JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Plant Sterols and Antioxidant Parameters in Enriched Beverages: Storage Stability

Marina González-Larena, Antonio Cilla, Guadalupe García-Llatas, Reyes Barberá, and María Jesús Lagarda*

Nutrition and Food Science Area, Faculty of Pharmacy, University of Valencia, Avda. Vicente Andrés Estellés s/n, 46100-Burjassot, Valencia, Spain

ABSTRACT: Plant sterols (PS) stability, antioxidant parameters, and color were studied during 6 months of storage at 4, 24, and 37 °C in three PS-enriched functional beverages. Beverages were skimmed milk with fruit juice and PS (MFJPS), fruit juice and PS (FJPS), and skimmed milk with PS (MPS). No loss in total PS content occurred during storage observing the same values at any given storage time point. Total carotenoids decreased 36% with storage at two months and then remained stable. Total polyphenols showed fluctuations throughout the storage, remaining stable at 6 months and reaching initial values. The antioxidant capacity (TEAC method) increased 18% at 6 months, and there was an increase in color over time and temperature, probably due to Maillard reaction compound formation. The increase in total antioxidant capacity might have helped PS maintenance throughout storage, these beverages being a good PS source even after 6 months of storage.

KEYWORDS: *sterol-enriched beverage, functional foods, phytosterols, antioxidants, storage stability*

INTRODUCTION

Plant sterols (PS) are currently used as functional food ingredients due to their protective effects, especially β-sitosterol, against colon, prostate and breast cancer, and their well-known total and low-density lipoprotein (LDL) cholesterol lowering activity.[1] Indeed, controlled clinical trials have demonstrated that PS consumption (2 g/d) results in a cholesterol reduction of approximately 10%.[2]

The first (and still the most common) PS-enriched commercial formulations were high fat food products.[1] However, the enrichment of spreads or high-fat foodstuffs with PS is contrary to dietary recommendations aimed at decreasing the risk of cardiovascular diseases. Hence, low-fat and nonfat alternatives need to be developed. In this sense, there are some examples of low-fat drinking foodstuffs enriched with PS, such as nonfat or low-fat milks and yogurts[3−7] and different beverages[8−10] including PS fortified orange juice-based beverages.[11,12]

Prevention of lipid oxidation during processing and storage is essential to maintain the quality and safety of PS-enriched foods since PS are susceptible to oxidation like all unsaturated lipids, and a decrease in initial PS content could occur as a result. The oxidative process of the lipid fraction in foods during long-term storage can be influenced by the packaging materials, storage temperature, oxygen availability, exposure to light, as well as by the antioxidant and pro-oxidant contents. In this context, one way of improving the oxidative stability of PS enrichment in a food would be their inclusion in a matrix with natural antioxidant compounds. In this sense, fruit−skimmed milk beverages, where PS enrichment is allowed,[13] appear as a suitable way to fulfill this requirement because they are appropriate for complying with health recommendations (restrictions of simple carbohydrates and saturated fatty acids (milk fat) and consumption of unsaturated fatty acids (used in the formation of PS esters)) and are good sources of other bioactive compounds such as polyphenols and vitamins, which also possess antioxidant activity.[14]

Although the PS profiles of several dairy products and juice beverages have been reported in the literature,[7,15,16] there are few studies regarding PS stability during storage in enriched beverages. Evaluations have been made of phytosterol-enriched whole milk powder stored at room temperature and at 38 °C for 12 months, milk enriched with free and esterified phytosterols and phytostanyl esters stored in the dark at room temperature and at 4 °C for 6 months,[5] and phytosterol-enriched milk subjected to Schaal oven conditions (24 h/65 °C, equivalent to one month of storage at room temperature).[6] However, as far as we are aware, no studies have evaluated PS stability in fruit−milk beverages, though it would be of special interest to check whether PS content is maintained stable during product shelf life to safeguard its potential bioactive properties. In addition, the relationship between PS stability and antioxidant parameters during storage is a relevant aspect that has not been addressed in food matrixes in literature so far. Besides, the stability of antioxidant parameters may be affected by processing and storage conditions (time and temperature) during the product shelf life.

The aim of the present study was to determine the effect of storage at 4, 24, and 37 °C during 6 months on PS stability (phytosterols/-stanols content) in three PS-enriched beverages. Furthermore, the evolution of antioxidant parameters (total carotenoids, total polyphenols, and total antioxidant capacity) and color measurements were determined complementarily to ascertain their protective role on PS stability in these functional beverages. These results will improve the knowledge on PS

Received: January 20, 2012
Revised: April 17, 2012
Accepted: April 18, 2012
Published: April 18, 2012

dx.doi.org/10.1021/jf3002679 | J. Agric. Food Chem. 2012, 60, 4725−4734

stability during storage and the food matrix effect upon bioactive compounds (PS and antioxidants), which could be of interest for both consumers and food scientists and the industry.

MATERIALS AND METHODS

Reagents. For plant sterol determination, standards used were 5β-cholestan-3α-ol (epicoprostanol) (purity ≥95%) used as internal standard (IS), (24*S*)-ethylcholest-5,22-dien-3-ol (stigmasterol) (purity 95%), (24*R*)-ethylcholest-5-en-3β-ol (β-sitosterol) (purity 95%), and 24α-ethyl-5α-cholestan-3β-ol (stigmastanol) (purity 97.4%) from Sigma Chemical Co. (St. Louis. MO, USA) and (24*R*)-methylcholest-5-en-3-ol (campesterol) (purity 98.6%) purchased from Steraloids (Newport, RI, USA). Chloroform, diethyl ether, methanol, anhydrous sodium sulfate, acetone, 2-propanol, and anhydrous pyridine were purchased from Merck & Co., Inc. (Whitehouse Station, NJ, USA). KOH was from Poch, S.A. (Sowinskiego, Poland), KCl from Panreac (Barcelona, Spain), hexane from J.T. Baker (Deventer, The Netherlands), and butylated hydroxytoluene (BHT) was from Sigma Chemical Co. (St. Louis, MO, USA). Silylating reagents: hexamethyldisilazane (HMDS) from Fluka (Buchs, Switzerland) and trimethylchlorosilane (TMCS) from Carlo Erba (Rodano, Italy). All reagents were of analytical grade.

For antioxidant parameters determination, the reagents used were dipotassium peroxodisulfate, absolute ethanol, methanol, acetone, sodium hydrogen carbonate from Merck (Barcelona, Spain), hexane from J.T. Baker (Deventer, The Netherlands), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (≥99% purity), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (97% purity) from Sigma Chemical Co. (Steinheim, Germany). Except for ABTS and Trolox, all the chemicals and reagents used in these experiments were of analytical grade. Millipore-Milli-Q distilled−deionized water (Millipore Ibérica, S.A., Barcelona, Spain) was used throughout the experiments.

Samples. Three beverages enriched with free microcrystalline PS from tall oil (0.8 g PS/100 mL beverage) as a source of PS were manufactured in a pilot plant by HERO Spain, S.A.: skimmed milk with fruit juice and PS (MFJPS), composed of reconstituted acidified skimmed milk, tangerine fruit juice from concentrate, banana puree, grape juice from concentrate, and PS; fruit juice and PS (FJPS), composed of tangerine fruit juice from concentrate, banana puree, grape juice from concentrate, PS, and water to substitute skimmed milk; and skimmed milk with PS (MPS), composed of reconstituted acidified skimmed milk, PS, and water to substitute fruit juices. The nutritional composition of samples is shown is Table 1. Fruit beverages were heat treated at 100−115 °C for 15−30 s to obtain microbiologically stable foodstuff, and were packed under aseptic conditions into sterile 100 mL polypropylene plastic containers. Samples were analyzed just after manufacture (time 0) and were then stored at 4, 24, or 37 °C and analyzed at regular time intervals of 2 months until 6 months. The storage time of 6 months was chosen because the manufacturer of PS-enriched beverages indicated that within this period the organoleptic properties in samples are preserved. In addition, this is the common and usual turnover period of this kind of products in sales points, where products are stored no longer than 6 months.

Table 1. Nutritional Composition of Plant Sterol-Enriched Beverages (Values Presented as per 100 g of Beverage)

	MFJPS	FJPS	MPS
kcal	72	30	48
proteins	2.7	0.5	1.6
carbohydrates	14.3	6.1	2.5
fat	0.4	0.2	0.2

Plant Sterols Determination. Lipids were extracted according to the procedure of Boselli et al.[17] A weight of sample (15 g) providing approximately 120 mg of PS was taken. Lipids were extracted with 80 mL of chloroform/methanol (1:1, v/v) containing 0.05% of BHT (which was added as antioxidant to avoid PS oxide artifact formation during saponification) and homogenized with a Polytron homogenizer (PT 2000, Kinematica AC, Switzerland) during 3 min at 250 W. After adding 40 mL of chloroform and homogenizing again, the sample was filtered (Whatman no. 1 90 mm, Maidstone, England) with a Buchner funnel. To the filtrate, 20 mL of 1 M KCl solution was added and refrigerated overnight. Then, the chloroform phase was concentrated in a rotary evaporator and dried with nitrogen. The lipid fraction was then reconstituted with hexane/2-propanol (4:1, v/v), dividing the sample into four aliquots for saponification. From here onward, the methodology employed and the chromatographic conditions used have been described previously by González-Larena et al.[18] For quantification, phytosterol calibration curves of campesterol (98.6−550.2 μg), stigmasterol (9.5−99.8 μg), and β-sitosterol (700.6−2398.8 μg) containing 200 μg of IS (epicoprostanol) were performed, with obtained linear regression coefficients of $r^2 > 0.985$ for all analytes. Quantification of phytostanols (campestanol and sitostanol) was performed using the response factor (R_f = 1.12) calculated for stigmastanol standard versus epicoprostanol (IS). The accuracy of the method was investigated by analyzing MFJPS, FJPS, and MPS after the addition of 90 μg of campesterol, 60 μg of stigmasterol, 900 μg of β-sitosterol, and 190 μg of sitostanol standards before the saponification step and calculating the recoveries under consideration of the PS content of the unspiked sample.[18] Accuracy, estimated by recovery assays and expressed as percentages as the mean ± standard deviation of three replicates, ranged for main plant sterols (β-sitosterol, β-sitostanol, and campesterol) between 100 and 105% for MPS and MFJPS samples, and between 79.3 and 82.7% for FJPS. In the case of stigmasterol, recovery percentages ranged between 126.4 and 75.9% for all samples studied.

Total Carotenoid Determination. For total carotenoid content determination, we used the colorimetric method described by Lee et al.[19] Briefly, 2.5 mL aliquots of beverages were mixed with 5 mL of the extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v/v) and shaken vigorously during 1 min. Then, the mixture was centrifuged for 5 min at 4000 rpm at 5 °C. The top layer of hexane containing the color was recovered and transferred to glass tubes protected from light and homogenized with a Pasteur pipet. After that, 1 mL of this supernatant was transferred to a 25 mL volumetric flask, and the volume was completed with hexane. Total carotenoid was determined by measuring the absorbance at 450 nm in a UV−vis spectrophotometer (Lambda 2, Perkin-Elmer, Ueberlingen, Germany). Total carotenoid content was calculated as mg of β-carotene/100 mL of beverage using an extinction coefficient of β-carotene, $E^{1\%} = 2505$.

Total Polyphenol Determination. Total polyphenolic compounds were determined spectrophotometrically with Folin−Ciocalteu reagent according to the method of Cilla et al.,[20] with slight modifications. Dilutions of 1:10 for fruit beverages with deionized water were used, followed by centrifugation at 11000 rpm for 5 min at 4 °C. For the colorimetric assay, to 100 μL of supernatant we added 3 mL of Na_2CO_3 solution (2% w/v) and 100 μL of Folin−Ciocalteu's phenols reagent (50% v/v). After incubation for 1 h in darkness at room temperature, absorbance detection was carried out at 765 nm using a UV−vis spectrophotometer. Results were expressed as milligrams of gallic acid equivalents (GAE) per liter. To remove possible interferences of milk in the Folin−Ciocalteu method, the value obtained for the MPS sample (with the same milk content as the MFJPS sample) was subtracted from that of the MFJPS sample.

Total Antioxidant Capacity (TAC) Determination. The TAC of the beverages was measured by using the Trolox equivalent antioxidant capacity (TEAC) method for hydrophilic (as described by Cilla et al.[20]) and lipophilic (as described by García-Alonso et al.[21]) extracts. Briefly, a stable stock solution of $ABTS^{\bullet+}$ was generated by reacting 7 mM ABTS with 140 mM potassium persulfate and allowing it to stand for 12−16 h in the dark at room temperature. The $ABTS^{\bullet+}$ working solution was prepared by diluting the stock solution with ethanol to

Table 2. Plant Sterol Contents (g/100 g of Sample) in PS-Enriched Beverages Just after Manufacture and after Storage[a]

	month	temperature	MFJPS	FJPS	MPS
campesterol	0		0.034 ± 0.001	0.040 ± 0.002	0.037 ± 0.004
	2	4 °C	0.037 ± 0.002	0.036 ± 0.001	0.037 ± 0.001
		24 °C	0.035 ± 0.003	0.039 ± 0.002	0.039 ± 0.002
		37 °C	0.038 ± 0.004	0.041 ± 0.002	0.037 ± 0.001
	4	4 °C	0.036 ± 0.002	0.040 ± 0.002	0.036 ± 0.002
		24 °C	0.037 ± 0.001	0.038 ± 0.001	0.042 ± 0.001
		37 °C	0.038 ± 0.001	0.037 ± 0.001	0.034 ± 0.002
	6	4 °C	0.037 ± 0.005	0.040 ± 0.001	0.038 ± 0.001
		24 °C	0.036 ± 0.001	0.038 ± 0.003	0.034 ± 0.001
		37 °C	0.038 ± 0.001	0.041 ± 0.001	0.038 ± 0.001
stigmasterol	0		0.010 ± 0.001	0.011 ± 0.001	0.010 ± 0.001
	2	4 °C	0.010 ± 0.001	0.010 ± 0.001	0.010 ± 0.001
		24 °C	0.010 ± 0.001	0.011 ± 0.001	0.011 ± 0.001
		37 °C	0.011 ± 0.001	0.011 ± 0.001	0.010 ± 0.001
	4	4 °C	0.010 ± 0.001	0.011 ± 0.001	0.010 ± 0.001
		24 °C	0.010 ± 0.001	0.010 ± 0.001	0.011 ± 0.001
		37 °C	0.011 ± 0.001	0.010 ± 0.001	0.009 ± 0.001
	6	4 °C	0.010 ± 0.001	0.011 ± 0.001	0.010 ± 0.001
		24 °C	0.010 ± 0.001	0.011 ± 0.001	0.009 ± 0.001
		37 °C	0.011 ± 0.001	0.011 ± 0.001	0.010 ± 0.001
β-sitosterol	0		0.486 ± 0.014	0.556 ± 0.036	0.514 ± 0.054
	2	4 °C	0.509 ± 0.025	0.505 ± 0.029	0.517 ± 0.015
		24 °C	0.495 ± 0.039	0.538 ± 0.036	0.551 ± 0.038
		37 °C	0.548 ± 0.064	0.561 ± 0.021	0.519 ± 0.021
	4	4 °C	0.496 ± 0.049	0.558 ± 0.031	0.507 ± 0.028
		24 °C	0.509 ± 0.013	0.520 ± 0.004	0.589 ± 0.008
		37 °C	0.503 ± 0.021	0.511 ± 0.017	0.492 ± 0.026
	6	4 °C	0.518 ± 0.071	0.566 ± 0.012	0.536 ± 0.026
		24 °C	0.496 ± 0.009	0.534 ± 0.039	0.477 ± 0.040
		37 °C	0.540 ± 0.020	0.569 ± 0.010	0.522 ± 0.009
campestanol	0		0.008 ± 0.001	0.009 ± 0.001	0.008 ± 0.001
	2	4 °C	0.008 ± 0.001	0.008 ± 0.001	0.008 ± 0.001
		24 °C	0.008 ± 0.001	0.009 ± 0.001	0.009 ± 0.001
		37 °C	0.009 ± 0.001	0.009 ± 0.001	0.008 ± 0.001
	4	4 °C	0.008 ± 0.001	0.009 ± 0.001	0.008 ± 0.001
		24 °C	0.008 ± 0.001	0.008 ± 0.001	0.009 ± 0.001
		37 °C	0.008 ± 0.001	0.008 ± 0.001	0.008 ± 0.001
	6	4 °C	0.008 ± 0.001	0.009 ± 0.001	0.009 ± 0.001
		24 °C	0.008 ± 0.001	0.009 ± 0.001	0.008 ± 0.001
		37 °C	0.008 ± 0.001	0.009 ± 0.001	0.008 ± 0.001
sitostanol	0		0.077 ± 0.002	0.088 ± 0.005	0.082 ± 0.008
	2	4 °C	0.079 ± 0.004	0.079 ± 0.004	0.081 ± 0.002
		24 °C	0.077 ± 0.006	0.083 ± 0.006	0.086 ± 0.006
		37 °C	0.085 ± 0.011	0.085 ± 0.002	0.081 ± 0.004
	4	4 °C	0.077 ± 0.008	0.086 ± 0.005	0.079 ± 0.005
		24 °C	0.080 ± 0.002	0.080 ± 0.002	0.090 ± 0.001
		37 °C	0.078 ± 0.003	0.080 ± 0.003	0.077 ± 0.004
	6	4 °C	0.080 ± 0.010	0.087 ± 0.002	0.083 ± 0.004
		24 °C	0.077 ± 0.001	0.083 ± 0.006	0.074 ± 0.001
		37 °C	0.084 ± 0.003	0.087 ± 0.001	0.081 ± 0.001

[a]Values expressed as the mean ± standard deviation (n = 3). MFJPS = skimmed milk with fruit juice; FJPS = fruit juice; MPS = skimmed milk.

attain an absorbance of 0.700 ± 0.020 at 734 nm, measured at 30 °C. Afterward, 2 mL of this solution was pipetted in a spectrophotometric cell, and the absorbance was read at this initial point of reaction (A_0) in a thermostatized UV−vis spectrophotometer (Lambda 2). Samples were diluted with distilled−deionized water to obtain a percentage inhibition of absorbance of approximately 50%. Then, 100 μL of hydrophilic, lipophilic, or the Trolox standard solutions (ethanolic solutions between 0 and 250 μM) was added, and the absorbance was measured at the end time of 3 min (A_f). Percentages of absorbance inhibition were calculated as follows: $(1 - A_f/A_0) \times 100$. Results for TAC (sum of hydrophilic plus lipophilic antioxidant capacity) were expressed as μM Trolox. To obtain the hydrophilic extract, beverages were diluted 1:10 with deionized water and centrifuged at 11000 rpm for 5 min at 4 °C. The supernatant was tested for TAC. The lipophilic extracts, in turn, were obtained by homogenizing 1 mL of beverage with 9 mL of the hexane/acetone/methanol mixture (2:1:1, v/v/v) for 30 min protected from light. Then, 7.5 mL of water was added, and the mixture was centrifuged at 5000 rpm for 5 min. The top layer

containing the lipophilic fraction was transferred into a 10 mL volumetric flask and the volume completed with hexane. Finally, a 1 mL aliquot of the lipophilic fraction was dried under a nitrogen stream and then reconstituted in 0.5 mL of 2-propanol before being analyzed for TAC.

Color Determination. Color was measured using a Hunter Labscan II Colorimeter (Hunter Associates Laboratory, Inc.). Results were expressed according to the CIELAB system with reference to illuminant D65 and a visual angle of 10°. The parameters determined were L* (luminosity or brightness: L* = 0 black and L* = 100 white), a* (red-green component: −a* = greenness and +a* = redness), and b* (yellow-blue component: −b* = blueness and +b* = yellowness). The difference in color between two samples (ΔE) was given by the expression: $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$.[22] To evaluate color changes, ΔE^* was calculated, where L*, a*, and b* values at the considered storage time were taken with respect to those obtained in just manufactured samples (time zero).

Statistical Analysis. The sample determinations were conducted in triplicate, with values reported as means ± standard deviation. Three-way (sample, temperature, and storage time) analysis of variance (ANOVA) was applied to the results obtained, followed by Tukey's posthoc test. A significance level of $p < 0.05$ was adopted for all comparisons. Statgraphics Plus, version 5.1 (Rockville, Maryland, USA), was used.

RESULTS AND DISCUSSION

Plant Sterols. The phytosterol and phytostanol contents in the beverages analyzed are reported in Table 2. Because of the fact that all matrixes were enriched with the same source of PS (tall oil), they showed the same PS profile. Likewise, an example of the PS profile of one of the samples analyzed is shown in Figure 1.

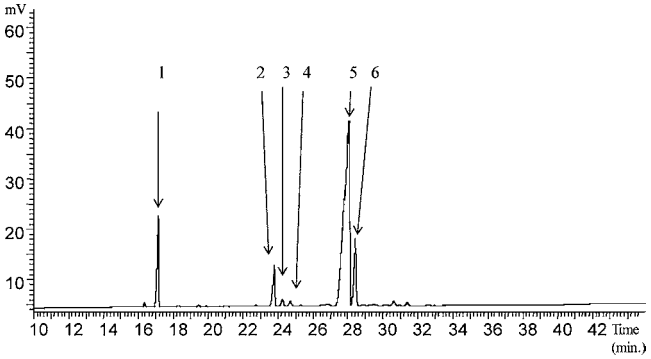

Figure 1. Plant sterol determination by GC-FID in skimmed milk with tangerine fruit juice and plant sterols (MFJPS). 1, epicoprostanol (IS); 2, campesterol; 3, campestanol; 4, stigmasterol; 5, β-sitosterol; 6, sitostanol.

Total PS contents ranged from 0.61 to 0.74 g/100 g sample, and β-sitosterol (mean value of 0.524 g/100 g sample) was the most abundant phytosterol, followed by campesterol (0.038 g/100 g) and stigmasterol (0.010 g/100 g). In addition, two phytostanols were detected: sitostanol (0.082 g/100 g) and, to a lesser extent, campestanol (0.008 g/100 g); however, no brassicasterol was detected. The PS profile obtained agrees with that of the tall oil ingredient used as a source of PS in the enrichment of the beverages, previously characterized by our group.[18] This profile also agrees with the observations of previous studies in relation to tall oil enriched beverages. In this sense, the PS profile reported by Maki et al.[8] was β-sitosterol (44%) > sitostanol (25%) > campesterol (12%) > campestanol (6%), and Jones et al.[9] found β-sitosterol to be the most abundant PS, followed by sitostanol and campestanol. However, no campesterol in the beverages of Jones et al.[9] and no stigmasterol in both studies were detected. Furthermore, the percentage of the different PS obtained in our samples complied with current legislation: < 80% β-sitosterol, < 40% campesterol, < 30% stigmasterol, < 3% brassicasterol, < 15% sitostanol, < 5% campestanol, and <3% other sterols/stanols.[13]

A three-way ANOVA (sample, temperature, and time of storage) was applied to total PS and individual PS contents in the three PS-enriched beverages. There were no significant differences considering time and temperature factors for total PS and individual PS. Therefore, no loss in initial total PS content had occurred during 6 months of storage, with values being the same at any given storage time point (see Table 2). Considering the sample factor, FJPS beverages showed higher values ($p < 0.05$) than the MFJPS samples for total PS content, with MPS samples not differing with both of them (Figure 2). Likewise, when each PS was considered individually, significant differences were found among samples. FJPS beverages showed a higher content ($p < 0.05$) than MFJPS samples for all individual PS studied. In addition, FJPS showed a greater content ($p < 0.05$) than MPS for all phytosterols (campesterol, stigmasterol, and β-sitosterol), but not for phytostanols (campestanol and sitostanol). Taking into account that PS were added with the same amount to the beverages, it seems that a probable matrix effect could explain the differences found among samples though these differences can be considered without physiological relevance.

Therefore, the samples studied (MFJPS, FJPS, and MPS) were stable under the assayed conditions. Some nonsignificant decreases in PS contents of FJPS and MPS samples at 24 °C were detected, with variations no greater than 9%. These slight decreases in PS content during storage were of the same order as those reported by other authors. A study with a commercial milk enriched with phytosterols treated under Schaal oven conditions (65 °C/24 h, equivalent to one month of storage at room temperature) reported values only 4% lower than the control, due to the antioxidants (such as vitamin E) included in the formulation, in contrast to the greater decrease (60%) observed with a drastic heating treatment (90 °C/15 min); in both cases, there were statistically significant differences among treatments and with the control.[6] Moreover, a study addressing the oxidative stability of PS in phytosterol-enriched heat-treated milk stored during 6 months at room temperature (20 °C) and in a refrigerator (4 °C)[5] showed rather similar changes at both temperatures, with PS decreases between 1.3% and 10%. In both studies, the authors found low levels of several phytosterol oxidation products, but this fact did not imply an apparent drop in phytosterols amount; in this sense, their results were in agreement with the fact that PS stability was well guaranteed during the shelf life of the product.

Antioxidant Parameters. Total carotenoids of beverages only containing fruit juice (MFJPS and FJPS) (since carotenoids content in MPS is negligible) enriched with PS during storage at 4, 24, and 37 °C over a 6 month period are presented in Table 3.

The mean total carotenoid contents of the samples analyzed in the present study comprised between 0.054 and 0.265 mg/100 mL, these being values that fall within the range of 0.009 to 26.1 mg/100 mL previously described for different juice−milk beverages containing fruits of citrus origin in their formulation and marketed in Spain.[23] An ANOVA with three factors

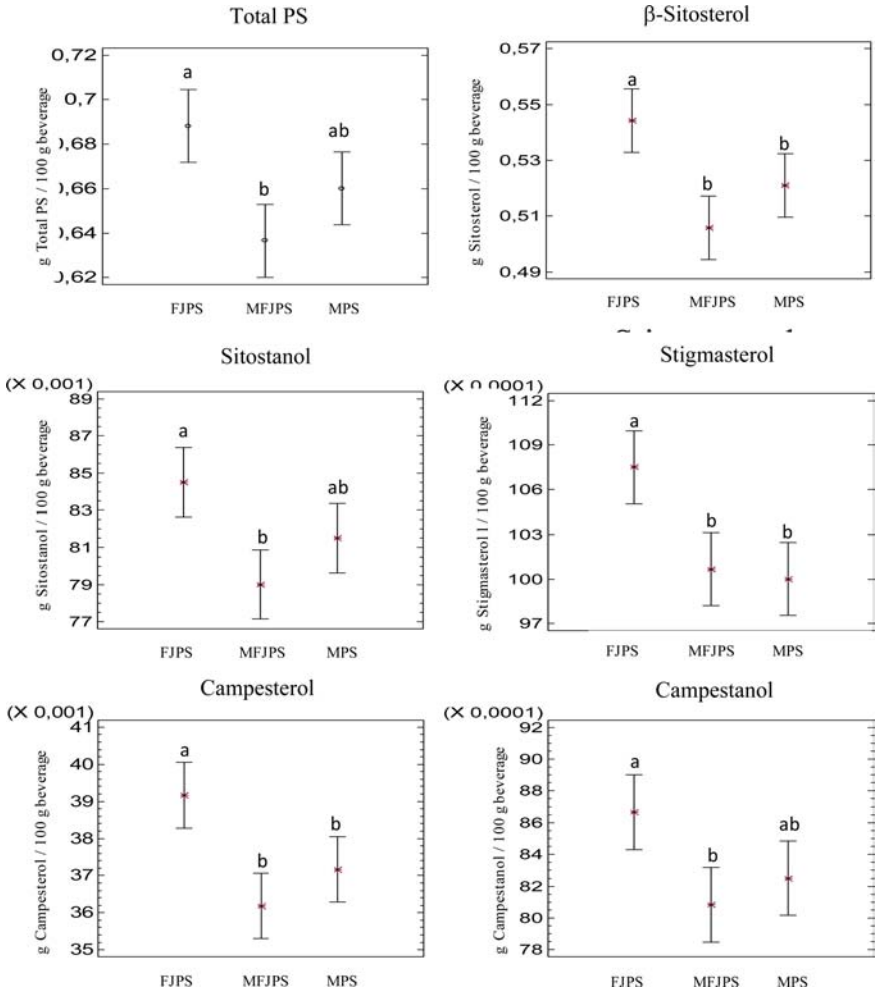

Figure 2. Total and individual PS contents (g/100 g beverage). Tukey's test: intervals for the means (sample factor). Noncoincidences of letters in each plot indicate statistically significant differences. MFJPS: skimmed milk with fruit juice and plant sterols. FJPS: fruit juice with plant sterols. MPS: skimmed milk with plant sterols.

Table 3. Total Carotenoids (mg *β*-Carotene/100 mL Beverage) and Total Polyphenols (mg GAE/100 mL Beverage) in PS-Enriched Beverages Just after Manufacture and after Storage[a]

sample	0 months		2 months	4 months	6 months
			Total Carotenoids		
MFJPS		4 °C	0.097 ± 0.013	0.131 ± 0.021	0.136 ± 0.007
	0.112 ± 0.011	24 °C	0.084 ± 0.004	0.082 ± 0.009	0.095 ± 0.008
		37 °C	0.067 ± 0.002	0.054 ± 0.006	0.059 ± 0.009
FJPS		4 °C	0.167 ± 0.004	0.178 ± 0.012	0.192 ± 0.013
	0.265 ± 0.043	24 °C	0.165 ± 0.008	0.137 ± 0.030	0.167 ± 0.010
		37 °C	0.148 ± 0.006	0.156 ± 0.014	0.164 ± 0.008
			Total Polyphenols		
MFJPS		4 °C	601.16 ± 24.06	554.56 ± 29.40	579.42 ± 15.94
	705.86 ± 9.44	24 °C	631.10 ± 26.28	630.67 ± 14.13	630.47 ± 23.39
		37 °C	613.63 ± 12.96	671.45 ± 38.54	603.67 ± 4.42
FJPS		4 °C	509.12 ± 30.24	542.60 ± 12.46	623.58 ± 15.94
	618.37 ± 40.33	24 °C	540.30 ± 26.46	529.01 ± 33.95	674.63 ± 15.32
		37 °C	613.88 ± 36.91	640.46 ± 26.22	666.97 ± 13.26

[a]Values are expressed as the mean ± standard deviation (n = 3). MFJPS: skimmed milk with fruit juice and plant sterols. FJPS: fruit juice with plant sterols. MPS: skimmed milk with plant sterols.

(sample, temperature, and time of storage) was applied to the results obtained (Figure 3). Considering the sample factor, FJPS beverages showed a total carotenoid content greater (p < 0.05) than that of MFJPS samples. In addition, the values decreased 36% (p < 0.05) with storage time at two months and then remained stable until the end of the storage period (6 months), the degradation of carotenoids being greater (p < 0.05) at 24 and 37 °C than at 4 °C. The greatest reduction in

dx.doi.org/10.1021/jf3002679 | J. Agric. Food Chem. 2012, 60, 4725−4734

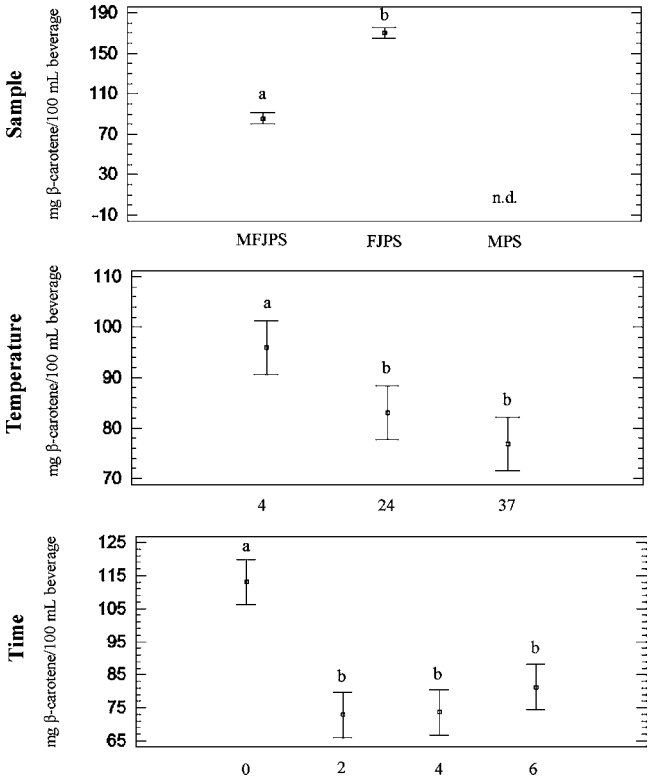

Figure 3. Total carotenoids (mg β-carotene/100 mL beverage). Tukey's test: intervals of the means (sample, temperature, and time factor). Noncoincidences of letters in each plot indicate statistically significant differences. MFJPS: skimmed milk with fruit juice and plant sterols. FJPS: fruit juice with plant sterols. MPS: skimmed milk with plant sterols.

total carotenoids (52%) (see Table 3) was observed in MFJPS stored for 4 months at 37 °C. This result is similar to that reported for a pasteurized orange juice−milk beverage stored for 42 days at 4 °C, with a 38% decrease in total carotenoids.[24] However, Plaza et al.[25] reported small losses of total carotenoids (<11%) in a pasteurized orange juice during 40 days of refrigerated storage at 4 °C. These authors relate the stability of carotenoids to the protection which ascorbic acid offers them against oxidation. In this sense, Choi et al.[26] reported that orange juice fortified with ascorbic acid (30 mg/100 mL) showed lower losses of total carotenoid content than the control juice (2.8% and 6.6%, respectively) after 7 weeks of storage at 4.5 °C. Nevertheless, in our study, the ascorbic acid contents were under 6 mg/100 mL in all just manufactured samples because ascorbic acid was not added to the beverages, and samples were thermally treated for pasteurization, these being facts that would explain a lesser protection from total carotenoids degradation, hence the differences found with the above-mentioned study.

Results concerning the changes in total polyphenols of beverages only containing fruit juice (MFJPS and FJPS) (since in MPS are negligible) enriched with PS during storage at 4, 24, and 37 °C over a 6 month period are presented in Table 3.

The mean total polyphenols of the PS-enriched beverages ranged between 509 and 706 mg GAE/L, in agreement with values reported for orange juices stored at 18, 28, and 38 °C during 6 months (507 to 684 mg CAE/L)[27] and for different juice−milk beverages containing fruits of citrus origin in their formulation (265 to 999 mg GAE/L).[23] According to the statistical analysis (three-way ANOVA) (Figure 4), MFJPS beverages had a higher ($p < 0.05$) total polyphenol content

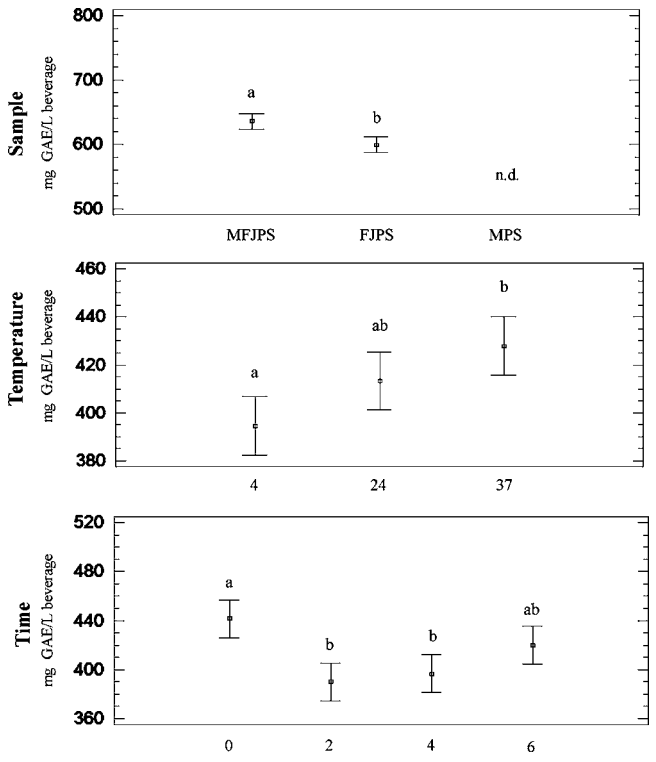

Figure 4. Total polyphenols (mg GAE/L). Tukey's test: intervals of the means (sample, temperature, and time factor). Noncoincidences of letters in each plot indicate statistically significant differences. MFJPS: skimmed milk with fruit juice and plant sterols. FJPS: fruit juice with plant sterols. MPS: skimmed milk with plant sterols.

than their counterpart sample without milk. However, considering the temperature factor, total polyphenols were surprisingly higher ($p < 0.05$) at 37 °C versus 4 °C. This same behavior has been reported for commercial tomato juices, whose total phenolic and flavonoid contents were found to be higher at 37 °C than at 8 and 22 °C after 8 months of storage.[21] These authors attribute this observation to the hypothetical formation of the Maillard reaction products via nonenzymatic browning that can react with Folin−Ciocalteu's reagent, resulting in an overestimation of phenolic compound content. In the case of the storage time factor, there were fluctuations in total polyphenols throughout the entire storage period. There was a decrease ($p < 0.05$) at two months that was maintained until four months, followed by recovery to initial values at six months. These results were in agreement with those of Klimczak et al.,[27] who reported a decline in total polyphenols of two commercial orange juices after four months of storage at 18, 28, and 38 °C, followed by a significant increase in these compounds at the end of storage (6 months), though without reaching the initial contents. Likewise, Piljac-Zegarac et al.[28] reported fluctuations in total polyphenols in different dark fruit juices during 29 days of refrigerated storage at 4 °C without a lowering of the initial phenolic content. These authors[27,28] consider that this phenomenon could be due to the formation of some compounds during storage that react with Folin−Ciocalteu's reagent, thus enhancing total phenolic content.

The total antioxidant capacity (TAC) of beverages enriched with PS is shown in Table 4.

dx.doi.org/10.1021/jf3002679 | J. Agric. Food Chem. 2012, 60, 4725−4734

Table 4. Total Antioxidant Capacity (μM Trolox) Determined by the TEAC Method in PS-Enriched Beverages Just after Manufacture and after Storage[a]

sample	0 months		2 months	4 months	6 months
MFJPS		4 °C	720.67 ± 74.44	883.71 ± 78.86	842.80 ± 45.61
	888.59 ± 9.92	24 °C	747.66 ± 74.23	1083.00 ± 83.23	1039.83 ± 94.17
		37 °C	825.97 ± 60.39	882.88 ± 44.54	870.94 ± 39.50
FJPS		4 °C	727.62 ± 72.42	590.87 ± 59.72	723.51 ± 29.68
	572.71 ± 24.64	24 °C	675.66 ± 62.50	712.25 ± 58.50	717.29 ± 45.06
		37 °C	766.23 ± 88.97	772.80 ± 52.70	931.82 ± 71.44
MPS		4 °C	10.26 ± 0.59	223.31 ± 10.65	206.34 ± 9.19
	107.87 ± 9.92	24 °C	15.24 ± 1.18	142.39 ± 1.74	202.40 ± 1.96
		37 °C	20.22 ± 4.68	135.19 ± 13.55	211.41 ± 3.19

[a]Values are expressed as the mean ± standard deviation (n = 3). MFJPS: skimmed milk with fruit juice and plant sterols. FJPS: fruit juice with plant sterols. MPS: skimmed milk with plant sterols.

The mean TAC ranged between 573 and 1083 μM Trolox for MFJPS and FJPS and between 10 and 223 μM Trolox for MPS. Results of fruit juice-containing beverages (MFJPS and FJPS) are coincident with those previously reported for different juice−milk beverages containing fruits of citrus origin in their formulation (610 to 3600 μM Trolox),[23] while results of milk beverage (MPS) agree with those for hydrophilic antioxidant capacity reported in UHT full-fat milk (118 μM Trolox), with negligible lipophilic antioxidant capacity values.[29] They furthermore also agree with the hydrophilic TEAC results of skimmed milk (~27 μM Trolox) of three beverages based on fruit juice, milk, and cereals.[30] In general, hydrophilic antioxidant activity accounts for >90% of the total antioxidant capacity of fruits and vegetables.[31] Accordingly, fruit juice-containing beverages (MFJPS and FJPS) showed values of hydrophilic antioxidant activity ranging between 87 and 98%. In the case of milk beverage (MPS), with the exception of the two months time period in which hydrophilic antioxidant capacity accounted for 100%, these values ranged between 61 and 79%.

The pronounced decrease in MPS at 2 months of storage could be related to the heat treatment of milk in the manufacture process which can promote an increase in its pro-oxidant activity, mainly due to the formation of novel oxidative molecules in the early stages of the Maillard reaction.[32] In turn, a sample containing fruit juice and milk (MFJPS) showed a low decrease in TAC at this time point probably due to the interaction between polyphenol and milk. Accordingly, it has been reported that these kinds of interactions may maintain the total antioxidant capacity of beverages subjected to thermal processing.[33]

Taking into account the statistical analysis applied (three-way ANOVA) (Figure 5), samples ranked in the following order: MFJPS > FJPS ≫ MPS (p < 0.05). There were no differences considering the temperature factor (p > 0.05), though in relation to the storage time factor, fluctuations were seen in TAC throughout the storage period. There was a nonsignificant descent at two months, followed by recovery (p < 0.05) of the initial values at four months, and a final increase was recorded at 18% (p < 0.05) in TAC versus the initial values at 6 months of storage. These results are in agreement with the 13% increase in TAC measured by the TEAC method, between the beginning and end of storage (4.5 months at 4 °C) of fruit beverages with/without iron and/or zinc and/or milk.[20] This fact seems to be related to the formation of novel compounds with antioxidant activity (later Maillard reaction products formed during prolonged storage possess potent antioxidant properties).[27,34] Besides, changes in the protein structure can increase the antioxidant capacity since casein and whey proteins have been regarded to have antioxidant activity.[35,36]

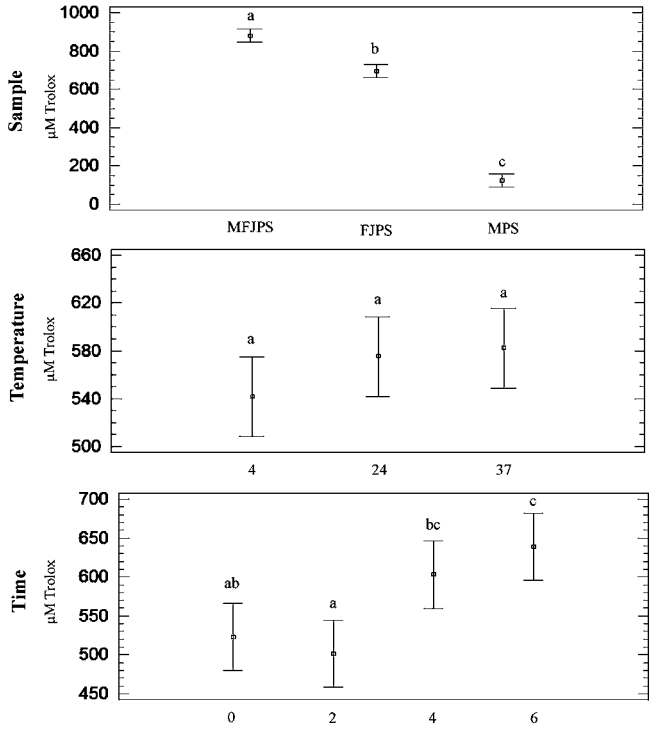

Figure 5. Total antioxidant capacity (μM Trolox) (TEAC method). Tukey's test: intervals of the means (sample, temperature, and time factor). Noncoincidences of letters in each plot indicate statistically significant differences. MFJPS: skimmed milk with fruit juice and plant sterols. FJPS: fruit juice with plant sterols. MPS: skimmed milk with plant sterols.

Increased browning during the production and/or storage of processed foods is influenced by diverse factors such as Maillard and enzymatic browning, ascorbic acid degradation, and the polymerization of anthocyanins with other phenolics.[37] Thus, in order to clarify whether increases in total polyphenols and TAC are related to browning derived from Maillard reaction product formation, color differences (ΔE) were determined (Table 5).

On considering the statistical analysis applied (three-way ANOVA), ΔE showed differences (p < 0.05) (Figure 6) between fruit juice-containing beverages (MFJPS and FJPS) and milk beverage (MPS), the latter showing the lowest changes. Concerning the temperature factor, there were

dx.doi.org/10.1021/jf3002679 | J. Agric. Food Chem. 2012, 60, 4725−4734

Table 5. Changes in Color in PS-Enriched Beverages Just after Manufacture and after Storage: Difference in Color (ΔE) Parameter[a]

sample		2 months	4 months	6 months
MFJPS	4 °C	2.28 ± 0.05	2.92 ± 0.08	3.08 ± 0.01
	24 °C	2.44 ± 0.01	4.96 ± 0.01	5.04 ± 0.01
	37 °C	5.80 ± 0.03	8.72 ± 0.01	10.89 ± 0.02
FJPS	4 °C	0.99 ± 0.08	2.31 ± 0.11	0.78 ± 0.02
	24 °C	3.28 ± 0.24	4.16 ± 0.10	7.40 ± 0.04
	37 °C	7.16 ± 0.06	12.24 ± 0.02	15.90 ± 0.02
MPS	4 °C	2.37 ± 0.11	2.46 ± 0.02	2.00 ± 0.02
	24 °C	2.45 ± 0.05	2.65 ± 0.09	1.36 ± 0.01
	37 °C	2.16 ± 0.01	1.67 ± 0.25	5.76 ± 0.01

[a]Values are expressed as the mean ± standard deviation (n = 3). MFJPS: skimmed milk with fruit juice and plant sterols. FJPS: fruit juice with plant sterols. MPS: skimmed milk with plant sterols.

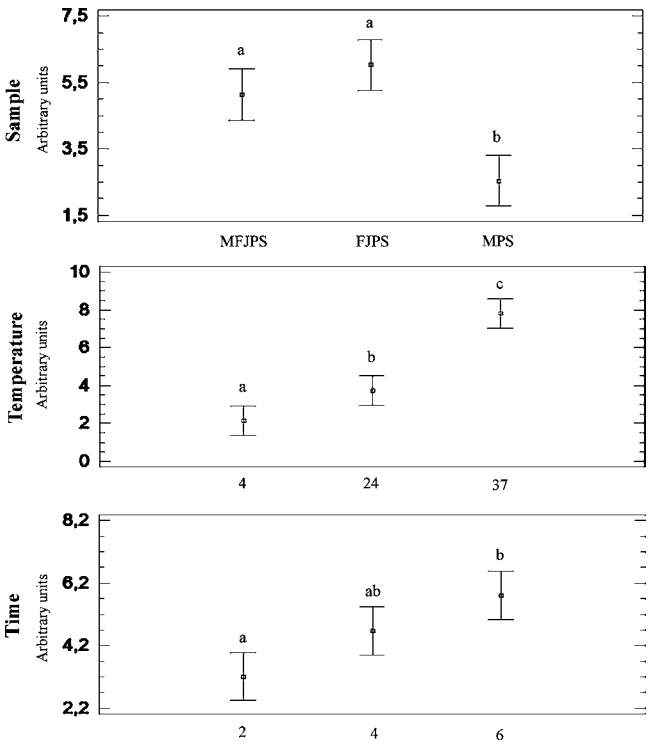

Figure 6. Color differences (ΔE). Tukey's test: intervals of the means (sample, temperature, and time factor). Noncoincidences of letters in each plot indicate statistically significant differences. MFJPS: skimmed milk with fruit juice and plant sterols. FJPS: fruit juice with plant sterols. MPS: skimmed milk with plant sterols.

temperature-dependent modifications in ΔE (being greatest at 37 °C). This fact is coincident with the temperature-dependent increase in total polyphenols (see Figure 4), which could correlate the increase in polyphenols at 37 °C with the formation of Maillard reaction products via nonenzymatic browning. Differences in ΔE also increased over time (with statistically significant differences ($p < 0.05$) between 2 and 6 months), in agreement with the results reported in a dessert made from dark-red concentrated juices resulting in a color change to a more brownish shade.[37] This observation can explain the progressive increase in total polyphenols and TAC seen from month 2 to month 6 (see Figures 4 and 5), ascribed to later Maillard reaction products formed during prolonged storage.

Another important issue to address is the linkage between PS stability and antioxidant parameters. To our knowledge, there are no studies relating PS stability in fruit−milk beverages with/without storage. However, it has been reported that natural antioxidants such as green tea catechins, α-tocopherol, and quercetin,[38] or rosemary and green tea extracts[39] exert protective effects upon the oxidative stability of PS during heating in food matrixes of fat origin such as lard, corn oil, and olive oil, or rapeseed oil, respectively; however, in these studies, storage has not been assessed.

The determination of total carotenoids, total polyphenols, total antioxidant capacity, and color can serve as complementary measures to check PS stability during product shelf life since depending on the evolution in the content of antioxidants in the whole period of study, the fate of PS content can be explained. In light of the results obtained in this study, the increase in total antioxidant capacity, due to the formation of Maillard reaction products, as proved by color parameters, might have helped in the maintenance of PS contents throughout the entire storage period. Nevertheless, authors have not found a correlation between PS contents and TAC values.

In summary, it can be concluded that the studied storage times and temperatures did not affect PS stability of the PS-enriched beverages, these beverages being an adequate PS source throughout the storage period studied. Therefore, the inclusion of these functional ingredients in a food matrix containing antioxidant compounds (polyphenols, carotenoids, and antioxidant proteins) would be a good choice for maintaining the initial PS contents, and the functional beverages could be ingested whenever during the period of their shelf life, making the most out of the potential health benefits of PS.

AUTHOR INFORMATION

Corresponding Author

*Tel: +34-963544909. Fax: +34-3544954. E-mail: m.j.lagarda@uv.es.

Funding

This study was financed by AGL2008-02591-C02-01 (CICYT-FEDER) and partially funded by Consolider Fun-C-Food CSD2007-00063. M.G.-L. holds a Danone grant from Instituto Danone (Spain). Thanks are also due to the Generalitat Valenciana (Spain) for financial support (GVACOMP2011-195) of the Bionutest group.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Hero España, S.A. for providing the beverages tested in the present study.

REFERENCES

(1) García-Llatas, G.; Rodríguez-Estrada, M. T. Current and new insights on phytosterol oxides in plant sterol-enriched food. *Chem. Phys. Lipids* **2011**, *164*, 607−624.

(2) Hernández-Mijares, A.; Bañuls, C.; Rocha, M.; Morillas, C.; Martínez-Triguero, M. L.; Víctor, V. M.; Lacomba, R.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J. Effects of phytosterol ester-enriched low-fat milk on serum lipoprotein profile in mildly hypercholesterolaemic patients are not related to dietary cholesterol or saturated fat intake. *Br. J. Nutr.* **2010**, *11*, 1−8.

(3) Clifton, P. M.; Noakes, M.; Sullivan, D.; Erichsen, N.; Ross, D.; Annison, G.; Fassoulakis, A.; Cehun, M. Cholesterol-lowering effects of plant sterol esters differ in milk, yoghurt, bread and cereal. *Eur. J. Clin. Nutr.* **2004**, *58*, 503−509.
(4) Noakes, M.; Clifton, P. M.; Doornbos, A. M. E.; Trautwein, E. A. Plant sterol ester-enriched milk and yoghurt effectively reduce serum cholesterol in modestly hypercholesterolemic subjects. *Eur. J. Nutr.* **2005**, *44*, 214−222.
(5) Soupas, L.; Huikko, L.; Lampi, A.-M.; Piironen, V. Oxidative stability of phytosterols in some food applications. *Eur. Food Res. Technol.* **2006**, *222*, 266−273.
(6) Menendez-Carreño, M.; Ansorena, D.; Astiasarán, I. Stability of sterols in phytosterol-enriched milk under different heating conditions. *J. Agric. Food Chem.* **2008**, *56*, 9997−10002.
(7) Barnsteiner, A.; Lubinus, T.; Di Gianvito, A.; Schmid, W.; Engel, K.-H. GC-based analysis of plant Stanyl fatty acid esters in enriched foods. *J. Agric. Food Chem.* **2011**, *59*, 5204−5214.
(8) Maki, K. C.; Shinnick, F.; Seeley, M. A.; Veith, P. E.; Quinn, L. C.; Hallissey, P. J.; Temer, A.; Davidson, M. H. Food products containing free tall oil-based phytosterols and oat beta-glucan lower serum total and LDL cholesterol in hypercholesterolemic adults. *J. Nutr.* **2003**, *133*, 808−813.
(9) Jones, P. J. H.; Vanstone, C. A.; Raeini-Sarjaz, M.; St-Onge, M.-P. Phytosterols in low- and nonfat beverages as part of a controlled diet fail to lower plasma lipid levels. *J. Lipid Res.* **2003**, *44*, 1713−1719.
(10) Shin, M. J.; Rim, S. J.; Jang, Y.; Choi, D.; Kang, S. M.; Cho, S. Y.; Kim, S. S.; Kim, D. K.; Song, K.; Chung, N. The cholesterol-lowering effect of plant sterol-containing beverage in hypercholesterolemic subjects with low cholesterol intake. *Nutr. Res. (N.Y.)* **2003**, *23*, 489−496.
(11) Devaraj, S.; Jialal, I.; Vega-López, S. Plant sterol-fortified orange juice effectively lowers cholesterol levels in mildly hypercholesterolemic healthy individuals. *Arterioscler., Thromb., Vasc. Biol.* **2004**, *24*, 25−28.
(12) Devaraj, S.; Autret, B. C.; Jialal, I. Reduced-calorie orange fruit beverage with plant sterols lowers C-reactive protein concentrations and improves the lipid profile in human volunteers. *Am. J. Clin. Nutr.* **2006**, *84*, 756−761.
(13) Commission Decision 2004/336/EC of 31 March 2004 authorizing the placing on the market of yellow fat spreads, milk based fruit drinks, yoghurt type products and cheese type products with added phytosterols/phytostanols as novel foods or novel food ingredients under Regulation (EC) No 258/97 of the European Parliament and of the Council. *Off. J. Eur. Comm.* **2004**, *L105*, 49−51.
(14) Normen, L; Frohlich, J. Role of Plant Sterols in Cholesterol Lowering. In *Phytosterols as Functional Food Components and Nutraceuticals*; Dutta, C., Ed.; Marcel Dekker Inc.: New York, NY, 2004; pp 243−315.
(15) Saraiva, D.; da Conceiçao Castilho, M.; da Rosário Martins, M.; Noronha da Silveira, M. I.; Ramos, F. Evaluation of phytosterols in milk and yogurts used as functional foods in Portugal. *Food Anal. Methods* **2011**, *4*, 28−34.
(16) Clement, L. M.; Hansen, S. L.; Costin, C. D.; Perri, G. L. Quantitation of sterols and steryl esters in fortified foods and beverages by GC/FID. *J. Am. Oil Chem. Soc.* **2010**, *87*, 973−980.
(17) Boselli, E.; Velazco, V.; Caboni, M. F.; Lercker, G. Pressurized liquid extraction of lipids for the determination of oxysterols in egg-containing food. *J. Chromatogr., A* **2001**, *917*, 239−244.
(18) González-Larena, M.; García-Llatas, G.; Vidal, M. C.; Sánchez-Siles, L. M.; Barberá, R.; Lagarda, M. J. Stability of plant sterols in ingredients used in functional foods. *J. Agric. Food Chem.* **2011**, *59*, 3624−3631.
(19) Lee, H. S.; Castle, W. S.; Coates, G. A. High performance liquid chromatography for the characterization of carotenoids in the new sweet orange (Earlygold) grown in Florida, USA. *J. Chromatogr., A* **2001**, *913*, 371−377.
(20) Cilla, A.; Perales, S.; Lagarda, M. J.; Barberá, R.; Clemente, G.; Farré, R. Influence of storage and *in vitro* gastrointestinal digestion on total antioxidant capacity of fruit beverages. *J. Food Compos. Anal.* **2011**, *24*, 87−94.
(21) García-Alonso, F. J.; Bravo, S.; Casas, J.; Pérez-Conesa, D.; Jacob, K.; Periago, M. J. Changes in antioxidant compounds during the shelf life of commercial tomato juices in different packaging materials. *J. Agric. Food Chem.* **2009**, *57*, 6815−6822.
(22) Calvo, C. Optical Properties. In *Handbook of Food Analysis. Physical Characterization and Nutrient Analysis*; Nollet, L. M. L., Ed.; Marcel Dekker, Inc.: New York, NY, 2004; Vol. *1*, pp 1−19.
(23) Zulueta, A.; Esteve, M. J.; Frasquet, I.; Frigola, A. Vitamin C, vitamin A, phenolic compounds and total antioxidant capacity of new fruit juice and skim milk mixture beverages marketed in Spain. *Food Chem.* **2007**, *103*, 1365−1374.
(24) Zulueta, A.; Barba, F. J.; Esteve, M. J.; Frígola, A. Effects on the carotenoid pattern and vitamin A of a pulsed electric field-treated orange juice-milk beverage and behaviour during storage. *Eur. Food Res. Technol.* **2010**, *231*, 525−534.
(25) Plaza, L.; Sánchez-Moreno, C.; De Ancos, B.; Elez-Martínez, P.; Martín-Belloso, O.; Cano, M. P. Carotenoid and flavanone content during refrigerated storage of orange juice processed by high-pressure, pulsed electric fields and low pasteurization. *LWT-Food Sci. Technol.* **2011**, *44*, 834−839.
(26) Choi, M. H.; Kim, G. H.; Lee, H. S. Effects of ascorbic acid retention on juice color and pigment stability in blood orange (*Citrus sinensis*) juice during refrigerated storage. *Food Res. Int.* **2002**, *35*, 753−759.
(27) Klimczak, I.; Malecka, M.; Szlachta, M.; Gliszczynska-Swiglo, A. Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. *J. Food Compos. Anal.* **2007**, *20*, 313−322.
(28) Piljac-Zegarac, J.; Valek, L.; Martinez, S.; Belscak, A. Fluctuations in the phenolic content and antioxidant capacity of dark fruit juices in refrigerated storage. *Food Chem.* **2009**, *113*, 394−400.
(29) Pulido, R.; Hernández-García, M.; Saura-Calixto, F. Contribution of beverages to the intake of lipophilic and hydrophilic antioxidants in the Spanish diet. *Eur. J. Clin. Nutr.* **2003**, *57*, 1275−1282.
(30) Perales, S.; Barberá, R.; Lagarda, M. J.; Farré, R. Antioxidant capacity of infant fruit beverages: influence of storage and *in vitro* gastrointestinal digestion. *Nutr. Hosp.* **2008**, *23*, 547−553.
(31) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem.* **2004**, *52*, 4026−4037.
(32) Calligaris, S.; Manzocco, L.; Anese, M.; Nicoli, M. C. Effect of heat-treatment on the antioxidant and pro-oxidant activity of milk. *Int. Dairy J.* **2004**, *14*, 421−427.
(33) Wegrzyn, T. F.; Farr, J. M.; Hunter, D. C.; Au, J.; Wohlers, M. W.; Skinner, M. A.; Stanley, R. A.; Sun-Waterhouse, D. Stability of antioxidants in an apple polyphenol-milk model system. *Food Chem.* **2008**, *109*, 310−318.
(34) Lee, H. S. Antioxidative activity of browning reaction products isolated from storage-aged orange juice. *J. Agric. Food Chem.* **1992**, *40*, 550−552.
(35) Elías, R. J.; Kellerby, S. S.; Decker, E. A. Antioxidant activity of proteins and peptides. *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 430−441.
(36) Clausen, M. R.; Skibsted, L. H.; Stagsted, J. Characterization of major radical scavenger species in bovine milk through size exclusion chromatography and functional assays. *J. Agric. Food Chem.* **2009**, *57*, 2912−2919.
(37) García-Alonso, F. J.; Periago, M. J.; Vidal-Guevara, M. L.; Cantos, E.; Ros, G.; Ferreres, F.; Abellán, P. Assessment of the antioxidant properties during storage of a dessert made from grape, cherry, and berries. *J. Food Sci.* **2003**, *68*, 1525−1530.
(38) Xu, G.; Guan, L.; Sun, J.; Chen, Z. Oxidation of cholesterol and *β*-sitosterol and prevention by natural antioxidants. *J. Agric. Food Chem.* **2009**, *57*, 9284−929.

(39) Kmiecik, D.; Korczak, J.; Rudzińska, M.; Michałowska, A. M.; Hęś, M. Stabilization of phytosterols in rapeseed oil by natural antioxidants during heating. *Eur. J. Lipid Sci. Technol.* **2009**, *111*, 1124−1132.