming the usefulness of the technique developed to evaluate the antioxidant effect of different F&V food products.

VI. CONCLUSIONS

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1. The consumer panel studies, performed by 200 untrained tasters of both genders aged from 20 to 69 years, contributed to the design of an optimised fruit and vegetable soup, containing 20% carrot, 20% tomato and 20% broccoli that shows good acceptance by the consumers. The daily consumption of the optimised soup increased the fruit and vegetable intake of the subjects by 180 g/d.

2. The raw material from the optimised soup was processed in different conditions and homogenised at high pressure in order to increase the retention and bioavailability of nutrients. These conditions created a functional food with an increased β-carotene and lycopene content of 40% compared with a reference soup containing the same proportion of carrot, tomato and broccoli, but with a lower olive oil content and processed in a traditional way.

3. Processing increased the β-carotene bioavailability of the optimised soup compared with the reference soup: in serum after a four week RCT, and in chylomicrons after the consumption of a single meal. These results agree with the higher β-carotene bioaccessibility of the optimised soup obtained by *in vitro* digestion. In both a pilot study and the large RCT, β-carotene levels in serum increased by more than 100% compared with basal values after consumption of the optimised soup.

4. The bioavailability and bioaccesibility of lycopene were not enhanced in the optimised soup. In fact, the concentration of lycopene in serum was seen to be significantly higher after consumption of the reference soup, whereas the concentration of this carotenoid in chylomicrons and the *in vitro* bioaccessibility data showed no differences between soups. Large RCTs may identify differences in lycopene bioavailability in foods that are not observed by other methods, such as postprandial studies and *in vitro* bioaccesibility assays.

5. In bioavailability studies in humans using a crossover design, it is necessary to specify the length of time between trials or washout period to ensure the studied compounds return to basal values. A 4-week washout period is sufficient for β-carotene bioavailability studies, while for lycopene, a longer period would be necessary.

6. In order to assess the antioxidant potential of the functional food studied, a new combined method was developed, partly in humans and partly in cell cultures, in which human hepatocarcinoma cell cultures (HepG2) were stimulated by postprandial chylomicrons obtained after the consumption of the optimised or reference soup. This method was seen to be useful for evaluating and differentiating the antioxidant capacity of different plant foods.

7. Consumption of the optimised soup had a beneficial effect on the oxidative status of the subjects. A reduction in the activity of antioxidant enzymes (GPx, GR, and SOD) and a slight decrease in lipid oxidation in serum compared with the reference soup were observed. These findings agree with those from the postprandial study, where chylomicrons in the optimised soup group exhibited significantly higher antioxidant activity in HepG2 cells than those from the reference group.

GENERAL CONSIDERATION

The consumption of fruit and vegetable products processed in a way that maximizes the retention and bioavailability of key nutrients such as carotenoids may help consumers to achieve the health benefits associated with a diet rich in fruit and vegetables. Further research, however, is necessary to improve our knowledge of the mechanism whereby processing modifies the bioavailability of specific carotenoids such as lycopene from different fruit and vegetable matrices; such knowledge would increase the nutritional and functional value of this type of food products.

VII. RESUMEN EN CASTELLANO/SUMMARY IN SPANISH

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El consumo de una dieta rica en frutas y verduras (F&V) se ha asociado con un menor riesgo de padecer enfermedades crónicas como las enfermedades cardiovasculares y algunos tipos de cáncer. Se estima que el bajo consumo de F&V en la Unión Europea causa anualmente más de 1 millón de muertes. Diversas organizaciones nacionales e internacionales abogan por un incremento de la ingesta de estos alimentos, sin embargo, su consumo en los países europeos es inferior a las recomendaciones (400-600 g diarios). El descenso de la ingesta de F&V observado en la población y sus consecuencias en la salud están contribuyendo a aumentar el interés por los aspectos positivos de la dieta. Los alimentos han asumido un nuevo estatus en el que, además de cumplir los requerimientos básicos nutricionales, deben tener beneficios adicionales para la salud, como por ejemplo prevenir o retrasar la aparición de enfermedades crónicas. Este hecho, junto con un cambio en el estilo de vida de los consumidores, que demandan productos más saludables y naturales, están influyendo el modo en el que la industria alimentaria elabora sus productos.

Algunos de los beneficios para la salud atribuidos a F&V se han relacionado con la combinación de fitoquímicos presentes en estos alimentos. Los carotenoides son fitoquímicos con actividad antioxidante que se han relacionado con la prevención de carcinogénesis y aterogénesis, impidiendo la oxidación de algunas biomoléculas importantes (DNA, proteínas, lípidos y LDLs). Sin embargo, las acciones biológicas de los carotenoides y sus posibles beneficios para la salud, se encuentran limitados por su biodisponibilidad en los alimentos. La biodisponibilidad es la fracción de un nutriente ingerido que es absorbido en el intestino y alcanza la circulación sistémica. En los carotenoides, se encuentra influenciada por el proceso de absorción y metabolismo de los mismos, pero también por la matriz, la formulación y las técnicas de procesado de los alimentos. Estos factores pueden afectar a los niveles séricos de carotenoides y, por tanto, a su actividad antioxidante en humanos. La industria alimentaria está desempeñando un papel importante en el desarrollo de nuevos alimentos vegetales con propiedades saludables, mediante el diseño de procesados que maximicen la retención de nutrientes claves de la materia prima (ej. carotenoides) con una biodisponibilidad incrementada. Todo ello, con el objetivo de contribuir a alcanzar los beneficios de una dieta rica en F&V.

Las técnicas de procesado de alimentos diseñadas para mejorar la biodisponibilidad de nutrientes, deben ser testadas en estudios en humanos. La determinación de la concentración de carotenoides en suero y quilomicrones humanos tras la ingesta de alimentos ricos en estos nutrientes, es un procedimiento sencillo que permite estimar la biodisponibilidad relativa de carotenoides. Además, los marcadores del estado de estrés oxidativo son comúnmente usados para estimar la actividad antioxidante de los carotenoides en estudios de intervención dietaria. Sin embargo, los estudios en humanos presentan limitaciones éticas y técnicas, suelen ser costosos, y permiten analizar un limitado número de muestras. Los modelos *in vitro* han sido desarrollados como una alternativa más simple, económica y reproducible que los estudios en humanos. Sin embargo, los modelos *in vitro* diseñados para determinar los posibles beneficios para la salud de los antioxidantes, deberían ser apoyados con estudios en humanos.

La presente tesis se enmarca en el proyecto europeo "Healthy Structuring" (VI Programa Marco). Dicho proyecto tiene como objetivo, desarrollar nuevos alimentos a partir de F&V, con una estructura y propiedades nutricionales mejoradas mediante la optimización de todos los pasos implicados en la manufactura, desde el cultivo de la materia prima hasta las condiciones de procesado. En este contexto, el objetivo de la presente tesis fue evaluar el efecto de sopas vegetales elaboradas mediante diferentes condiciones de procesado, sobre la biodisponibilidad de β-caroteno y licopeno, así como en el estado oxidativo en humanos. Para ello se realizaron dos paneles de evaluación sensorial; un estudio piloto; un estudio de intervención dietaria a largo plazo y un estudio postprandial. Además los quilomicrones obtenidos en el estudio postprandial se utilizaron para la estimulación de células HepG2 y evaluación del efecto antioxidante en cultivos celulares.

En el primer panel de evaluación sensorial, realizado por 100 catadores no entrenados de ambos sexos y de 20 a 69 años, se analizaron 5 sopas, 5 salsas y 5 bebidas, todas ellas elaboradas con zanahoria, tomate, y brócoli. El formato más aceptado por los participantes fue la sopa. Además, una de las sopas (sopa #2) fue el producto mejor valorado de los 15 evaluados y se seleccionó como alimento de referencia a partir del cual se elaboraron diferentes prototipos de una sopa optimizada. En un segundo panel, 100 voluntarios evaluaron 8 prototipos de sopa optimizada y 1

sopa de referencia. La sopa optimizada con mayor aceptación en el panel (sopa optimizada #8) y que además presentó una mayor retención y bioaccesibilidad de β-caroteno y licopeno, fue seleccionada y producida para la realización de un estudio piloto (sopa optimizada piloto).

En el estudio de intervención a largo plazo y en el ensayo postprandial se estudió una sopa de referencia y una optimizada, elaboradas con la misma proporción de zanahoria, tomate y brócoli (20/20/20), pero con distinto contenido de aceite de oliva (2.5 y 5% en la sopa referencia y optimizada, respectivamente) y procesado. La sopa de referencia fue elaborada mediante técnicas de procesado convencional, sometiendo toda la materia prima de forma conjunta a un tratamiento térmico. En la sopa optimizada, la zanahoria, el tomate y el brócoli fueron pre-procesados de forma distinta en función del tipo de nutriente cuya retención y biodisponibilidad se quería incrementar. Posteriormente, toda la materia prima fue mezclada, triturada y sometida a homogeneización a alta presión (HPH). Los dos tipos de procesado dieron lugar a dos productos con apariencia y textura diferentes. La sopa de referencia presentó un aspecto heterogéneo con partículas pequeñas, mientras que la sopa optimizada mostró una textura más suave y homogénea.

Se realizó un estudio piloto en el que se estudió el efecto del consumo diario de la sopa optimizada piloto, con una alta bioaccesibilidad *in vitro* de carotenoides, sobre la concentración sérica de β-caroteno y licopeno, y los niveles de marcadores de estrés oxidativo en varones jóvenes. Los datos obtenidos de los niveles séricos de ambos carotenoides serían utilizados para el cálculo del tamaño muestral de un estudio de intervención a largo plazo, realizado posteriormente. Además, se estudió si un periodo de lavado de 4 semanas es suficiente para que los valores de β-caroteno y licopeno alcanzados tras la suplementación con la sopa, retornaran a los niveles basales. Esta información sería de utilidad para futuros estudios *crossover* con este tipo de alimentos. Para ello, 14 varones jóvenes consumieron 300 mL/día de la sopa optimizada piloto durante 4 semanas, seguido de un periodo de lavado de 4 semanas. La bioaccesibilidad *in vitro* de β-caroteno y licopeno en la sopa fue 55 y 43%, respectivamente. Se analizaron las concentraciones séricas de β-caroteno y licopeno basales, tras 3 y 4 semanas de suplementación y tras 4 semanas de lavado. También se analizaron los niveles de marcadores de estrés oxidativo, antes y después de la suplementación.

La concentración sérica de β-caroteno alcanzada tras la suplementación fue significativamente mayor que los valores basales (0.33 ± 0.05 μmol/L) tras 3 (0.69 ± 0.06 μmol/L) y 4 semanas (0.78 ± 0.10 μmol/L) del consumo de la sopa ($P < 0.001$). La concentración de licopeno en suero fue también significativamente mayor que los valores basales (0.26 ± 0.08–0.56 ± 0.04 μmol/L y 0.60 ± 0.04 μmol/L, tras 3 y 4 semanas, respectivamente) ($P < 0.001$). La mayor concentración de ambos carotenoides se observó tras 4 semanas de suplementación, sin embargo los niveles no fueron significativamente diferentes de los encontrados a las 3 semanas. El periodo de lavado de 4 semanas disminuyó la concentración sérica de ambos carotenoides aunque sólo el β-caroteno retornó a los valores basales. Tras la suplementación, la actividad de la enzima glutation peroxidasa aumentó significativamente con respecto a los valores basales, mientras que la actividad superóxido dismutasa disminuyó significativamente sólo a las 3 semanas. Así, la concentración en suero de β-caroteno y licopeno aumentó más de un 100% tras 3 y 4 semanas de consumo de la sopa optimizada piloto. Los marcadores de oxidación no mostraron variaciones claras, excepto en el caso de la glutation peroxidasa. El periodo de lavado de 4 semanas fue suficiente para devolver los niveles de β-caroteno a las concentraciones basales, pero no en el caso del licopeno, lo que podría ser importante en estudios que evalúen la biodisponibilidad de ambos carotenoides.

A partir de la información obtenida en el estudio piloto, se diseñó un estudio de intervención a largo plazo en individuos sanos (34 mujeres y 35 varones). El objetivo fue comparar el efecto de la ingesta diaria de dos sopas procesadas de forma diferente (sopa de referencia y sopa optimizada) sobre la biodisponibilidad de β-caroteno y licopeno, y marcadores del estado de estrés oxidativo y riesgo cardiovascular. La concentración sérica de β-caroteno tras la suplementación fue significativamente mayor en los sujetos que consumieron la sopa optimizada que en aquellos que consumieron la de referencia (0.41 ± 0.05 *vs.* 0.24 ± 0.03 μM, respectivamente), mientras que la concentraciones sérica de licopeno fueron mayores en el grupo de referencia (0.06 ± 0.02 *vs.* 0.16 ± 0.02 μM). Así, la sopa optimizada, mostró una mayor biodisponibilidad de β-caroteno, mientras que la biodisponibilidad de licopeno fue mayor en la sopa de referencia.

Por otro lado, se investigó en un estudio postprandial, el efecto del consumo de una única dosis de la sopa de referencia y optimizada sobre la concentración de β-caroteno y licopeno en quilomicrones humanos. Diez sujetos varones participaron en el estudio, en el que consumieron la sopa de referencia y optimizada separados por un periodo de lavado de 28 días. Las concentraciones de β-caroteno y licopeno en los quilomicrones aislados de las muestras de sangre fueron similares para ambos grupos. Sin embargo, solo el 50% de los sujetos se consideró respondedor a carotenoides. Los cálculos basados en los respondedores mostraron que, la concentración de β-caroteno fue significativamente mayor en los quilomicrones del grupo que consumió la sopa optimizada (área bajo la curva (ABC): 0.36 ± 0.04 μmol/L * h) en comparación con el grupo de referencia (ABC: 0.10 ± 0.05 μmol/L * h, $P = 0.007$); mientras que en el caso del licopeno, no se observaron diferencias significativas. Así, el consumo de una única dosis de sopa reveló una mayor biodisponibilidad de β-caroteno en la sopa optimizada, resultados que concuerdan con los obtenidos en el estudio de intervención a largo plazo.

El efecto del consumo de las sopas sobre el estado oxidativo de los sujetos fue evaluado en el estudio de intervención a largo plazo. La actividad de las enzimas antioxidantes disminuyó significativamente con el consumo de ambas sopas, siendo el descenso más pronunciado con la sopa optimizada. Además, el cambio en los niveles séricos de homocisteína tendió a ser mayor con la sopa optimizada (-1.67 ± 0.63 *vs.* 0.02 ± 0.17 μM, $P = 0.06$). Así, el estado antioxidante de los sujetos fue modulado fundamentalmente mediante la reducción de la actividad de las enzimas antioxidantes, siendo el efecto mayor en los sujetos que consumieron la sopa optimizada.

En otro estudio, se desarrolló un nuevo método para evaluar el efecto antioxidante de alimentos vegetales, en el que se estimularon células HepG2 con quilomicrones postprandiales humanos obtenidos tras la ingesta del alimento en estudio. Tres sujetos en ayunas consumieron 600 mL de la sopa optimizada piloto y tras 5 horas se les proporcionó una comida que no contenía carotenoides. Se tomaron muestras de sangre basales (en ayunas) y cada hora, durante 9 horas (10 muestras/sujeto en total). Los quilomicrones se aislaron de las muestras de suero y se usó una fracción de quilomicrones para la cuantificación de los carotenoides, y otra para la estimulación de las células HepG2. Demostramos el efecto antioxidante de los quilomicrones

postprandiales en las células HepG2 cuando la concentración de carotenoides en quilomicrones con respecto a los valores basales fue máxima (a las 3 h). Así, el método desarrollado puede ser de utilidad para evaluar del efecto antioxidante de alimentos vegetales en un sistema biológico.

Utilizando ésta técnica, en el estudio postprandial se observó que los quilomicrones de los sujetos del grupo que consumió la sopa optimizada presentaron una actividad antioxidante significativamente mayor (menor fluorescencia) en células HepG2 que los del grupo de la sopa de referencia (ABC [todos los sujetos] = 897.7 ± 25.5 *vs.* 979.8 ± 25.6% of fluorescencia/tiempo basal ∗ h, respectivamente). Estos resultados están en concordancia con lo observado en el estudio de intervención a largo plazo. Así, la estimulación de células HepG2 con quilomicrones postprandiales humanos mostró ser una técnica de utilidad para evaluar el efecto antioxidante de alimentos vegetales elaborados con diferente procesados y/o con distintas matrices.

1. CONCLUSIONES

Del trabajo desarrollado en esta tesis se obtuvieron las siguientes conclusiones:

1. Los estudios de evaluación sensorial, en los que han participado 200 catadores no entrenados de ambos sexos y de 20 a 69 años, han permitido diseñar una sopa vegetal optimizada, elaborada con 20% de zanahoria, 20% de tomate, y 20% de brócoli, que presenta una alta palatabilidad y gran aceptación por parte del consumidor. El consumo diario de esta sopa optimizada ha supuesto un incremento en la ingesta de verduras de 180 g/día en los sujetos.

2. Las materias primas de la sopa optimizada han sido procesadas bajo diferentes condiciones y homogeneizadas a alta presión, con el objetivo de incrementar la retención y biodisponibilidad de nutrientes. Estas condiciones han permitido obtener un alimento funcional con contenidos de β-caroteno y licopeno aumentados en un 40% con respecto a una sopa de referencia, elaborada con la misma proporción de vegetales pero con menor cantidad de aceite de oliva y procesada de modo convencional.

3. El procesado de la sopa optimizada ha aumentado de forma efectiva la biodisponibilidad del β-caroteno con respecto a la sopa de referencia, tanto en suero en estudios de intervención a largo plazo (4 semanas), como en quilomicrones tras el consumo de una única dosis. Estos resultados concuerdan además, con la mayor bioaccesibilidad del β-caroteno en la sopa optimizada, obtenida mediante digestión *in vitro*. El aumento de β-caroteno en suero tras el consumo de la sopa optimizada fue superior al 100% con respecto a los valores basales, tanto en el estudio piloto como en el estudio de intervención a largo plazo.

4. La biodisponibilidad y la bioaccesibilidad del licopeno no han mejorado en la sopa optimizada. De hecho, la concentración de licopeno en suero ha sido significativamente mayor con la sopa de referencia, mientras que la concentración de este carotenoide en quilomicrones y la bioaccesibilidad *in vitro* no han mostrado diferencias entre ambas sopas. Los estudios de intervención a largo plazo en humanos pueden mostrar diferencias en la biodisponibilidad del licopeno de distintos alimentos, no observadas con otros métodos tales como el estudio postprandial y la bioaccesibilidad *in vitro*.

5. En estudios de biodisponibilidad en humanos, con diseños cruzados y aleatorios, es necesario precisar la duración entre ensayos o periodo de lavado, para asegurar así el retorno a los valores basales del compuesto en estudio. Un periodo de lavado de 4 semanas es suficiente para estudios de biodisponibilidad del β-caroteno, mientras que en el caso del licopeno sería necesario un periodo de mayor duración.

6. Con el fin de valorar el potencial antioxidante del citado alimento funcional, se ha desarrollado además, un nuevo método combinado, parte en humanos y parte en cultivos celulares, consistente en la obtención de quilomicrones postprandiales tras el consumo de las sopas, optimizada o de referencia, y la aplicación de éstos en cultivos de células de hepatocarcinoma humano (HepG2). Dicho método ha mostrado su utilidad para valorar y diferenciar la capacidad antioxidante de distintos alimentos vegetales.

7. El consumo de la sopa optimizada ha tenido un efecto beneficioso en el estado oxidativo de los sujetos. Se ha observado una mayor reducción en la actividad de las enzimas antioxidantes (GPx, GR, y SOD) y una ligera disminución de la oxidación lipídica en suero respecto a la sopa de referencia. Estos resultados concuerdan con los del estudio postprandial, donde los quilomicrones del grupo que consumió la sopa optimizada han mostrado una actividad antioxidante en células HepG2 significativamente mayor a la observada con los quilomicrones del grupo de referencia.

CONSIDERACIÓN GENERAL

El consumo de alimentos vegetales elaborados mediante procesados que maximizan la retención y biodisponibilidad de nutrientes clave como los carotenoides, puede contribuir a alcanzar los efectos beneficiosos asociados a una dieta rica en frutas y verduras. Es necesario, sin embargo, continuar investigando en los mecanismos por los cuales el procesado puede modificar la biodisponibilidad de carotenoides específicos como el licopeno, presentes en diferentes matrices vegetales; lo que podría incrementar el valor nutricional y funcional de este tipo de alimentos.

VIII. LITERATURE CITED

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IX. ANNEX

IX. ANNEX

1. SCIENTIFIC PRODUCTION RESULTING FROM THE PRESENT THESIS

1.1 Publications

1.1.1 Articles

-1. **Rebeca Martínez-Tomás**, Francisca Pérez-Llamas, María Sánchez-Campillo, Daniel González-Silvera, Ana I. Cascales, Manuel García-Fernández, José Á. López-Jiménez, Salvador Zamora Navarro, María I. Burgos, Fernando López-Azorín, Anna Wellner, Francisco Avilés Plaza, Lucy Bialek, Marie Alminger, Elvira Larqué. *Daily intake of fruit and vegetable soups processed in different ways increases human serum β-carotene and lycopene concentrations and reduces levels of several oxidative stress markers in healthy subjects.* **Food Chem 2012; 134 (1): 127- 133.**

-2. **Rebeca Martínez-Tomás**, Elvira Larqué, Daniel González-Silvera, María Sánchez-Campillo, María Isabel Burgos, Anna Wellner, Soledad Parra, Lucy Bialek, Marie Alminger, Francisca Pérez-Llamas. *Effect of the consumption of a fruit and vegetable soup with high in vitro carotenoid bioaccessibility on serum carotenoid concentrations and markers of oxidative stress in young men.* **Eur J Nutr 2012; 51 (2): 231- 239.**

-3. María Sánchez-Campillo, Elvira Larqué, Daniel González-Silvera, **Rebeca Martínez-Tomás**, Manuel García-Fernández, Francisco Avilés, Anna Wellner, Lucy Bialek, Soledad Parra, Marie Alminger, Salvador Zamora, Francisca Pérez-Llamas. *Changes in the carotenoid concentration in human postprandial chylomicron and antioxidant effect in HepG2 caused by differently processed fruit and vegetable soups.* **Food Chem 2012; 133 (1): 38-44.**

-4. Sánchez-Campillo, María; Pérez-Llamas, Francisca; González-Silvera, Daniel; **Martínez-Tomás, Rebeca**; Burgos, M Isabel; Avilés, Francisco; Wellner, Anna; Parra, Soledad; Bialek, Lucy; Alminger, Marie; Larqué, Elvira. *Cell based assay to quantify the antioxidant effect of food derived carotenoids enriched in postprandial human chylomicrons.* **J Agric Food Chem 2010; 58 (20): 10864- 10868.**

-5. Sandy Van Buggenhout, Lilia Ahrné, Marie Alminger, Anna Andrys, Mia Benjamin, Lucy Bialek, Graham Cleaver, Ines Colle, Maud Langton, Elvira Larqué, Lien Lemmens, Anders Löfgren, Patricia Lopez-Sanchez, Francisca Pérez-Llamas, **Rebeca Martínez-Tomás**, Jim Robertson, Sebastian Schalow, Cecilia Svelander, Nikolaus Wellner, Marc Hendrickx, Keith Waldron. *Structural design of natural plant-based foods to promote nutritional quality.* **Trends Food Sci Technol 2012; 24 (1): 47-59.**

-6. Marie Alminger, Cecilia Svelander, Anna Wellner, **Rebeca Martinez-Tomas**, Lucy Bialek, Elvira Larque, Francisca Perez-Llamas. *Applicability of in vitro models in predicting the in vivo bioavailability of lycopene and β-carotene from differently processed soups.* **Food and Nutrition Sciences 2012; 3 (4): 477-489.**

-7. Burgos Alves MI, Avilés Plaza F, **Martínez-Tomás R**, Sánchez-Campillo M, Larqué E, Pérez-Llamas F, Martínez Hernández P, Parra Pallarés S. *Oxidized LDL and*

its correlation with lipid profile and oxidative stress biomarkers in young healthy Spanish subjects. **J Physiol Biochem 2010; 66 (3): 221-227.**

1.2 Presentations in congresses

1.2.1 International congresses

-1. **Martínez-Tomás R**, Larqué E, González-Silvera D, Sánchez-Campillo M, García-Fernández M, Cascales AI, Burgos MI, Avilés F, Wellner A, López-Jiménez JA, Bialek L, Parra S, Alminger M, Zamora S, Pérez-Llamas, F. *Effect of vegetable soup consumption on the serum levels of carotenoids and oxidative status in healthy adults.* **European PhD Conference in Food Science and Technology BerlinFOOD2010. Berlin, Germany. 2010. Oral presentation.**

-2. **Martínez-Tomás R**, Larqué E, González-Silvera D, Sánchez-Campillo M, Cascales AI, Burgos MI, Wellner A, López-Jiménez JA, Bialek L, Parra S, Alminger M, Pérez-Llamas, F. *Effect of two vegetable soups with different technological processing on serum carotenoids and oxidative status in healthy subjects*. **2010 EFFoST Annual Meeting. Dublin, Irland. 2010. Poster.**

-3. **Martínez-Tomás, Rebeca**; Larqué, Elvira; Burgos, María Isabel; Avilés, Francisco; González, Daniel Sánchez-Campillo, María; García, Manuel; Wellner, Anna; Bialek, Lucy; Parra, Soledad; Alminger, Marie; Pérez-Llamas, Francisca. *Effect of intake of a functional vegetable soup on serum concentrations of carotene and folate and markers of oxidative stress in healthy men*. **EGEA Conference, 6th edition. Brussels, Belgium. 2010. Poster.**

-4. Sánchez-Campillo M, Larqué E, González-Silvera D, **Martínez-Tomás R**, García-Fernández M, Avilés F, Wellner A, Bialek L, Parra S, Alminger M, Zamora S, Pérez-Llamas, F. *Carotenoid response and antioxidant effect of postprandial human chylomicrons after the intake of reference and optimized vegetable food products in HepG2 cells.* **2010 EFFoST Annual Meeting. Dublin, Irland. 2010. Poster.**

-5. Sánchez-Campillo, María; González, Daniel; **Martínez-Tomás, Rebeca**; Burgos, María Isabel; López, José Ángel; Cascales, Ana Isabel; Avilés, Francisco; Wellner, Anna; Parra, Soledad; Bialek, Lucy; Alminger, Marie; Larqué, Elvira; Pérez-Llamas, Francisca. *Method to evaluate in vitro antioxidant effect of postprandial carotenoid enriched chylomicrons on human hepatocytes.* **EGEA Conference, 6th edition. Brussels, Belgium. 2010. Poster.**

-6. G. J. Cleaver, L.Bialek; **R. Martínez-Tomás**, E. Larqué, JA López-Jiménez, F. Pérez-Llamas. *Food Neophobia and Consumer Responses to Novel Food Products.8th Pangborn Sensory Science Symposium.* **Florence, Italy. 2009. Poster.**

1.2.2 National congresses (Spain)

-1. **R. Martínez-Tomás**, E. Larqué Daza, M.I. Burgos Alves, F. Avilés Plaza, M. Sánchez-Campillo Muñoz, D. González Silvera, A. Wellner, L. Bialek, S. Parra Pallarés, M. Alminger, F. Pérez Llamas. *Effect of the consumption of a functional vegetable soup on the antioxidant status in young men.* Publication: **Nutr Hosp 2010;**

25 (1): 113. **II Congress of the Spanish Federation of Nutrition, Food and Dietetics (FESNAD). Barcelona. 2010. Poster.**

-2. **Martínez-Tomás R**, Larqué E, González D, Sánchez-Campillo M, García M, Cascales AI, Burgos MI, Avilés F, Wellner A, López-Jiménez JA, Bialek L, Parra S, Alminger M, Zamora S, Pérez-Llamas, F. *Comparative study of the effect of the consumption of two vegetables soups, reference and functional, on carotenoid serum levels and oxidative status in healthy adults.* **National Conference of Food. Sevilla. 2010. Poster.**

-3. Romero R, Burgos M. I, **Martinez-Tomás R**, Sánchez-Campillo M, González D, Cascales A. I, García M, Avilés F, Parra S, Larqué E, Pérez-Llamas F, Martínez P. *Oxidative stress: normal values in a young population.* Publication: **Journal of Clinical Laboratory 2010; 3: 351**. **IV National Congress of Clinical Laboratory. Zaragoza. 2010. Poster.**

-4. J. García Salas, M. Burgos Alves, R. Romero Zambrano, **R. Martínez Tomás**, F. Avilés Plaza, E. Larqué, F. Pérez Llamas, P. Martínez Hernández y S. Parra Pallarés. *Correlation of the oxidized low-density lipoprotein and the lipid profile. IV National Congress of Clinical Laboratory.* Publication: **Journal of Clinical Laboratory 2010; 3: 351. IV National Congress of Clinical Laboratory. Zaragoza. 2010. Poster.**

1.3 Other achievements

1.3.1 Awards

-1. Winner of the 2010 EGEA Poster. "*Effect of intake of a functional vegetable soup on serum concentrations of carotene and folate and markers of oxidative stress in healthy men*". EGEA Conference, 6th edition. Brussels, Belgium. 2010.

1.4 Stays in other laboratories performed during the realization of the present Thesis

-1. Centre: Department of Chemical and Biological Engineering, Food science, Chalmers University of Technology. Göteborg, Sweden. Topic: Quantification of β-carotene and lycopene in serum and in chylomicrons by HPLC. Duration: 3 months. 2010.

2. APORTACIÓN EN LOS TRABAJOS/*CONTRIBUTION REPORT*

Artículo I/*Paper I*. "Effect of the consumption of a fruit and vegetable soup with high in vitro carotenoid bioaccessibility on serum carotenoid concentrations and markers of oxidative stress in young men" [Eur J Nutr (2012) 51:231–239]

La primera autora Rebeca Martínez Tomás (RMT), participó en la ejecución del estudio, en el análisis de las muestras y en el análisis e interpretación de los datos; redactó el manuscrito para su publicación.

The first author Rebeca Martínez Tomás (RMT), participated in the carrying out of the study, in the analysis of the samples and in the analysis and interpretation of the data; wrote the manuscript for publication.

Artículo II/*Paper II*. "Cell-based assay to quantify the antioxidant effect of food-derived carotenoids enriched in postprandial human chylomicrons" [J. Agric. Food Chem. 2010, 58, 10864–10868]

La coautora RMT, participó la ejecución del estudio y en el trabajo de laboratorio; contribuyó al análisis e interpretación de los datos y a la redacción del manuscrito para su publicación.

The coauthor RMT, participated in the carrying out of the study and in the laboratory work; contributed to the analyses and interpretation the data and the writing of the manuscript for publication.

Artículo III/*Paper III*. "Daily intake of fruit and vegetable soups processed in different ways increases human serum β-carotene and lycopene concentrations and reduces levels of several oxidative stress markers in healthy subjects" [Food Chemistry 134 (2012) 127–133]

La primera autora Rebeca Martínez Tomás (RMT), participó en la ejecución del estudio, en el análisis de las muestras y en el análisis e interpretación de los datos; redactó el manuscrito para su publicación.

The first author RMT, participated in the carrying out of the study, the analysis of the samples and in the analysis and interpretation of the data; wrote the manuscript for publication.

Artículo IV/*Paper IV*. "**Changes in the carotenoid concentration in human postprandial chylomicron and antioxidant effect in HepG2 caused by differently processed fruit and vegetable soups" [Food Chemistry 133 (2012) 38–44]**

La coautora RMT, participó en la ejecución del estudio y en el análisis de las muestras; contribuyó al análisis e interpretación de los datos y a la redacción del manuscrito para su publicación.

The coauthor RMT, participated in the carrying out of the study and in the analysis of the samples; contributed to the analyses and interpretation of the data and the writing of the manuscript for publication.

"Señor, dame serenidad para aceptar las cosas que no puedo cambiar, valor para cambiar las cosas que puedo y sabiduría para poder diferenciarlas."

Karl Paul Reinhold Niebuhr (1892- 1971) Teólogo y político estadounidense

"*La mayoría de los fiascos nos vienen por querer adelantar la hora de los éxitos."*

Amado Nervon (1870-1919) Poeta y prosista mexicano