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Bioavailability of β-Cryptoxanthin in the Presence of Phytosterols: In Vitro and in Vivo Studies

Fernando Granado-Lorencio,*,[†,‡] Encarnación Donoso-Navarro,[‡] Luis Manuel Sánchez-Siles,[§] Inmaculada Blanco-Navarro,[†,‡] and Belen Pérez-Sacristán[†]

[†]Unidad de Vitaminas and [‡]Servicio de Bioquímica Clínica, Hospital Universitario Puerta de Hierro-Majadahonda, 28222 Madrid, Spain
[§]Research and Development Department, Hero Institute for Nutrition, Alcantarilla, 30820 Murcia, Spain

ABSTRACT: Bioactive compounds are used in the design and development of new food products with potential health benefits, although little is known regarding their bioavailability and interactions. This study assessed the stability, in vitro bioaccessibility, and human bioavailability of β-cryptoxanthin from β-cryptoxanthin-rich drinks with and without added phytosterols developed for this purpose. The developed drinks showed no difference in the content of β-cryptoxanthin, and they were stable over 6 months. In vitro, hydrolysis of β-cryptoxanthin esters and the amount of free β-cryptoxanthin at duodenal and micellar phases were similar regardless of the presence of phytosterols. In the human study, the daily intake provoked significant increments of β-cryptoxanthin in serum regardless of the type of the drink. In conclusion, in vitro and in vivo human studies have shown that the bioavailability of β-cryptoxanthin is not significantly affected by the presence of phytosterols when they are simultaneously supplied in a drink.

KEYWORDS: β-cryptoxanthin, in vitro bioaccessibility, in vivo bioavailability, phytosterols, intervention study

■ INTRODUCTION

Bioavailability is a critical feature in the assessment of the role of micronutrients in human health, and the approaches to this issue include in vitro and in vivo methods. Comparison and predictive value of in vitro digestion models are complicated because it is difficult to ascertain which one provides the most accurate bioaccessibility values unless they can be compared with in vivo human studies.[1,2] Moreover, although in vitro systems may provide relevant information regarding the factors influencing the bioavailability of nutrients from foods, these models cannot totally simulate in vivo human situations, especially those concerning biological variability, timing of consumption, and serum responses and biological effects upon regular intake.

β-Cryptoxanthin is a major dietary provitamin A carotenoid mostly provided by citrus fruits.[3,4] Epidemiological studies suggest a potential beneficial role of carotenoids in general, and β-cryptoxanthin in particular, in bone health, and both dietary intake and serum levels of β-cryptoxanthin have been inversely related to different bone and joint disorders (e.g., knee osteoarthritis, rheumatoid arthritis).[5−8] These observational data are consistent with in vitro, animal, and human studies showing that β-cryptoxanthin displays a unique anabolic effect on bone calcification[9,10] and modulates bone remodelling markers.[11]

Fruits and vegetables have been convincingly associated with a lower risk for developing cardiovascular diseases,[12,13] possibly in relation with their antioxidant nutrients (i.e., carotenoids). In this sense, serum carotenoids, and specifically β-cryptoxanthin, have been associated with markers of inflammation, oxidative stress, and endothelial function.[14,15] Moreover, β-cryptoxanthin may be also beneficial in preventing vascular disease by exerting effects related to brachial-ankle pulse wave velocity (an independent predictor of cardiovascular mortality)[16] and carotid intima-media thickness.[17,18] Also, within the scope of cardiovascular disease prevention, numerous clinical trials have shown that phytosterols/stanols and their esters are effective in reducing circulating cholesterol levels in humans when they are incorporated into a broad range of food matrices.[19]

Currently, the food industry is playing a key role in the design and development of new products with several components that, when simultaneously present in foods, may interact. Nevertheless, although this may be relevant for food quality and stability of micronutrients in foods, little is known regarding the effects on their bioavailability. In this context, we aimed to develop and assess a β-cryptoxanthin-rich drink with added phytosterols. To better understand the potential effect of phytosterols on the bioavailability of β-cryptoxanthin, we used a complementary approach, an in vitro model and an in vivo human study. Specifically, our aim was (1) to assess the content and stability of β-cryptoxanthin in a β-cryptoxanthin-enriched milk-based fruit drink, with and without added phytosterols; (2) to evaluate in vitro the bioaccessibility of β-cryptoxanthin in the presence of phytosterols; and (3) to assess the bioavailability in vivo of β-cryptoxanthin by measuring the serum response upon regular intake of these drinks.

■ MATERIALS AND METHODS

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), and phospholipase A_2 (EC 232.637.7)) used in the in vitro digestion and standards for carotenoid analysis were purchased from Sigma-Aldrich

Table 1. Composition of the Drinks Used in the in Vitro and in Vivo Bioavailability Studies[a] (Mean, CI_{95%})

type of drink	lutein (μg/100 mL)	zeaxanthin (μg/100 mL)	α-cryptoxanthin (μg/100 mL)	β-cryptoxanthin (μg/100 mL)	α-carotene (μg/100 mL)	trans-β-carotene (μg/100 mL)	phytosterols added[b] (g/100 mL)
β-cryptoxanthin-enriched drink	8.2 (4.8, 11.6)	12.7 (7.3, 18.1)	33.0 (30.2, 37.4)	300 (251, 349)	4.5 (3.0, 6.0)	12.0 (8.5, 15.5)	0
β-cryptoxanthin plus phytosterols-enriched drink	6.5 (4.9, 8.0)	9.4 (8.1, 10.6)	34.0 (29.4, 38.7)	299 (262, 337)	3.5 (2.6, 4.5)	10.8 (8.9, 12.6)	0.8

[a] Milk-based fruit drinks (skimmed milk (50%), mandarin juice (48%), banana puree (1%), grape juice (1%)). Data referred to carotenoid content in saponified extracts. No significant differences were found between drinks. [b] As free sterols (information supplied by the manufacturer (Hero S.A).

(Madrid, Spain), VWR Internacional Eurolab (Mollet del Vallés, Spain), and Carlo Erba (Madrid, Spain).

Phytosterols (PSs) Source. PSs were free plant sterols from pine tree. Because PSs are insoluble in water, the ingredient was presented in microencapsulated powder form to be water dispersible and suitable for use in low-fat beverages. The PSs used in the study were analyzed by gas chromatography with mass spectrometric detection and rendered a composition of 78.9% β-sitosterol, 11.9% sitostanol, and 7.1% campesterol.[20]

β-Cryptoxanthin and PS-Enriched Drinks. To assess the in vitro bioaccessibility and in vivo bioavailability of β-cryptoxanthin, two milk-based fruit drinks were used: (1) β-cryptoxanthin-enriched milk-based fruit drink and (2) β-cryptoxanthin-enriched milk based fruit drink with added PSs. Qualitative and quantitative contents of the drinks were approached on the basis of technological and scientific criteria, and the final product was specifically developed (not commercially available, Table 1) to provide ca. 750 μg of β-cryptoxanthin and 2 g of PSs/250 mL tetra brick.

Drinks were prepared at the pilot plant of the Hero Global Technology Center located in Alcantarilla, Murcia, Spain, according to the controls required by the law and adequacy for human consumption, as requested by the Comité Etico de Investigación Clínica (CEIC). Briefly, skimmed milk powder was dissolved in water. In a separate tank, microencapsulated water-dispersible PSs were mixed with the reconstituted fruit juices using a high-speed mixer device. The milk phase was acidified by the addition of the PSs-enriched fruit juice base and, to prevent protein destabilization, a high-methoxyl pectin was added. The resulting mixed phases were heated to 70 °C and homogenized at 150 bar in two steps (100 + 50). After homogenization, the product was pasteurized at 90 °C during 30 s by indirect heat exchanger, cooled to 20 °C, and filled aseptically in 250 mL tetra bricks. The process and conditions were identical for the β-cryptoxanthin-enriched drink.

In Vitro Digestion. To assess the in vitro bioaccessibility of β-cryptoxanthin from the drinks, a static gastrointestinal digestion model previously applied to fruits and vegetables was used.[21] The protocol was applied to the drinks at different time intervals (0, 3, and 6 months) to evaluate the effect of storage at room temperature. Briefly, samples of fruit juice (in triplicate) were transferred to a flask, and a saliva solution, at pH 6.5, containing organic and inorganic components and α-amylase was added, after which they were incubated in a shaking water bath (37 °C, at 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 incubated for 1 h. Duodenal juice and bile solution were introduced after neutralization of the pH (at 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 each step of the digestion (food, duodenal, and micellar phase), aliquots (1 mL) were collected in duplicate, extracted before (to assess free form) and after chemical (KOH) hydrolysis (to evaluate total content), and analyzed by high-performance liquid chromatography. Using this model, stability during digestion (recovery), degree of β-cryptoxanthin ester hydrolysis,

and incorporation into aqueous—micellar phase of free and total β-cryptoxanthin were determined.

In Vivo Study. The human bioavailability study consisted of the supplementation of the diet with 1 × 250 mL milk-based fruit drink/day for 4 weeks with a wash-out period of 4 weeks between. By using a computer-based table of pseudorandom numbers, volunteers ($n = 38$) were allocated to receive either intervention in a random order. Enrollment criteria included age (mean, confidence intervals (CI) 95%), 55 (54, 56), body mass index (mean, CI 95%), 25.6 (24.8, 26.4), no hormone, anabolic, or antiresorptive therapy, and no use of cholesterol-lowering drugs, ω-3 fatty acids, or phytosterol-containing foods or supplements. Additionally, inclusion criteria include serum levels of vitamins A, E, and D and biochemical and hemotological profile within reference ranges (except for total and LDL cholesterol, which were considered as an inclusion criteria as it was a "target" end point of the intervention study).

Subjects were provided with a list of fruits, vegetables, juices, and beverages rich in the compounds of interest (i.e., β-cryptoxanthin) to avoid during the intervention period to minimize interferences from the habitual diet and maximize the expected response. Overnight fasting blood samples were collected before and after each supplementation period (days 0 and 28) for the analysis of β-cryptoxanthin in serum (used as exposure marker for assessing the response). The study protocol was approved by the Comité Ético de Investigación Clínica of the Hospital Universitario Puerta de Hierro-Majadahonda (Madrid, Spain), and all subjects were informed and gave their signed consent. This trial was registered at ClinicalTrials.gov (ClinicalTrials.gov Identifier: NCT 01074623).

Safety Considerations. For phytosterols, the amounts added were selected on the basis of effective and safe doses as shown in different intervention trials.[19] For β-cryptoxanthin, the doses were approached considering the average content of selected rich sources (i.e., orange, mandarin) and the intake achievable from a balanced diet.[22] No significant changes in the nutritional status of the volunteers were expected due to the following facts: the final amount of β-cryptoxanthin provided by the experimental drinks (ca. 750 mg/brick) was similar to that contained in 300—400 g of fresh orange,[22] the intervention protocol implied no changes in the habitual dietary and lifestyle patterns (except for avoiding major dietary contributors to β-cryptoxanthin intake), and the limited duration of the study (4 weeks). Still, hypercarotenemia, carotenodermia, and hepatic, renal, and hematological indices were monitored.

Phytochemical Analysis. β-Cryptoxanthin and other carotenoids in the milk-based fruit drinks were extracted and analyzed by HPLC.[21,23] To analyze β-cryptoxanthin in serum, samples were processed as routinely performed in clinical practice. Briefly, 0.5 mL of serum was mixed with 0.5 mL of ethanol containing internal standard (retinyl acetate), vortexed, and extracted twice with 2 mL of methylene chloride/hexane (1:5). Organic phases were pooled, evaporated to dryness, and reconstituted to be injected (THF/EtOH) onto an ultraperformance liquid chromatograph (UPLC Aquity System, Waters).[24] Detection was carried out by a photodiode array (Waters Associates, Milford, MA) set
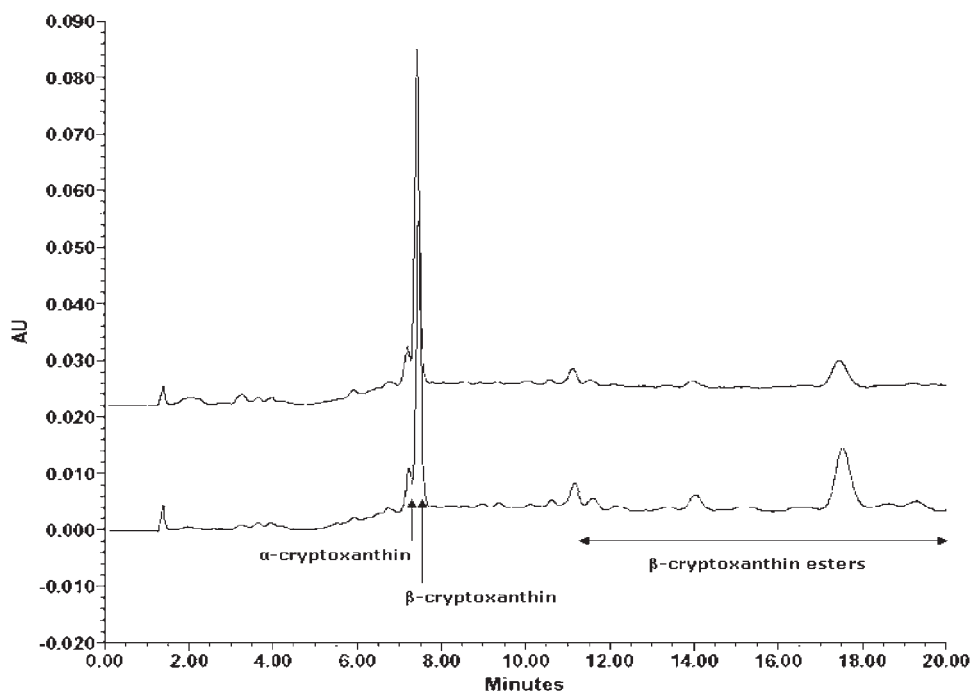
Figure 1. Carotenoid profile (450 nm) of unsaponified extracts of the β-cryptoxanthin-enriched beverage before (lower chromatogram) and after in vitro digestion (duodenal phase; upper chromatogram). During the in vitro digestion, a decrease in β-cryptoxanthin esters with a simultaneous increase in free β-cryptoxanthin can be observed.

at 450 nm and identification by comparison of retention times with those of authentic standards and online UV−visible spectra. In both systems, lutein ((3R,3′R,6′R)-β,ε-carotene 3,3′-diol)/zeaxanthin ((3R,3′R)-β, β-carotene 3,3′-diol), α-cryptoxanthin ((3′R,6′R)-β,ε-carotene 3′-ol), β-cryptoxanthin ((3R)-β,β-carotene 3-ol), lycopene (ψ,ψ-carotene), γ-carotene (β,ψ-carotene), α-carotene ((6′R)-β,ε-carotene), β-carotene (β,β-carotene), phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene), and phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene) can be simultaneously determined.

Samples from each individual (before and after both interventions) were analyzed on the same day to reduce analytical variability. The short- and long-term precision and accuracy of the analytical methods are verified periodically through our participation in the Fat-Soluble Quality Assurance Program conducted by the National Institute of Standards and Technology (NIST; Gaithersburg, MD).

Statistics. In vitro results were interpreted on the basis of data from crude and saponified extracts. To achieve consistency in the results from in vitro experiments, the parameters evaluated (i.e., degree of hydrolysis) were also expressed as percentages of the total amounts initially present in the drinks (saponified extracts) and descriptive statistics were used (mean, 95% CI). For in vivo (human) responses, differences in serum concentrations for each intervention period and differences due to the type of the drink consumed were assessed using parametric methods (ANOVA and post hoc Scheffé test). Statistical significance was set at $p < 0.05$, and the calculations were performed with SPSS 8.0 statistical software for Windows (SPSS Inc., Chicago, IL).

■ RESULTS

β-Cryptoxanthin Content, Stability, and in Vitro Bioaccessibility. The composition of the drinks is shown in Table 1. There were no significant differences in the initial content of β-cryptoxanthin between both types of drinks, and the amounts of free and total β-cryptoxanthin were not significantly different after 6 months of storage (329 ± 5 versus 321 ± 10 μg/100 mL

for total β-cryptoxanthin in the drink with no PSs ($p = 0.15$); 297 ± 6 versus 320 ± 16 μg/100 mL for total β-cryptoxanthin in the drink with added PSs ($p = 0.51$)). Similarly, the relative contribution of free to total β-cryptoxanthin was maintained over time (23.4% at 0 months versus 22.9% at 6 months for the β-cryptoxanthin-enriched drink ($p = 0.68$); 23.9% at 0 months versus 21.6% at 6 months for the drink containing β-cryptoxanthin plus PSs ($p = 0.25$)).

During the in vitro digestion (Figure 1), amounts and percentages of free forms at duodenal and micellar phases were not different between both drinks (Table 2), and the net hydrolysis of β-cryptoxanthin esters (percentage of free forms at duodenal phase minus that initially present) was similar regardless of the presence of phytosterols in the media (ca. 24.7 versus 22.1% for β-cryptoxanthin and β-cryptoxanthin plus PSs drinks, respectively). Only total content (and percentage of recovery) at duodenal phase showed significant differences between drinks during in vitro digestion (Table 2). Also, considering the amounts of free and total β-cryptoxanthin recovered in the micellar phases, a substantial amount of β-cryptoxanthin monoesters was also micellarized (ca. 38−45% of the total content) regardless of the presence of phytosterols in the media.

In Vivo Bioavailability. Baseline serum levels of β-cryptoxanthin were not different at the start of each intervention period (Table 3). Regardless of the type of drink consumed, the daily consumption of the drink provoked significant increases in the serum levels of β-cryptoxanthin (Figure 2), approaching mean values close to 55 μg/dL at the end of each intervention period (28 days). Additionally, the percentage of change (final/basal), the final concentrations achieved, and the cholesterol-adjusted final concentrations in serum did not differ significantly according to the type of drink, suggesting that the

Table 2. Content and Percentage of Recovery of β-Cryptoxanthin under in Vitro Conditions (Mean, CI$_{95\%}$)[a]

	β-cryptoxanthin			
type of drink	total content (μg/100 mL)	free β-cryptoxanthin (μg/100 mL)	% of free form	% of recovery
β-cryptoxanthin-enriched drink ($n = 6$)	300 (251, 349)	68 (59, 78)	23 (20, 26)	100
duodenal phase ($n = 9$)	255 (219, 292)	145 (116, 173)	48 (40, 55)	84 (76, 92)
micellar phase ($n = 9$)	248 (207, 288)	154 (139, 168)	49 (42, 56)	83 (70, 93)
β-cryptoxanthin plus PSs-enriched drink ($n = 6$)	299 (262, 337)	65 (60, 69)	22 (19, 24)	100
duodenal phase ($n = 9$)	306 (266, 346)[b]	131 (117, 144)	44 (39, 49)	106 (100, 110)[c]
micellar phase ($n = 9$)	269 (252, 287)	148 (137, 159)	50 (45, 55)	90 (84, 97)

[a] Total content determined in saponified extracts (used as reference values = 100%). Free β-cryptoxanthin and percentage of free form determined in unsaponified extracts and expressed against total initial content (saponified extracts). Percentage of recovery determined in saponified extracts and expressed against the total initial content. [b] Significantly different from the β-cryptoxanthin-enriched drink ($p < 0.05$) (ANOVA). [c] Significantly different from the β-cryptoxanthin-enriched drink ($p < 0.001$) (ANOVA).

Table 3. β-Cryptoxanthin Response in Serum upon Regular Consumption of the Drinks ($n = 36$) (Mean, CI$_{95\%}$)

	serum β-cryptoxanthin (μg/dL)	cholesterol-adjusted β-cryptoxanthin ((μg/mg) $\times$ 100)	% of change (final, basal)	net increment (μg/dL)
β-cryptoxanthin-enriched drink				
basal	16.9 (13.7, 20.1)	8.0 (6.5, 9.5)		
final (4 weeks)	56.5 (50.0, 63.0)[a]	25.6 (22.7, 28.6)[a]	310 (222, 398)	36.4 (29.9, 43.0)
β-cryptoxanthin plus PSs-enriched drink				
basal	17.8 (13.9, 21.6)	7.9 (6.1, 9.6)		
final (4 weeks)	53.2 (47.3, 59.0)[a]	25.3 (22.7, 27.8)[a]	282 (198, 365)	34.5 (29.9, 39.2)

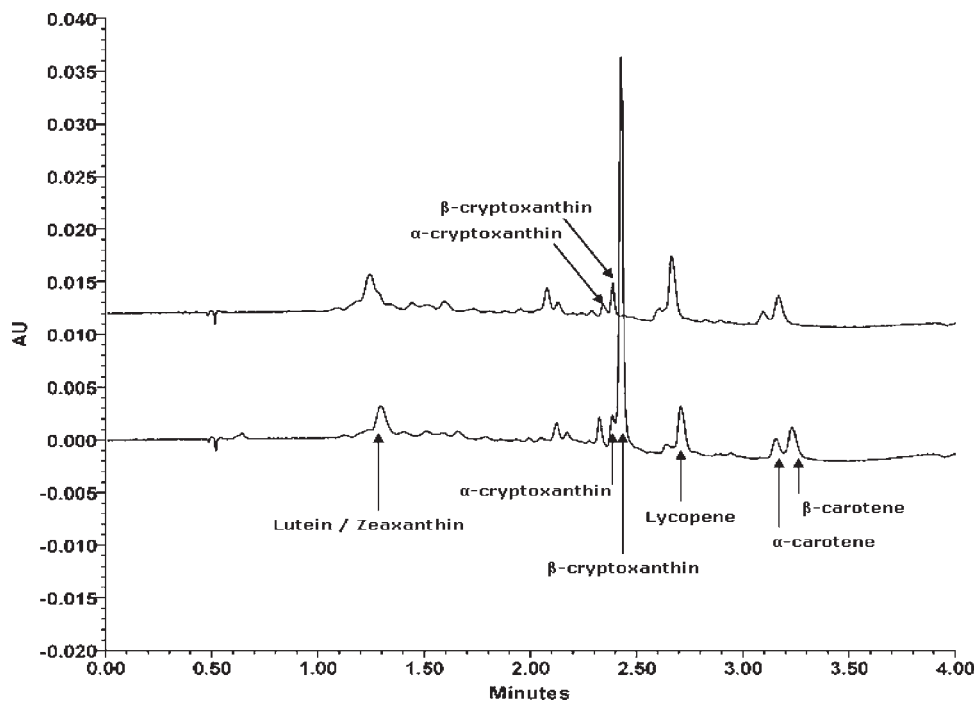[a] Significantly different from basal concentrations (One-way ANOVA, $p < 0.001$).

Figure 2. Serum carotenoid profile (450 nm) in a volunteer before (upper chromatogram) and after supplementation with β-cryptoxanthin-enriched drinks (lower chromatogram). A significant increase in the serum concentrations of β-cryptoxanthin is detected upon consumption of the beverage.

simultaneous presence of phytosterols in the drink did not modify the response of β-cryptoxanthin in serum upon regular intake.

During the intervention study, no changes in hepatic, renal, or hematological markers of the subjects were found, and no hypercarotenemia or carotenodermia was observed or reported.

11822

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■ DISCUSSION

The food industry is increasingly developing, using emerging technologies and new food packaging processes, and marketing new products, although information about how these processes affect the bioavailability of the nutrients is limited. In the present study, we developed a milk-based fruit drink containing two bioactive compounds (i.e., β-cryptoxanthin and phytosterols) and assessed the bioavailability of β-cryptoxanthin by using a complementary approach comprising in vitro and in vivo studies. Within the context of functional foods, bioactive components should be characterized as they are consumed.[25] Thus, we measured free and total content of β-cryptoxanthin in the drinks and showed its stability over 6 months, supporting the adequacy of this food matrix as a carrier of bioactive compounds for human consumption.

Food- and host-related factors may affect the bioavailability and bioaccessibility of carotenoids, ranging from 0.1% to almost 100%.[21,26] Overall, our results regarding the degree of ester hydrolysis and the bioaccessibility of β-cryptoxanthin (ca. 50%) under in vitro conditions are concordant with previous findings in citrus fruits.[21,27,28] Additionally, the presence of free and ester forms of β-cryptoxanthin in the micellar phase is consistent with former observations,[21,29] confirming the importance of assessing both chemical forms in estimating the bioaccessibility in vitro.

Our results also suggest that, when simultaneously present in the drink, phytosterols do not affect significantly the degree of hydrolysis of β-cryptoxanthin esters or its incorporation into the micellar phase. Moreover, because the amount of β-cryptoxanthin in the micellar phase may be indicative of the maximum amount available for absorption,[2] on the basis of these results, a similar serum response could be expected after consumption of the drinks with and without phytosterols, as observed in the human study. In this sense, it is accepted that long-term use of phytosterols results in a reduction in the serum levels of the most lipophilic carotenoids (i.e., α-carotene, β-carotene, and lycopene).[30,31] However, our data show that, upon regular consumption, the in vivo serum response of β-cryptoxanthin is not significantly affected by the simultaneous presence of phytosterols, confirming that β-cryptoxanthin bioavailability is not substantially affected when consumed as part of a phytosterol-rich source.[32] Interestingly, this effect is consistent with our in vitro observations supporting the utility of the in vitro model to partly assess the factors affecting the bioavailability of carotenoids and to predict the in vivo response.[2,26] Finally, the present data show the adequacy of both drinks to increase the serum levels of β-cryptoxanthin above the 95th percentile of the reference populations,[33] levels epidemiologically associated with health benefits,[5−8] supporting its efficacy as a carrier for bioactive components with potential health benefits.

In conclusion, our results support the adequacy and stability of a drink containing β-cryptoxanthin and phytosterols. Using in vitro and in vivo studies, we have shown that the bioavailability of β-cryptoxanthin is not significantly affected by the presence of phytosterols when simultaneously ingested in the drink and, additionally, we also provide data supporting its safety at the doses used. Finally, the present study supports the use of in vitro models to assess food-related factors affecting the bioavailability of carotenoids and its potential value to predict in vivo responses.

■ AUTHOR INFORMATION

Corresponding Author
*Postal address: Unidad de Vitaminas, Servicio de Bioquímica Clínica, Hospital Universitario Puerta de Hierro-Majadahonda, c/Maestro Rodrigo 2 (Majadahonda), Madrid, Spain. Phone: +34-91- 191 7578. Fax: +34-91-191 6806. E-mail: fgranado.hpth@ salud.madrid.org or granadof@hotmail.es.

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11823

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11824

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