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Lutein bioavailability from lutein ester-fortified fermented milk: *in vivo* and *in vitro* study☆,☆☆

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Abstract

We assessed the bioavailability of lutein from lutein-fortified fermented milk using *in vivo* and *in vitro* approaches. Twenty-four volunteers were randomized to take lutein-fortified fermented milk at two levels of fortification. Single-dose bioavailability study (2×100 ml, ca. 8 or 16 mg of lutein) was performed using a three-point approach (baseline, 3.5 and 6.5 h). Multiple-dose study consisted of consuming one serving/day (ca. 4 or 8 mg/100 ml) for 14 days. Blood samples for biochemical, hematological and lutein analysis were drawn at baseline, Day 7 and Day 14. *In vitro* bioaccessibility was assessed by a static gastrointestinal digestion model. Lutein content, *in vitro* ester hydrolysis and micellarization, and lutein concentrations achieved in serum were analyzed by HPLC. *In vivo*, post-prandial response was higher using the high content fermented milk, but the percentage of absorption was not different according to the dose consumed. Net increments at Day 7 and Day 14 were significantly higher on consuming the high-dose milk as well. *In vitro*, lutein ester hydrolysis was incomplete regardless of the amount initially present. Free lutein released was higher using the high-dose fermented milk, but the percentage of hydrolysis was similar at both levels of fortification. In the micellar phase, the percentage of free and total lutein was not different according to the dose. Our results support the suitability of the fermented milk as a carrier of lutein esters and an *in vivo* dose-dependent effect upon regular consumption and suggest the usefulness of *in vitro* models to provide relevant information to predict *in vivo* responses.

Keywords: Lutein; Bioavailability; Human study; *In vitro* study; Fermented milk

1. Introduction

Lutein, a plant pigment that is among the most well-known carotenoids, is also one of those most widely distributed in frequently consumed fruits and vegetables [1,2]. Humans are not capable of synthesizing carotenoids *de novo* and, thus, their presence in human tissues is entirely of dietary origin. Lutein, together with zeaxanthin, is especially abundant at the centre of the retina (macula) and, in fact, they are commonly referred to as macular pigments [3].

Lutein has no provitamin A activity in humans, although it displays other biological activities that have attracted so much attention in relation to human health that it has been proposed they could fit the criteria for conditionally essential nutrients [4]. Interest in lutein has dramatically increased even when there are no human pathological conditions associated with deficiency or toxicity specifically related to lutein. Thus, although there are no established recommendations of intake [5], the increasing commercial availability and use of lutein-containing supplements, both at a population level and as recommended by some physicians, have led to the assessment of the risk associated with their consumption [6].

Age-related cataracts and maculopathy are the major causes of blindness among the elderly population throughout

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the world, and delaying the development of these two conditions would enhance the quality of life for older persons while reducing the economic burden imposed by these conditions [7]. It has been proposed that lutein and zeaxanthin may prevent light-initiated oxidative damage to the retina and, thus, protect against age-related deterioration [3,7]. In this context, concentrations of lutein and zeaxanthin in serum and tissues (i.e., macula) increase significantly upon the ingestion of lutein-rich foods and lutein capsules [8–11], and parallel an increase in macular pigment optical density and an improvement in visual function [10–13].

Nowadays, the idea that proper nutrition and, possibly, the use of supplements, along with healthy lifestyles, may provide the least costly and most practical means to delay age-related cataracts and maculopathy is becoming more widely accepted [3,13,14]. In fact, intervention studies using commercially available lutein supplements are now performed. Simultaneously, the industrial production, the development of new deliverable forms (i.e., water soluble) and the applications in the food market, beverages and supplements are all increasing [15].

Milk is an effective delivery vehicle for micronutrients (i.e., vitamin A, E) with a long-standing tradition of safety [16], and fortification of frequently consumed foods has been proven to be an effective and low-cost way to increase the micronutrient supply and reduce the incidence of micronutrient deficiencies [17–19]. In this context, fortified dairy products represent a growing market that is of interest to those sectors of the population with unbalanced diets and increased needs (i.e., lutein). However, information on the bioavailability of micronutrients (natural or added) from dairy products is very limited [20].

Lutein is a promising biologically active component in the food industry, but the new EU regulations regarding the nutrition and health qualifications of foods (EU no. 1924/2006) impose new approaches to support nutritional and healthy qualifications. Within this framework, we aimed to assess the relative *in vivo* bioavailability of lutein from fortified fermented milk using a combined approach, i.e., single and multiple doses. Moreover, since both food and host-related factors (i.e., genetics, nutritional status) may influence the lutein bioavailability [21–23] and to approach all these aspects better, we also performed complementary *in vitro* studies to assess the bioaccessibility of lutein from the fortified fermented milk.

2. Subjects and methods

2.1. Standards and reagents

All reagents for preparing organic and inorganic solutions — taurocholate salts and enzymes (i.e., α-amylase; EC 3.2.1.1), pepsin from porcine stomach (EC 3.4.23.1), human pancreatic lipase (EC 3.1.1.3), cholesterol esterase (EC 3.1.1.13), phospholipase A_2 (EC 232.637.7) used in the *in vitro* digestion and standards for analysis of vitamin A (all-*trans*-retinol, product code 95144), vitamin E (α-tocopherol, product code T-3251) and lutein (product code X-6250) in blood — were purchased from Sigma Aldrich (Madrid, Spain), VWR Internacional Eurolab (Mollet del Vallés, Spain) and Carlo Erba (Madrid, Spain). Stock solutions of vitamin A, vitamin E and lutein were prepared by dissolving different amounts of the compounds in ethanol, and concentrations were calculated on the basis of absorptivity values ($E^{1\%}$ 1 cm; retinol, 1835 at 325 nm; α-tocopherol, 71 at 292 nm; lutein, 2550 at 445 nm).

2.2. Fermented milk

Lutein-fortified fermented milk (Lutein esters mix, Cognis GmbH) (100-ml bottles) was prepared according to the protocols and controls required for human consumption. According to the manufacturer, fermented milk was prepared using partially skimmed milk and lactic ferments, and provided 84 kcal, 2.4 g of protein, 15.5 g of carbohydrates and 1.7 g of fat per bottle (100 ml). Transport and storage were maintained at 4°C throughout the study. Fermented milk was prepared in two different occasions to contain lutein esters at low (equivalent to ca. 4 mg free lutein/100 ml) and high dose (ca. 8 mg free lutein/100 ml).

2.3. Subjects and bioavailability study

Twenty-four apparently healthy volunteers (12 men and 12 women, 18–30 years) were singled out by nonprobabilistic sampling. All the participants were required (inclusion criteria) to have biochemical and hematological profiles and serum levels of vitamin A, E and carotenoids within accepted reference ranges [24]. The use of vitamin and/or herbal supplements, dieting, pregnancy, chronic medication and intercurrent disease or infection that could alter the bioavailability or status of the compounds of interest were used as exclusion criteria.

The study consisted of two combined bioavailability (single-dose and multiple-dose) tests using lutein ester-fortified fermented milk at two levels of fortification (ca. 4 and 8 mg/100 ml per day). After compliance with the inclusion criteria, subjects were allocated to receive either low-dose (n=12, six women and six men) or high-dose (n=12, six women and six men) intervention in a random order by a computer-based table of pseudo-random numbers. Subjects were provided with a list of lutein/zeaxanthin-rich foods (i.e., fruits, vegetables, juices, beverages, eggs and byproducts) to avoid during the 24 h prior to the intervention and during the multiple-dose study in order to minimize interferences from the habitual diet. The participants were asked to fill a Semiquantitative Food Frequency Questionnaire at the end of the intervention period to check the compliance with these criteria. The questionnaire was specifically designed and tested to provide the frequency of consumption of the major dietary contributors to carotenoid intake (as the

major determinant of carotenoid serum levels) over the previous 2 weeks of blood collection [25].

Single-dose bioavailability study was performed on Day 1 (low or high dose) using a three-point approach [26,27]. After overnight fasting, subjects were cannulated and a blood sample was obtained (baseline). Subjects were provided with a standard breakfast [two slices of bread spread with lard (10 g) plus two slices of ham] and the lutein-fortified fermented milk (2×100 ml, total dose supplied was ca. 8 or 16 mg of lutein) and were allowed to stand free until the second (3.5 h, expected peak concentration) and third (6.5 h, expected baseline recovery) blood sample collection. During this time, volunteers were asked to avoid the ingestion of any food or liquid except water.

Multiple-dose bioavailability study consisted of the intake of one serving (ca. 4 and 8 mg) per day for the following 14 days (ca. total dose 60 and 120 mg for the low- and high dose, respectively, during the study). Fasting blood samples for biochemical, hematological, vitamin and lutein analysis were drawn at baseline (Day 0), Day 7 and Day 14. Compliance was tested by personal interview (i.e., counting the servings not consumed, if any), using serum levels of lutein at mid-points (Day 7) and evaluating the Food Frequency Questionnaire at the end of the intervention. Modifications of dietary and lifestyle habits (i.e., dieting, smoking habit, alcohol, antibiotic consumption), and the presence of fever and infections were also monitored through personal interviews and by the Food Frequency Questionnaire.

The study protocol was approved by the Comité Ético de Investigación Clínica of the Hospital Universitario Puerta de Hierro. All subjects were informed and gave their signed consent before their inclusion in the study.

2.4. Risk/benefit assessment

Doses for fortification were chosen by considering the average content of selected rich sources (i.e., spinach), the consumption levels in different European countries [1] and the suggested safety levels of intake [6]. Based on these data, the final doses were established at approximately one and two times the upper level of intake in our population [1]. These amounts (ca. 4–8 mg/day) were similar to that contained in 150–200 g of (cooked) spinach [1], and thus no adverse effects were expected. Also, because of the intervention protocol implied no changes in the habitual dietary patterns, except for avoiding major dietary contributors to lutein intake and the limited duration of the study (2 weeks), no significant changes in the nutritional status of the volunteers were expected either. Moreover, despite the amounts of lutein provided being much below the suggested safety levels of intake (20 mg/day) [6], subjects were monitored for general biochemical indexes in routine clinical practice including lipid profile and hepatic function and hematological indexes. In addition, because of the novelty of the food to be used, biochemical markers of vitamin A and E nutritional status were also assessed (retinol and α-tocopherol concentrations in serum, respectively).

2.5. Blood sample analysis

Analyses of lutein in the post-prandial period (single-dose study) were performed in serum samples as lutein is likely to exchange among lipoproteins during the post-prandial period [28,29], and thus analyses of this fraction may not be an accurate estimate of the "true" relative absorption. Lutein in serum was analyzed as described by Olmedilla et al. [24]. Using this method, *trans*-lutein, zeaxanthin, 13/15-*cis*-lutein, among other carotenoids, can be determined simultaneously. Identification of compounds was carried out by comparing retention times with those of authentic standards and on-line UV–visible spectra.

Samples from each individual (obtained before and after the intervention) were analyzed on the same day to reduce analytical variability. The short- and long-term precision and accuracy of the analytical method are periodically verified through our participation in the Fat-Soluble Quality Assurance Program conducted by the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). Biochemical hematological parameters (inclusion, baseline and at the end of interventions) were monitored by the General Biochemistry and Hematology Laboratories of the hospital according to quality-controlled standardized methods.

2.6. Analysis of lutein in the fermented milk

The lutein ester-fortified fermented milk prepared for the human studies was tested for the lutein content by spectrophotometer and HPLC measurements using different protocols of extraction and on different days during the study. Up to six different mixtures of solvents and conditions were assayed for extraction including hexane/methylene chloride (using vortex: n=1, in triplicate; and ultrasonic bath: n=2, in triplicate) [24,30], tetrahydrofuran/methanol (n=2, in duplicate) [31], acetone (overnight at 4°C, n=2 in duplicate), ethanol (overnight at 4°C, n=11 in duplicate) and direct chemical hydrolysis (n=1, in duplicate) of the matrix [30]. For quality control and accuracy of lutein content in the fermented milk, analysis were also performed, using two extraction protocols [24,31], on a Reference Standard Material (Slurried Spinach; SRM 2385, NIST) with certified values for lutein content.

2.7. In vitro bioaccessibility

To assess the *in vitro* bioaccessibility of lutein from fermented milk, a static gastrointestinal digestion model previously applied to fruits and vegetables was used [32]. Low- and high-content fortified fermented milk were analyzed (n=3) in triplicate to evaluate stability during digestion (i.e., recovery), the extent of ester hydrolysis and the percentage of micellization. Briefly, samples (in triplicate) of approximately 5 ml were transferred to a flask

and a saliva solution containing organic and inorganic components and α-amylase was added after which the samples were incubated in a shaking water bath (37°C, 95 OPM) for 5 min. Gastric juice with organic and inorganic solutions, mucin, bovine serum albumin and pepsin from porcine stomach was added. The pH was adjusted to 1.1 and the solution was incubated for 1 h. Duodenal juice (containing porcine pancreatin) and bile solutions were introduced after neutralization of the pH (7.8), and the human pancreatic lipase, colipase, cholesterol esterase, phospholipase A_2 and taurocholate salts were added. The final mixture was incubated for 2 h at 37°C. Analysis was performed in samples collected from the starting material, duodenal phase and after transference into supernatants (micellar phase). At each step, aliquots (1 ml) were collected in duplicate, extracted before and after chemical (KOH) hydrolysis to estimate free and total lutein content, respectively, and analyzed by high-performance liquid chromatography [24].

2.8. Statistical analysis

During the intervention, only one volunteer (a woman) dropped out of the study (cause: dieting and weight loss) so that 23 subjects were included in the statistical analysis. The areas under the curves (three-point AUC) of the postprandial responses in serum vs. time (6.5 h) were calculated by the trapezoidal method after correction for baseline concentrations. Percentages of relative absorption during the study (6.5 h) were calculated on the basis of the AUC values for lutein in serum, correcting for plasma volume (assuming 4% body weight) and expressed against the dose supplied, as determined by HPLC analysis. For each dose, differences at each time point were assessed by paired *t* test and ANOVA (before and after log transformation of the data) as well as by nonparametric tests (Wilcoxon signed ranks test, Mann–Whitney *U* test). Statistical significance was set at $P<.05$ and the analysis was performed using the SPSS 8.0 statistical software for Windows (SPSS Inc., Chicago, IL, USA).

For the *in vitro* digestion model, in order to allow the comparability of the results from different experiments, the parameters evaluated (i.e., stability, extent of hydrolysis) were also expressed as percentages of the initial content in the food.

3. Results

3.1. Lutein content in the fermented milk

The fermented milk was analyzed by spectrophotometer and HPLC. Although preliminary technical problems were observed (i.e., turbidity, apparent incomplete extraction), a total of six extraction protocols were assayed and the final results provide fairly consistent results. For comparative purposes, the best recovery was obtained using overnight extraction (at 4°C in ethanol) and content values obtained were 4.0 mg of lutein/100 ml (95% CI, 2.9–5.1) ($n=5$ assays, in duplicate) and 8.2 mg/100 ml (95% CI, 7.7–8.7) ($n=6$ assays, in duplicate) for the low and high dose, respectively. HPLC analysis of the extracts (saponified extracts) confirmed the concentrations by spectrophotometer readings and showed an average contribution of the all-*trans*-isomer of lutein of 72–74% in the fermented milk. Accuracy of the data generated was assessed by analyzing the lutein content in the SRM 2385. With the use of two extraction protocols [24,31], the analysis rendered values of 32.2±3.2 and 34.1 ±0.9 mg/kg, respectively (certified value, 32.9±6.5 mg/kg).

3.2. Human study

At the start of the intervention, there was no statistical difference in the mean serum levels of lutein according to the dose to be ingested ($P=.43$; ANOVA of log-transformed data). The AUC (0–6.5 h) calculated for each dose, the percentage of absorption (adjusted for plasma volume) and the net increments (baseline corrected) at each time point are shown in Table 1. Post-prandial response was higher using the high-content fermented milk, although, in relative terms, the percentage of absorption was not different according to the dose consumed. Similarly, net increments achieved at 6.5 h, 7 days and 14 days were higher on consuming the high-dose milk, reaching statistical significance only upon regular consumption of the fermented milk (Days 7 and 14). However, within subject, both levels of fortification were capable of incrementing significantly the levels of lutein in serum ($P<.005$ and $P<.001$ for low and high dose, respectively; Wilcoxon signed ranks test and *T* test of log-transformed data). No changes in the biochemical or hematological profile of the subjects were observed during the study. No significant changes in the serum status

Table 1
Response in serum in the combined bioavailability study

Single-dose study

	3-point AUC (μmol/l per 6.5 h)	% Absorption (0–6.5 h)[a]
Low dose	0.124 (0.062 to 0.186)	2.48 (1.19 to 3.77)
High dose	0.216 (0.126 to 0.305)	2.14 (1.17 to 3.11)
ANOVA	0.08	0.30

Single dose and multiple dose study
Net increments (baseline corrected) (μmol/L)

	At C_{max} (3.5 h)	At 6.5 h	At 7 days	At 14 days
Low dose	0.009 (−0.011 to 0.033)	0.038 (0.019 to 0.057)	0.147 (0.098 to 0.196)	0.163 (0.109 to 0.218)
High dose	0.001 (−0.046 to 0.049)	0.069 (0.031 to 0.107)	0.452 (0.369 to 0.535)	0.560 (0.440 to 0.679)
ANOVA[b]	0.77	0.13	<0.001	<0.001

Values are shown as mean (95% CI).
[a] Adjusted for plasma volume (4% body weight). Dose used for calculations were ca. 8 mg (low dose) and 16 mg (high dose).
[b] Differences between doses.

of vitamin A (retinol) or E (α-tocopherol) were found either, and no hypercarotenemia or carotenodermia was observed or reported.

3.3. In vitro bioaccessibility

Total lutein content (in saponified extracts) showed a very high stability under simulated gastrointestinal conditions, both at low and high level of content, with an average of 95–100% of the initial content recovered at the end of the duodenal phase. At this stage, hydrolysis of lutein esters was incomplete regardless of the amount initially present (low or high dose), although the amount of free lutein released was higher using the high-dose fermented milk (Fig. 1). Interestingly, the degree of ester hydrolysis (expressed as percentage) was very close at both levels of fortification, although it was slightly higher at the low content [mean (95% CI), 17% (14–20) and 14% (12–16) for the low and high content, respectively; P=.039]. In the micellar phase, however, the percentage of free (nonsaponified extracts) and total lutein (saponified extracts) recovered was not different according to the initial dose in the fermented milk (P=.38 and P=.27) (Fig. 1). Overall, the present data suggest that, in relative terms, both the degree of ester hydrolysis and the percentage of transference into the micellar phase were fairly consistent at the levels of fortification used in the present study.

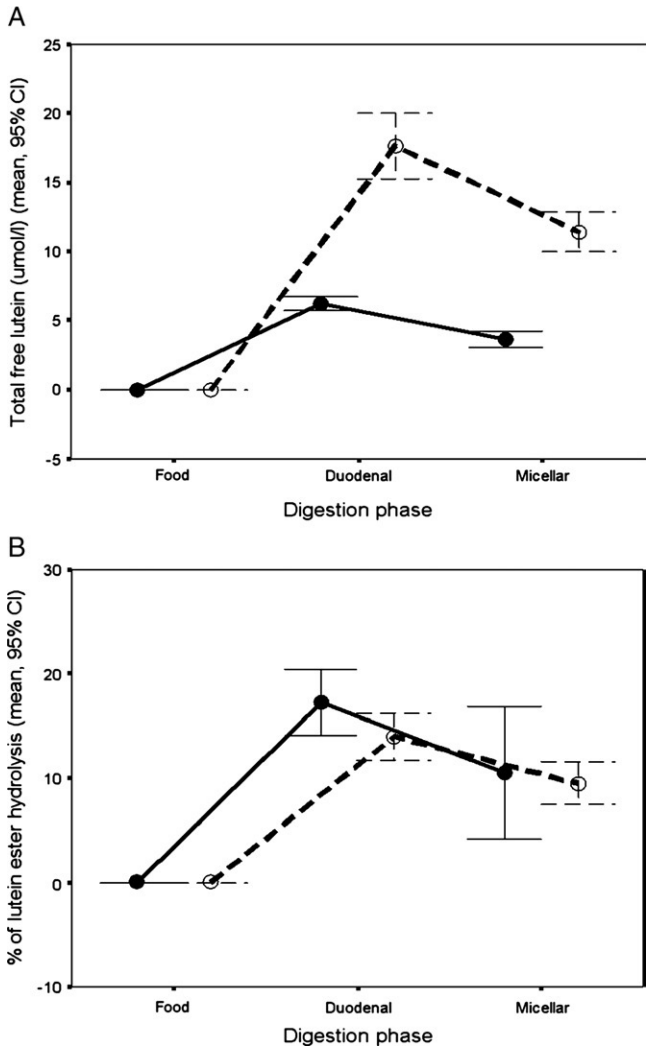

Fig. 1. *In vitro* digestion model. Amount of total free lutein (*trans*+*cis*) (A) and percentage of ester hydrolysis (B) during *in vitro* gastrointestinal digestion of lutein ester-fortified milk. Low dose (circle) and high dose (open circle).

4. Discussion

The food industry is increasingly developing, modifying and using emerging technologies and food packaging processes, and marketing new products. However, little is known about their effect on the bioavailability of the nutrients. Within this context, our complementary approach provides useful information regarding the *in vitro* bioaccessibility and *in vivo* bioavailability of lutein from fortified fermented milk and at nutritional, not pharmacological, levels of consumption. In addition, by determining other clinical indicators, we also provide other potentially relevant information regarding the adequacy and safety of the approach and the doses used (i.e., interactions and side-effects on nutritional and biochemical markers).

Serum lutein concentrations show a wide variability, both within subjects and among groups, although, on a population basis, the concentration of lutein in serum is widely used as the "best available" method to establish exposure and assess the nutritional status [33,34]. Our results show that the regular consumption of lutein ester-fortified fermented milk, at the level of fortification and consumption used (ca. 4–8 mg/day), may increase the serum levels of lutein above the 90 percentile of the reference ranges in the US and European populations (>0.50 μmol/L) [5,34]. These concentrations have been suggested as relevant for different physiological and clinical end points [9], and recent data seem to support their adequacy and safety [10,11,35].

To assess the bioavailability of lutein from fortified fermented milk, we used a combined *in vivo* approach. The extent to which the chylomicron post-prandial model may be applied to study xanthophylls bioavailability (i.e., lutein) has been questioned because of the potential transference to other lipoprotein fractions during the post-prandial state [28,29] and thus this model may underrepresent the true extent of lutein absorption [28]. To account for these effects, we used serum samples and a post-prandial three-point approach, based on the lesser invasiveness of the method and its power to predict the relative response (at C_{max}) of the subjects, especially for comparative purposes (i.e., between foods or doses) [26,27]. In this sense, our results using the three-point approach clearly showed differences for AUC at both levels of fortification, supporting its validity to provide relevant data for comparative purposes as previously reported for other food components.

Food and host-related factors may affect the bioavailability and bioaccessibility of carotenoids, ranging from 0.1% to almost 100% [36], and depend on several factors including microconstituent species, chemical form and amount present, food matrix and food processing [21,28]. As mentioned, *in vitro* methods may be appropriate for studying preabsorptive processes, but their validity, as an index of absorbability and/or bioavailability, should be validated under *in vivo* situations [23,37], although *in vitro* results may not fully explain the *in vivo* response [37,38]. Using the same chemical form (lutein esters), we have shown the efficacy of the fermented milk as a carrier for lutein esters at different levels of fortification. Interestingly, this effect is consistently observed both *in vitro* and *in vivo*. As shown, under *in vitro* conditions, the higher the amount of lutein esters in the milk, the higher the amount of free lutein released and micellarized, predicting a higher *in vivo* response even when the percentage of hydrolysis was similar for both doses. Consistently, *in vivo*, we obtained a greater post-prandial AUC and a higher response at Days 7 and 14 with the high dose, but with a similar percentage of absorption, as predicted by the *in vitro* model. Thus, *in vitro* and *in vivo* data are concordant and support the validity of the complementary approaches to assess at least in part the factors affecting the bioavailability of carotenoids, as previously reported [23,36,38].

In summary, the present results support the suitability of fermented milk as a carrier of lutein esters and the efficacy of this food-based approach to improve the status of lutein in control subjects. Moreover, our data also support an *in vivo* dose-dependent effect as well as the usefulness of *in vitro* models to provide relevant information to predict *in vivo* responses. Overall, the present approach and the information provided may be relevant in the design and evaluation of the nutritional and health effects of novel, potentially functional, food products.

References

[1] O'Neill ME, Carroll Y, Corridan B, Olmedilla B, Granado F, Blanco Y, Van den Berg H, Hininger Y, Roussell AM, Chopra M, Southon S, Thurnham DI. A European Carotenoid Database to assess carotenoid intakes and its use in a five-country comparative study. Br J Nutr 2001; 85:499–507.

[2] Granado F, Blázquez S, Olmedilla B. Changes in carotenoid intake in Spanish population over the period 1964–2004. Public Health Nutr 2007;10:1018–23.

[3] Krinsky NI, Landrum JT, Bone RA. Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. Annu Rev Nutr 2003;23:171–203.

[4] Semba RD, Dagnelie G. Are lutein and zeaxanthin conditionally essential nutrients for eye health? Med Hypotheses 2003;61:465–72.

[5] Institute of Medicine, Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids. Washington DC: National Academy Press; 2000.

[6] Shao A, Hathcock JN. Risk assessment for the carotenoids lutein and lycopene. Regul Toxicol Pharmacol 2006;45:289–98.

[7] Chiu ChJ, Taylor A. Nutritional antioxidants and age-related cataract and macular degeneration. Exp Eye Res 2007;84:229–45.

[8] Olmedilla B, Granado F, Southon S, Wright AJA, Blanco I, Gil-Martínez E, et al. A European multicenter, placebo-controlled intervention trial with a-tocopherol, carotene rich palm-oil, lutein or lycopene at dietary achievable levels. Clin Sci 2002;102:447–56.

[9] Granado F, Olmedilla B, Blanco I. Nutritional and clinical relevance of lutein in human health. Br J Nutr 2003;90:487–502.

[10] Trieschman M, Beatty S, Nolan JM, Hense HW, Heimes B, Austermann U, et al. Changes in macular pigment optical density and serum concentrations of its constituent carotenoids following supplemental lutein and zeaxanthin: The LUNA study. Exp Eye Res 2007;84:718–28.

[11] Schalch W, Cohn W, Barker FM, Köpcke W, Mellerio J, Bird AC, et al. Xanthophyll accumulation in the human retina during supplementation with lutein and zeaxanthin—the LUXEA (LUtein Xanthophyll Eye Accumulation) Study. Arch Biochem Biophys 2007;458:128–35.

[12] Olmedilla B, Granado F, Blanco I, Vaquero M. Lutein, but not a-tocopherol, supplementation improves visual function in patients with age-related cataracts: a 2 years double blind placebo-controlled pilot study. Nutrition 2003;19:21–4.

[13] Richer S, Devenport J, Lang JC. LAST II: Differential temporal responses of macular pigments optical density in patients with atrophic age-related macular degeneration to dietary supplementation with xanthophylls. Optometry 2007;78:213–9.

[14] Coleman H, Chew E. Nutritional supplementation in age-related macular degeneration. Curr Opin Ophthalmol 2007;18:220–3.

[15] Nutraingredients. http://www.Nutraingredients.com/. Accessed on May 12th, 2008.

[16] Hayes KC, Pronczuk A, Perlman D. Vitamin E in fortified cow milk uniquely enriches human plasma lipoproteins. Am J Clin Nutr 2001; 74:211–8.

[17] Berner LA, Clydesdale FG, Douglass JS. Fortification contributed greatly to vitamin and mineral intakes in the United States, 1989–1991. J Nutr 2001;131:2177–83.

[18] Serra-Majem L, Ortega R, Aranceta J, Entrala A, Gil A. Fortified foods: criteria for vitamin supplementation in Spain. Public Health Nutr 2001;4:1331–4.

[19] Dary O, Mora JO. Food fortification to reduce vitamin A deficiency: international vitamin A consultative group recommendations. J Nutr 2002;32:2927S–33S.

[20] Herrero-Barbudo C, Olmedilla-Alonso B, Granado-Lorencio F, Blanco-Navarro I. Bioavailability of vitamins A and E from whole and vitamin-fortified milks in control subjects. Eur J Nutr 2006;45: 391–8.

[21] West CE, Castenmiller JJJM. Quantification of the "SLAMENGHI" factors for carotenoid bioavailability and bioconversion. Int J Vitam Nutr Res 1998;68:371–7.

[22] Faulks RM, Southon S. Challenges to understanding and measuring carotenoid bioavailability. Biochim Biophys Acta 2005;1740:95–100.

[23] Failla M, Chitchumroonchokchai C. In vitro models as tools for screening the relative bioavailabilities of provitamin A carotenoids in foods. Technical Monographs Series 2005. N° 3. HarvestPlus.

[24] Olmedilla B, Granado F, Gil-Martínez E, Blanco I, Rojas-Hidalgo E. Reference levels of retinol, a-tocopherol and main carotenoids in serum of control and insulin-dependent diabetic Spanish subjects. Clin Chem 1997;43:1066–71.

[25] Granado-Lorencio F, Olmedilla-Alonso B, Blanco-Navarro I, Botella-Romero F, Simal-Antón A. Assessment of carotenoid status and the relation to glycaemic control in type I diabetics: a follow-up study. Eur J Clin Nutr 2006;60:1000–8.

[26] Guerci B, Paull JL, Hadjadj S, Durlach V, Verges B, Attia A, Girardgloba A, Drouin P. Analysis of the postprandial lipid metabolism: use of a 3-point test. Diabetes Metab 2001;27: 449–57.

[27] Herrero-Barbudo C, Granado-Lorencio F, Olmedilla-Alonso B. Suitability of 3 point versus 7-point postprandial retinyl palmitate AUC in human bioavailability studies. Eur J Nutr 2008;47:55–8.

[28] Parker RS, Swanson JE, You CS, Edwards AJ, Huang T. Bioavailability of carotenoids in human subjects. Proc Nutr Soc 1999;58: 155–62.
[29] Pateau I, Chen H, Goh NMY. Interactions in the postprandial appearance of β-carotene and canthaxanthin in plasma triacylglycerol-rich lipoproteins in humans. Am J Clin Nutr 1997;66:1133–43.
[30] Herrero-Barbudo MC, Granado-Lorencio F, Blanco-Navarro I, Olmedilla-Alonso B. Retinol, a- and g-tocopherol and carotenoids in natural and vitamin-fortified dairy products commercialized in Spain. Int Dairy J 2005;15:521–6.
[31] Granado F, Olmedilla B, Blanco I, Rojas-Hidalgo E. Carotenoid composition in raw and cooked Spanish vegetables. J Agric Food Chem 1992;40:2135–40.
[32] Granado-Lorencio F, Olmedilla-Alonso B, Herrero-Barbudo C, Blanco-Navarro I, Pérez-Sacristán B, Blázquez-García S. In vitro bioaccessibility of carotenoids and tocopherols from fruits and vegetables. Food Chem 2007;102:641–8.
[33] Van den Berg H, Heseker H, Lamand M, Sandstrom B, Thurham DI. Flair concerted action n° 10 status papers. Introduction, conclusions and recommendations. Int J Vitam Nutr Res 1993;63:247–51.
[34] Olmedilla B, Granado F, Southon S, Wright AJA, Blanco I, Gil-Martínez E, et al. Baseline serum concentrations of carotenoids, vitamins A, E and C in control subjects from five European countries. Br J Nut 2001;85:227–38.
[35] Rosenthal JM, Kim J, de Monasterio F, Thompson DJ, Bone RA, Landrum JT, et al. Dose-ranging study of lutein supplementation in persons aged 60 years or older. Invest Ophthalmol Vis Sci 2006;47: 5227–33.
[36] Reboul E, Richelle M, Perrot E, Desmoulins-Mazelet C, Pirisi V, Borel P. Bioaccessibility of carotenoids and vitamin E from their main dietary sources. J Agric Food Chem 2006;54:8749–55.
[37] Oomen A, Hack A, Minekus M, Zeijdner E, Conelis C, Schoeters G, Verstraete W, De Wile T, Wragg J, Rompelberg C, Sips A, Van Wijnen J. Comparison of five in vitro digestion models to study the bioaccessibility of soil contaminants. Environ Sci Technol 2002;36: 3326–34.
[38] Granado F, Olmedilla B, Herrero C, Pérez-Sacristán B, Blanco I, Blázquez S. Bioavailability of lutein, β-carotene and tocopherols from broccoli: in vivo and in vitro assessment. Exp Biol Med 2006;231: 1733–8.