JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Changes in Water-Soluble Vitamins and Antioxidant Capacity of Fruit Juice−Milk Beverages As Affected by High-Intensity Pulsed Electric Fields (HIPEF) or Heat during Chilled Storage

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ABSTRACT: The effect of high-intensity pulsed electric fields (HIPEF) or thermal processes and refrigerated storage on water-soluble vitamins and antioxidant capacity of beverages containing fruit juices and whole (FJ-WM) or skim milk (FJ-SM) was assessed. Peroxidase (POD) and lipoxygenase (LOX) inactivation as well as color changes were also studied. High vitamin C retention was observed in HIPEF and thermally treated beverages, but a significant depletion of the vitamin during storage occurred, which was correlated with antioxidant capacity. HIPEF treatment did not affect the concentration of group B vitamins, which also remained constant over time, but thermally treated beverages showed lower riboflavin (vitamin B_2) concentration. With regard to enzyme activity, thermal processing was more effective than HIPEF on POD and LOX inactivation. The color of the beverages was maintained after HIPEF processing and during storage. Consequently, HIPEF processing could be a feasible technology to attain beverages with fruit juices and milk with high vitamin content and antioxidant potential.

KEYWORDS: vitamin C, group B vitamins, peroxidase, lipoxygenase, fruit juice, milk, high-intensity pulsed electric fields, thermal processing, storage

INTRODUCTION

Consumers' awareness of the role of nutrition in health and well-being is the main reason for the success of the functional foods market. Functional foods provide properties beyond nourishing purposes, because they contain one or more compounds that provide enhancing functions in the organism, promoting welfare and health or reducing the risk of several diseases.[1] Functional milks and dairy beverages are one of the most diversified categories among functional foods, including probiotics, prebiotics, and enriched or enhanced milks.[2] Thus, beverages based on fruit juices and milk are widely accepted due to the well-known antioxidant properties of fruits as well as the health benefits of milk. However, successful development and marketing of these type of products with high added value require that industrial and academic advances are brought together.[3] In fact, several studies have demonstrated that high temperature applied during thermal processing negatively affects the vitamin content of fruit juices or milk.[4,5] Therefore, overcoming bioactive losses caused by traditional preservation technologies is a major challenge with regard to functional foods to fulfill consumer demands. Hence, the use of high-intensity pulsed electric fields (HIPEF) is being investigated as a nonthermal technology to stabilize food products, maintaining their nutritional and bioactive properties.[6,7] In this sense, several research papers suggest the ability of HIPEF to retain antioxidant compounds in fruit juices. Thus, high vitamin C retention in HIPEF-treated orange juice[8−14] or blended beverages with orange juice has been demonstrated.[15−17] On the other hand, only a few studies are published dealing with the influence of HIPEF on group B vitamins. Results indicate that HIPEF is likely to maintain the group B vitamin concentration in milk[18] or in orange−milk beverages.[19]

Peroxidase (POD) (EC 1.11.1.7) catalyzes the oxidation of a wide range of natural substances with aromatic groups. This enzyme contributes to deteriorative changes in flavor, texture, color, and nutrition in processed fruits and vegetables.[20] On the other hand, lipoxygenase (LOX) (EC 1.13.11.12), an enzyme present in most plant-based foods, is involved in the oxidation of polyunsaturated fatty acids containing a *cis*,*cis*-1,4-pentadiene.[21] Moreover, LOX has been associated with quality deterioration because of its negative effects on pigments during storage and its role in off-flavor and odor production[22] and antioxidant status of plant-based foods.[23] HIPEF processing has been successfully applied with the aim to inactivate both LOX[22,24−26] and POD[27−31] in diverse plant-based food products.

Limited information is available concerning the effect of HIPEF on bioactive compounds present in heterogeneous food matrices such as blended fruit juices with milk. Therefore, the aim of this work was to investigate the effect of HIPEF (35 kV/cm for 1800 μs, 4 μs bipolar square wave pulses at 200 Hz) and thermal (90 °C 60 s) processing on the content of vitamin C and group B vitamins (niacin, thiamin, and riboflavin) as well as the antioxidant capacity of a fruit juice beverage with whole or skim milk. Moreover, POD and LOX activities and color changes in the beverages were assessed.

MATERIALS AND METHODS

Beverage Preparation. Orange, mango, kiwi, and pineapple fruits were purchased in a local supermarket (Lleida, Spain). Fruit was

Received: March 21, 2011
Revised: August 12, 2011
Accepted: August 16, 2011
Published: August 16, 2011

sanitized in a 200 ppm sodium hypochlorite solution for 2 min, rinsed with tap water, and dried by hand. Then the juice was extracted from each fruit separately and mixed with commercial pasteurized whole (3.5% fat) or skim (0.3% fat) bovine milk with the following proportions: orange (30%), kiwi (25%), mango (10%), pineapple (10%), milk (17.5%), and sugar (7.5%).[32] Fruit juice−whole (FJ-WM) or −skim (FJ-SM) milk beverages were filtered through a cloth, and their pH was adjusted to 3.35 with citric acid. Samples were stored in 25 mL sterile plastic flasks at 4 ± 1 °C in the absence of light and with minimal headspace volume. Because of microbial growth, the treated and untreated samples were stored for no more than 56 and 14 days, respectively.

HIPEF Processing. A continuous flow bench-scale system OSU-4F (The Ohio State University, Columbus, OH), delivering square-wave pulses, was used for HIPEF processing. On the basis of previous work,[32] the HIPEF process was conducted at 35 kV/cm electric field strength for 1800 μs, a pulse frequency of 200 Hz, and 4 μs bipolar pulses. Electric field strength and pulse duration and frequency were controlled through a pulse generator (model 9410, Quantum Composers, Inc., Bozeman, MT) and measured with an oscilloscope (TEKScope, Tektronix Inc., Beaverton, OR). The samples were pumped through the system at a flow rate of 760 mL/min with a variable gear pump (model 752210-25, 106 Cole Palmer Instrument Co., Vermon Hills, IL). The system was composed of eight collinear treatment chambers serially connected, each with two stainless steel electrodes separated by 0.292 cm. Each treatment chamber has a diameter of 0.23 cm and a volume of 0.0121 cm^3. Between each treatment chamber the product was refrigerated in an ice−water bath so that the temperature of the product always was below 40 °C, which was measured with thermocouples at the inlet and outlet of each treatment chamber.

Thermal Treatment. Beverages were thermally treated at 90 °C for 1 min to ensure the inactivation of spoilage microorganisms and to simulate a conventional preservative treatment based on the literature.[33] The samples were pumped with a peristaltic pump (model D-21 V, Dinko, Barcelona, Spain) at a flow rate of 40 mL/min and passed through a tubular stainless steel heat exchanger coil system (0.037 cm^2 section and 1100 cm long) submerged in a hot water bath settled at 90 °C (Universitat de Lleida, Lleida, Spain). Then the heated beverages were immediately cooled in a water bath with ice passing through a stainless steel coil.

Vitamin C. The vitamin C content of fruit juice−milk beverages was determined by HPLC. The extraction procedure was conducted in accordance with the validated method proposed by Odriozola-Serrano et al.[34] A sample of 10 mL of the beverages was mixed with 10 mL of a solution containing 4.5% w/v metaphosphoric acid and 0.72% w/v DTT. The mixture was centrifuged at 22100*g* for 15 min at 4 °C (Centrifuge Avanti J-25, Beckman Instruments Inc., Fullerton, CA), and the supernatant was filtered through a Whatman filter paper. Subsequently, the samples were filtered with a Millipore 0.45 μm membrane and stored at −40 °C until the moment of analysis. An aliquot of 50 μL was injected into a HPLC system equipped with a reverse-phase C18 Spherisorb ODS2 (5 μm) stainless steel column (4.6 mm × 250 mm) and an isocratic mobile phase consisting on Milli-Q water adjusted to pH 2.6 with a 0.01% sulfuric acid solution flowing at 1 mL/min. The vitamin C detection was carried out with a 486 absorbance detector (Waters, Milford, MA) working at 245 nm. A calibration curve with ascorbic acid (Scharlau Chemie, S.A., Barcelona, Spain) was built for vitamin C quantification, and results were expressed as milligrams of vitamin C per 100 mL of beverage.

Group B Vitamins. Group B vitamins were extracted from the beverages with acid and further enzymatic hydrolysis and analyzed by HPLC following the method proposed by Viñas et al.[35] A 10 mL sample of the beverages was placed in an amber flask and mixed with 25 mL of HCl (0.1 mol/L), homogenized with an ultrasonic processor for 30 s, and then heated in a water bath at 90 °C for 30 min. When the mixture was cold, the pH was adjusted to 4 with sodium acetate (1 mol/L), and 0.1 g of taka-diastase was added. It was incubated at 50 °C with agitation for 2 h. To stop the reaction, 1 mL of trichloroacetic acid (50% w/v) was added and heated at 90 °C for 10 min. After the sample had cooled, the pH was adjusted to 6 with potassium hydroxide (10 mol/L), and it was quantitatively transferred to a 50 mL calibrated flask using potassium dihydrogenphosphate buffer (10 mmol/L) at pH 6. Aliquots were centrifuged at 3468*g* for 10 min at 4 °C (Centrifuge AvantiTM J-25, Beckman Instruments Inc.), filtered through a Millipore 0.45 μm membrane, and stored at −40 °C until the moment of analysis. An aliquot of 20 μL was injected into the HPLC system, which was equipped with a 600 controller, a 486 absorbance detector scanning from 200 to 350 nm, and a 717 Plus autosampler with cooling system (Waters). Group B vitamins were separated using a C18 Nova-Pack (4 μm) stainless steel analytical column (3.9 × 150 mm) (Waters). A gradient elution was used consisting on an initial isocratic step with potassium dihydrogenphosphate buffer (10 mmol/L) at pH 6 for 13 min followed by a linear gradient to acetonitrile buffer (6:94 v/v) during 1 min. This mixture was held for 6 min. Then a second linear gradient to acetonitrile buffer (12:88 v/v) in 1 min was held for 10 min. Afterward, the initial conditions were established in 1 min and held for 25 min. The flow rate of the mobile phase was always 1 mL/min, and total run time was 57 min. Vitamins were identified by comparison of their spectral data and retention times with those of reference standards (Sigma-Aldrich) and quantified by integration of peak areas and comparison to calibration curves. Results were expressed as milligrams of vitamin per 100 mL of beverage.

Antioxidant Capacity. The antioxidant capacity of fruit juice−milk beverages was studied through the evaluation of free radical-scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method described by Odriozola-Serrano et al.[36] An aliquot of 0.01 mL of the beverages was mixed with 3.9 mL of methanolic DPPH (0.025 g/L) and 0.09 mL of distilled water in a spectrophotometer cuvette. The homogenate was mixed by inversion and kept in darkness for 30 min at room temperature. Absorbance of the samples was measured with a spectrophotometer at 515 nm against a blank of methanol without DPPH. Results were expressed as percentage of inhibition of the radical DPPH, which can be related to the decrease in absorbance with respect to the control value (DPPH initial absorption value).

Enzyme Activity. *POD.* POD activity was measured on the basis of the method proposed by Elez-Martínez et al.[27] To obtain the enzymatic extract, a sample of 10 mL of the beverages was mixed with 20 mL of sodium phosphate (0.2 M) buffer at pH 6.7. The homogenate was centrifuged at 24000*g* for 15 min at 4 °C (Centrifuge Avanti J-25, Beckman Instruments Inc.), and the supernatant was filtered through a Whatman filter paper. The POD activity was determined spectrophotometrically (Cecil CE 2021 spectrophotometer, Cecil Instruments Ltd., Cambridge, U.K.) by measuring the *p*-phenylenediamine oxidation at 485 nm. In a 1 cm path cuvette were placed 2.7 mL of sodium phosphate (0.05 M) buffer at pH 6.5, 0.2 mL of *p*-phenylenediamine (1% w/v), 0.1 mL of hydrogen peroxide (1.5% w/v), and 0.1 mL of the enzymatic extract. The POD activity was determined by measuring the reaction rate during 5 min at 20 °C. POD activity was defined as the change in absorbance per minute and milliliter of enzymatic extract.

LOX. LOX activity was measured following a modified procedure described by Indrawati et al.[37] The beverage was centrifuged (10000*g*, 15 min) at 4 °C and filtered through a Whatman no. 1 filter. The enzymatic extract was composed by 1 mL of the filtered supernatant and 19 mL of distilled water. A solution of linoleic acid (0.017 mL/L) was prepared by mixing 0.05 mL of ethyl alcohol (95% v/v), 0.05 mL of linoleic acid, and 15 mL of borate buffer (0.2 mol/L) and further pH adjustment to 9.0 at 25 °C with NaOH (1 mol/L). To obtain the

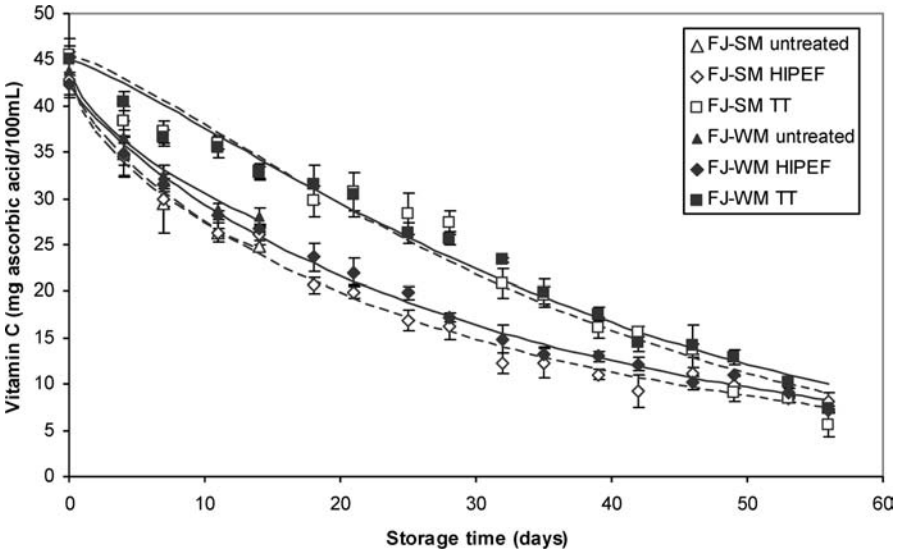

Figure 1. Vitamin C concentration (mg ascorbic acid/100 mL) during storage at 4 °C of fruit juice−whole (FJ-WM) or −skim (FJ-SM) milk beverages treated by high-intensity pulsed electric fields (HIPEF) or heat (TT). The plotted lines correspond to the data fitted to a Weibull equation for fruit juice-skim (- - -) or whole (—) milk beverages. Data shown are the mean ± standard deviation ($n = 4$).

substrate a 5 mL aliquot of the described linoleic acid solution was dissolved with 20 mL of borate buffer (0.2 mol/L) and 5 mL of distilled water. In a quartz cuvette were mixed 2 mL of the substrate linoleic acid solution, 0.95 mL of borate buffer (0.2 mol/L), and 0.05 mL of enzyme extract. Oxidation of linoleic acid was analyzed spectrophotometrically at 234 nm at 25 °C (Cecil CE 2021 spectrophotometer, Cecil Instruments Ltd.). A blank was prepared without the enzyme extract. LOX activity was determined by measuring the initial rate of the reaction, which was computed from the linear portion of the plotted curve. LOX activity was defined as a change of 1 unit in absorbance per minute and milliliter of enzymatic extract.

Percentage of residual POD and LOX activity was calculated with regard to the untreated beverage immediately after preparation (eq 1).

$$\mathrm{RA} = 100\frac{A_t}{A_0} \tag{1}$$

where A_t and A_0 are activities of either POD or LOX in the treated and untreated beverages, respectively.

Color. The color of beverages was measured directly with a Minolta CR-400 colorimeter (Konica Minolta Sensing, Inc., Osaka, Japan) at room temperature set up for illuminant D_{65} at a 10° observer angle and calibrated with a standard white plate. CIE L^*, a^*, and b^* values were determined. Hue angle was calculated using eq 2:

$$h = \mathrm{arctg}\left(\frac{b^*}{a^*}\right) \tag{2}$$

Statistics and Modeling. The depletion of vitamin C content and antioxidant capacity of beverages during refrigerated storage was adjusted to a Weibull equation (eq 3)

$$C = C_0 \exp(-(k_\alpha t)^\gamma) \tag{3}$$

where C is the vitamin C content or antioxidant capacity, C_0 is the initial concentration of vitamin C or antioxidant capacity, t is the storage time (days), k_α is the kinetic factor (days^{-1}), and γ is the shape factor. $\gamma < 1$ indicates a concavity upward and $\gamma > 1$, a concavity downward.

The adjusted regression coefficients (R^2 adj) and accuracy factor (A_f, eq 4) of the model were calculated to evaluate the fitting of the model to experimental data.

$$A_f = 10^{\sum\left|\log\left(\frac{\text{predicted}}{\text{observed}}\right)\right| / n} \tag{4}$$

Log(predicted/observed) is the logarithmic relationship between the predicted and experimental values, and n is the number of observations.

Experiments were conducted in duplicate, and two repetitions were performed of each analysis to obtain the mean value. The analysis of variance (ANOVA) was carried out using Statgraphics Plus v. 5.1 Windows package (Statistical Graphics Co., Rockville, MD). To establish significant differences ($p \leq 0.05$) between treatments and formulations, the least significant difference (LSD) test was made at a 5% significance level.

RESULTS AND DISCUSSION

Vitamin C Content. The contents of vitamin C of untreated FJ-SM and FJ-WM beverages were 43.08 and 43.72 mg/100 mL, respectively (Figure 1). These values are in the range of those observed by Zulueta et al.[38] in fruit juice and milk mixed beverages marketed in Spain. In the present work, we observed 99.5 and 97.0% vitamin C retention after applying HIPEF treatment to the FJ-SM and FJ-WM beverages, respectively. Other authors have also reported high vitamin C retention after HIPEF processing in orange juice[8−13] or blended beverages with orange juice.[15−17] Elez-Martínez and Martín- Belloso[39] observed vitamin C retention above 87.5% in orange juice after HIPEF treatment at different conditions. Furthermore, they observed higher vitamin C retention at low-intensity electric pulses, low frequency, short treatment time, and bipolar mode.

The Weibull model accurately described the degradation kinetics of vitamin C during refrigerated storage (Figure 1), with A_f values ranging from 1.01 to 1.11 and R^2 from 95.5 to 99.2, respectively (Table 1). Other authors have used Weibullian equations to predict vitamin C depletion during storage of plant-based products with good accuracy.[40,41] Vitamin C degradation rate was faster in HIPEF-treated and untreated beverages

Table 1. Estimated Parameters of Weibull Distribution Function Proposed To Describe the Depletion of Vitamin C in Fruit Juice−Whole (FJ-WM) or −Skim (FJ-SM) Milk Beverages during Storage Time at 4 °C Treated by High-Intensity Pulsed Electric Fields (HIPEF) or Heat (TT)[a]

	C_0	k_α	γ	R^2	A_f
FJ-SM HIPEF	42.85 ± 0.07	0.03587 ± 0.00024	0.79 ± 0.01	98.6 ± 0.5	1.06
FJ-SM TT	45.56 ± 0.10	0.02613 ± 0.00068	1.27 ± 0.01	95.5 ± 0.5	1.11
FJ-SM untreated	43.70 ± 2.62	0.03149 ± 0.01079	0.66 ± 0.16	98.3 ± 1.6	1.02
FJ-WM HIPEF	42.40 ± 1.42	0.03145 ± 0.00181	0.87 ± 0.03	99.2 ± 0.1	1.04
FJ-WM TT	45.00 ± 2.64	0.02487 ± 0.00185	1.23 ± 0.22	97.6 ± 0.5	1.06
FJ-WM untreated	43.72 ± 2.64	0.02467 ± 0.00717	0.73 ± 0.23	99.2 ± 0.1	1.01

[a] Values are expressed as the mean ± standard deviation. C_0 is the initial concentration of vitamin C; k_α is the kinetic factor (days^{-1}); γ is the shape factor; and A_f is the accuracy factor.

Table 2. Estimated Parameters of Weibull Distribution Function Proposed to Describe the Depletion of Antioxidant Capacity in Fruit Juice−Whole (FJ-WM) or −Skim (FJ-SM) Milk Beverages during Storage Time at 4 °C Treated by High-Intensity Pulsed Electric Fields (HIPEF) or Heat (TT)[a]

	C_0	k_α	γ	R^2	A_f
FJ-SM HIPEF	21.00 ± 0.23	0.00982 ± 0.00243	0.52 ± 0.12	94.9 ± 0.5	1.03
FJ-SM TT	20.83 ± 0.40	0.01014 ± 0.00009	0.78 ± 0.01	90.3 ± 0.6	1.05
FJ-SM untreated	21.33 ± 2.18	0.01428 ± 0.00177	0.80 ± 0.23	99.1 ± 1.0	1.01
FJ-WM HIPEF	22.25 ± 1.05	0.01350 ± 0.00105	1.14 ± 0.26	93.3 ± 0.8	1.03
FJ-WM TT	21.53 ± 2.00	0.01320 ± 0.00090	1.67 ± 0.68	94.6 ± 3.5	1.03
FJ-WM untreated	22.56 ± 0.06	0.05474 ± 0.00465	9.12 ± 2.65	95.1 ± 9.2	1.00

[a] Values are expressed as the mean ± standard deviation. C_0 is the initial antioxidant capacity; k_α is the kinetic factor (days^{-1}); γ is the shape factor; A_f is the accuracy factor.

than in those thermally treated, as shown by kinetic factors of the model (Table 1). This fact could be explained by lower POD inactivation, as discussed later (Figure 3) and faster microbial growth during storage in HIPEF-treated beverages, as observed in previous work,[32] in comparison with thermal processing. Consequently, beverages treated with HIPEF retained 50% of the initial vitamin C content during 21 days, whereas thermally processed samples retained it for 28 days. In accordance with our work, Zulueta et al.[16] also observed faster degradation kinetics in HIPEF-treated orange juice−milk beverage regarding heat processing. Otherwise, Elez-Martínez et al.[14] reported that orange juice treated by HIPEF retained more vitamin C than heat-processed juice for 56 days at 4 °C. This fact proved that complex food matrices such as blended beverages do not behave as simple products such as fruit juices when they are processed by HIPEF.

Group B Vitamins. The concentration of group B vitamins in FJ-SM or FJ-WM beverages after processing and during storage is presented in Table 3. The concentrations of niacin, thiamin, and riboflavin are within the range of those reported by the USDA nutrient database for standard reference (USDA, 2010) in milk and fruits used in the formulation of the present blended beverages.

In general, niacin and thiamin contents in FJ-WM or FJ-SM beverages were not significantly affected by the treatment applied. However, it was observed that HIPEF-treated and untreated beverages showed significantly higher riboflavin levels than those thermally treated, immediately after processing and during storage time. Accordingly, Rivas et al.[19] reported no significant changes on group B vitamins (pantothenic acid, biotin, folic acid, and riboflavin) in a fortified orange juice−milk beverage treated by HIPEF. Nevertheless, these authors reported up to 18−23% loss in group B vitamin content after a heat treatment (95 °C for 45 s). Moreover, Riener et al.[42] did not find differences in the group B vitamin content between HIPEF-treated and untreated milk. In addition, Bendicho et al.[18] found no significant changes in thiamin and riboflavin levels in skim milk or skim milk ultrafiltrate after applying HIPEF or thermal treatments. Most data suggest that niacin is stable to heat processing,[43] whereas thiamin is a thermolabile vitamin.[44] However, according to Lešková et al.,[45] losses of thiamin become noticeable after treatments at high temperature for several minutes. By comparison of the existing scientific evidence about the effect of heat or HIPEF on water-soluble vitamins it could be concluded that, in general, these compounds are more heat- than HIPEF-sensitive.

On the other hand, the type of milk used in the formulation significantly affected the content of thiamin and riboflavin of beverages, because FJ-WM beverages had higher levels of these vitamins in comparison with FJ-SM. According to the USDA nutrient database, whole milk has slightly higher amounts of group B vitamins in comparison with skim milk. Likewise, riboflavin in its pure form is known to be water insoluble,[46] and it might be partly lost during skimming of milk.

Group B vitamin concentration remained practically constant during 56 days at 4 °C in all of the studied samples, thus evidencing no oxidative degradation of these compounds. The results obtained by Rivas et al.[19] confirmed the stability of group B vitamins during storage (81 days at 4 °C) in a blended beverage with orange juice and milk treated by HIPEF.

Antioxidant Capacity. The antioxidant capacities of untreated FJ-SM and FJ-WM beverages were 21.3 ± 1.9 and 22.5 ± 0.2%, respectively. There were no significant differences in the

Table 3. Group B Vitamins (Niacin, Thiamin, and Riboflavin) Content of Fruit Juice−Whole (FJ-WM) or −Skim (FJ-SM) Milk Beverages Treated by High-Intensity Pulsed Electric Fields (HIPEF) or Heat (TT) during Storage at 4 °C[a]

			group B vitamins (mg/100 mL)		
storage time (days)	beverage	process	niacin	thiamin	riboflavin
0	FJ-SM	untreated	0.9233 ± 0.0098 C	0.706 ± 0.010 B	0.123 ± 0.011 B
		HIPEF	0.9218 ± 0.0081 C	0.691 ± 0.046 B	0.117 ± 0.009 AB
		TT	0.9110 ± 0.0079 C	0.634 ± 0.010 A	0.104 ± 0.013 A
	FJ-WM	untreated	0.8835 ± 0.0041 B	0.687 ± 0.020 B	0.189 ± 0.009 D
		HIPEF	0.8556 ± 0.0024 A	0.697 ± 0.015 B	0.187 ± 0.015 D
		TT	0.8660 ± 0.0239 A	0.645 ± 0.010 A	0.155 ± 0.007 C
7	FJ-SM	untreated	0.9153 ± 0.0173 B	0.774 ± 0.032 B	0.137 ± 0.010 B
		HIPEF	0.9166 ± 0.0036 B	0.818 ± 0.060 B	0.101 ± 0.013 A
		TT	0.9051 ± 0.0152 AB	0.805 ± 0.091 B	0.094 ± 0.006 A
	FJ-WM	untreated	0.9098 ± 0.0036 B	0.762 ± 0.017 B	0.191 ± 0.006 C
		HIPEF	0.8921 ± 0.0050 A	0.681 ± 0.008 A	0.184 ± 0.016 C
		TT	0.9119 ± 0.0041 B	0.772 ± 0.044 B	0.184 ± 0.016 C
14	FJ-SM	untreated	0.8829 ± 0.0027 ABC	0.641 ± 0.010 A	0.122 ± 0.023 A
		HIPEF	0.8938 ± 0.0309 BC	0.804 ± 0.086 BC	0.115 ± 0.006 A
		TT	0.8672 ± 0.0195 A	0.686 ± 0.085 A	0.123 ± 0.024 A
	FJ-WM	untreated	0.9030 ± 0.0070 C	0.887 ± 0.008 C	0.222 ± 0.015 B
		HIPEF	0.8775 ± 0.0017 AB	0.784 ± 0.053 B	0.209 ± 0.012 B
		TT	0.8932 ± 0.0044 BC	0.855 ± 0.051 BC	0.206 ± 0.015 B
21	FJ-SM	HIPEF	0.9079 ± 0.0055 B	0.610 ± 0.048 A	0.117 ± 0.017 A
		TT	0.9262 ± 0.0106 C	0.711 ± 0.072 B	0.125 ± 0.021 A
	FJ-WM	HIPEF	0.8756 ± 0.0103 A	0.692 ± 0.032 B	0.193 ± 0.019 C
		TT	0.8887 ± 0.0092 A	0.676 ± 0.049 AB	0.157 ± 0.011 B
28	FJ-SM	HIPEF	0.9057 ± 0.0062 C	0.648 ± 0.073 A	0.120 ± 0.005 A
		TT	0.8966 ± 0.0026 B	0.677 ± 0.011 A	0.141 ± 0.007 B
	FJ-WM	HIPEF	0.8824 ± 0.0050 A	0.708 ± 0.056 A	0.179 ± 0.004 C
		TT	0.8835 ± 0.0047 A	0.663 ± 0.035 A	0.175 ± 0.004 C
35	FJ-SM	HIPEF	0.8996 ± 0.0071 A	0.696 ± 0.044 A	0.136 ± 0.008 A
		TT	0.8935 ± 0.0049 A	0.694 ± 0.010 A	0.131 ± 0.008 A
	FJ-WM	HIPEF	0.8890 ± 0.0149 A	0.729 ± 0.046 AB	0.162 ± 0.013 B
		TT	0.9286 ± 0.0224 B	0.762 ± 0.050 B	0.166 ± 0.006 B
42	FJ-SM	HIPEF	0.8630 ± 0.0255 A	0.608 ± 0.060 A	0.124 ± 0.006 A
		TT	0.8580 ± 0.0077 A	0.616 ± 0.057 A	0.110 ± 0.009 A
	FJ-WM	HIPEF	0.9476 ± 0.0141 C	0.611 ± 0.035 A	0.177 ± 0.011 C
		TT	0.9095 ± 0.0036 B	0.732 ± 0.014 B	0.162 ± 0.010 B
49	FJ-SM	HIPEF	0.8412 ± 0.0178 A	0.567 ± 0.067 B	0.150 ± 0.014 B
		TT	0.8342 ± 0.0109 A	0.465 ± 0.051 A	0.121 ± 0.015 A
	FJ-WM	HIPEF	0.8749 ± 0.0247 B	0.684 ± 0.011 C	0.186 ± 0.002 C
		TT	0.9039 ± 0.0153 C	0.710 ± 0.053 C	0.171 ± 0.008 C
56	FJ-SM	HIPEF	0.8383 ± 0.0151 A	0.536 ± 0.033 A	0.128 ± 0.008 B
		TT	0.8411 ± 0.0073 A	0.515 ± 0.087 A	0.088 ± 0.004 A
	FJ-WM	HIPEF	0.8856 ± 0.0042 B	0.648 ± 0.017 B	0.172 ± 0.012 C
		TT	0.8912 ± 0.0044 B	0.644 ± 0.025 B	0.171 ± 0.010 C

[a] Values are expressed as the mean ± standard deviation. Different letters in the same column for each storage day indicate significant differences among beverages ($p < 0.05$).

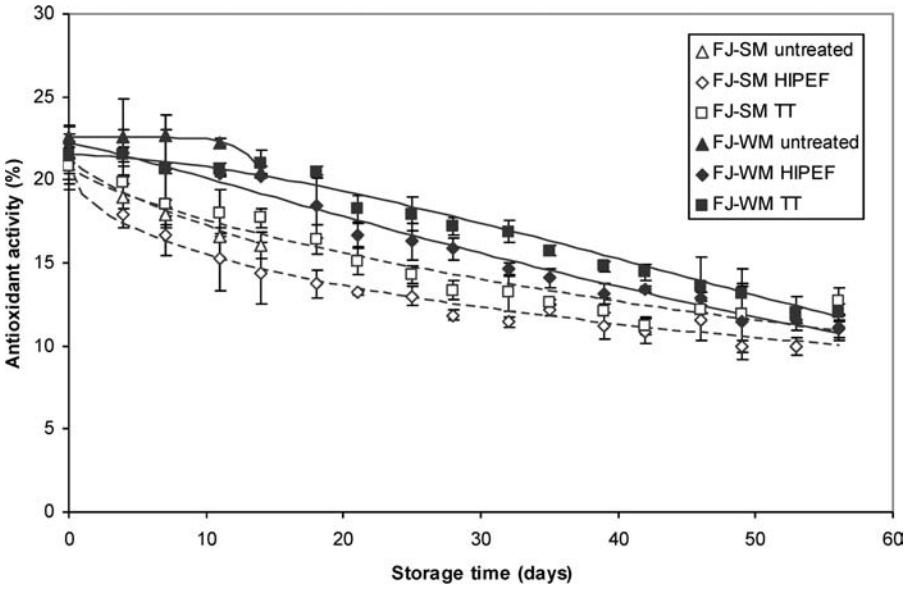

Figure 2. Antioxidant activity (%) during storage at 4 °C of fruit juice−whole (FJ-WM) or −skim (FJ-SM) milk beverages treated by high-intensity pulsed electric fields (HIPEF) or heat (TT). The plotted lines correspond to the data fitted to a Weibull equation for fruit juice-skim (- - -) or whole (—) milk beverages. Data shown are the mean ± standard deviation (n = 4).

antioxidant activities of treated and untreated beverages, immediately after processing. Similarly, Morales-de la Peña et al.[17] found no differences in the antioxidant capacities between HIPEF and thermally treated fruit juice−soy milk beverages with regard to those untreated.

The Weibull model described the depletion of antioxidant capacity of the beverages during refrigerated storage well with A_f and R^2 values between 1.00 and 1.05 and between 93.3 and 99.1, respectively (Table 2; Figure 2). A higher antioxidant activity was observed in thermally treated beverages in comparison with untreated or HIPEF-treated ones throughout storage time. Antioxidant capacity values of beverages had a statistically significant correlation coefficient (R^2 = 75.5) with their vitamin C content, indicating that vitamin C is a major contributor to the overall antioxidant potential of these type of products.

Both immediately after processing and during storage, beverages formulated with whole milk showed higher antioxidant capacity than those formulated with skim milk, regardless of the treatment applied. In addition, it is intriguing that FJ-WM beverages showed lower antioxidant capacity depletion rates in comparison with FJ-SM (Table 2). Accordingly, Zulueta et al.[47] found a positive correlation between the fat content of raw milk and its antioxidant activity. They observed a significantly higher antioxidant activity in whole milk than in low-fat or skim milk. These results reveal contribution from lipophilic antioxidants acting as radical scavengers in food products.

Enzyme Activity. *POD Activity.* Immediately after processing, both HIPEF and thermal treatments significantly decreased the POD activity in the beverages (Figure 3). HIPEF processing reduced the initial POD activity up to 53.6 ± 11.8% in FJ-WM beverage and up to a 25.7 ± 2.7% in FJ-SM beverage. Elez-Martínez et al.[27] observed that orange juice POD inhibition was greater when the electric field strength, the treatment time, the pulse frequency, and the pulse width increased, reaching POD total inactivation at 35 kV/cm for 1500 μs in bipolar mode. In contrast, Morales-de la Peña et al.[17] reported a 71% of POD relative activity in a fruit juice−soy milk beverage after treatment by HIPEF (kV/cm for 1400 μs). Thus, it can be stated that HIPEF effectiveness against POD largely depends on the food matrix. On the other hand, heat-treated beverages showed lower POD relative activity in comparison with those HIPEF-treated, reaching values of 6.4 ± 5.4 and 18.2 ± 1.5% in FJ-WM and FJ-SM beverages, respectively. Other authors have observed a partial POD inactivation after thermal processing of citrus juices[48] due to the presence of several POD isoenzyme fractions with different thermostabilities.[49]

With regard to the effectiveness in POD inactivation, we found a significant interaction between HIPEF processing and the type of milk used in the formulation, with lower POD relative activity in the beverage formulated with skim milk (25.7 ± 2.7%) than with whole milk (53.6 ± 11.8%). Likewise, Bendicho et al.[50] observed higher inactivation of a protease from *Bacillus subtilis* when HIPEF was applied in skim milk in comparison with whole milk. This fact proves the protective effect of fat content in milk against electrical stress.

The initial POD activity in all of the studied samples remained practically constant during refrigerated storage, regardless of the treatment applied, thus evidencing an irreversible HIPEF or heat-induced stress in POD present in the mixed beverages. Accordingly, Zhong et al.[28] observed significant changes in the secondary structure of POD in buffered solution after being HIPEF treated, causing activity loss after treatment.

LOX Activity. HIPEF processing had a minor effect on LOX inactivation in FJ-SM or FJ-WM beverages because there were no differences between untreated and HIPEF-treated beverages (Figure 4). In contrast, thermal treatment reduced LOX activity significantly in both beverages after processing; however, LOX activity was still evident. Moreover, heat treatment was more effective regarding LOX inactivation in the beverage formulated with whole milk compared with the beverage formulated with skim milk. The weak effectiveness of HIPEF on LOX inactivation in mixed beverages contrasts with results reported by other authors. Min et al.[24] reported higher LOX inactivation levels in tomato juice treated by HIPEF, up to 20% of residual activity, at

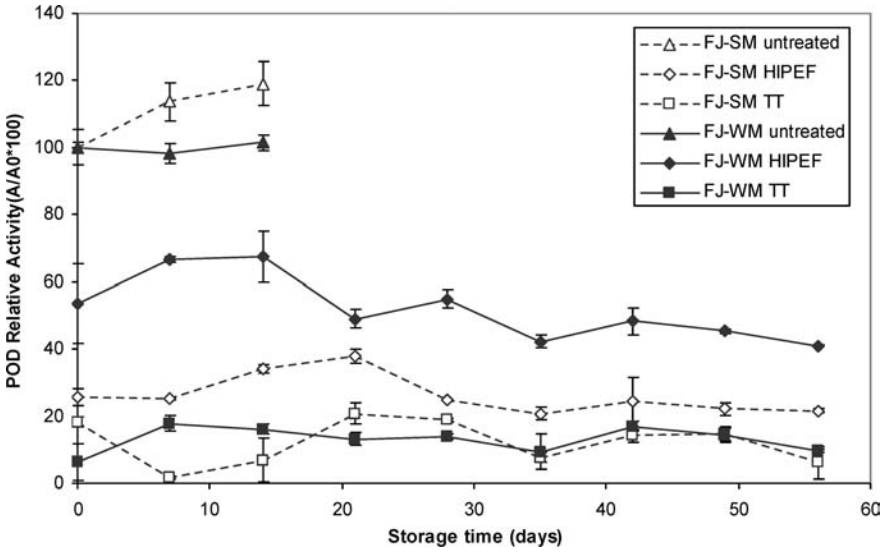

Figure 3. Peroxidase (POD) relative activity (%) during storage at 4 °C in fruit juice—whole (FJ-WM) or —skim (FJ-SM) milk beverages treated by high-intensity pulsed electric fields (HIPEF) or heat (TT). Data shown are the mean ± standard deviation (n = 4).

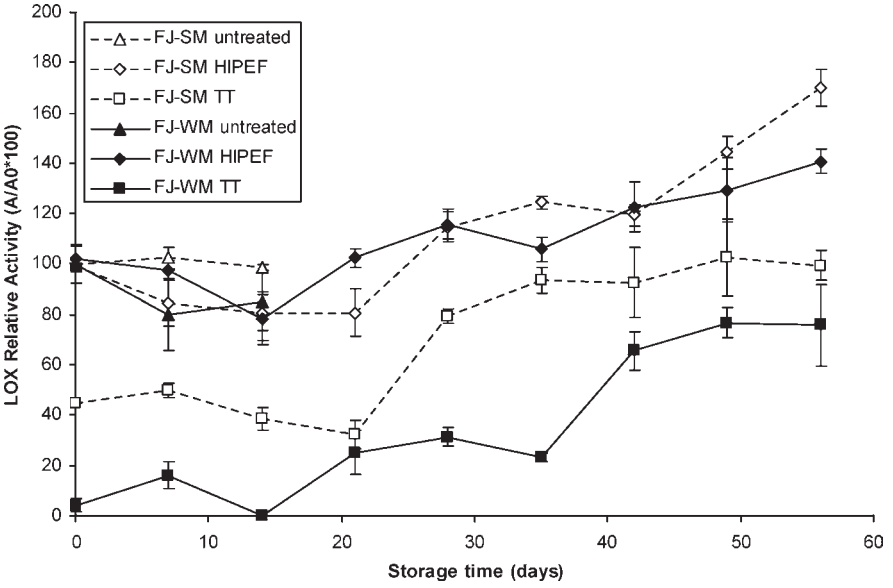

Figure 4. Lipoxygenase (LOX) relative activity (%) during storage at 4 °C in fruit juice—whole (FJ-WM) or —skim (FJ-SM) milk beverages treated by high-intensity pulsed electric fields (HIPEF) or heat (TT). Data shown are the mean ± standard deviation (n = 4).

higher electric field strength and treatment time. Li et al.[51] also found that the higher the electric field strength, treatment time, and pulse frequency and width, the higher the LOX inactivation, reaching a maximum of 88% LOX inactivation in soy milk. However, LOX has shown to be more HIPEF-resistant than other enzymes.[26] In addition, the level of enzyme inactivation by HIPEF can also be affected by food matrix, because some enzymes might be protected from HIPEF-induced damage by the presence of casein micelles in the treatment medium.[52]

A significant increase in LOX activity from the third week of storage was observed in all of the studied samples, regardless of the type of milk used in the formulation and the treatment applied. This fact proves that neither HIPEF nor thermal processing produced irreversible changes in LOX activity in mixed beverages. The effects of HIPEF on proteins include the association or dissociation of functional groups, movements of charged chains, and changes in alignment helices.[53] Luo et al.[54] observed significant changes on purified LOX secondary (α-helix and β-sheet) and tertiary structure after a HIPEF treatment. Unfortunately, there are no studies to illustrate whether these changes are irreversible or not during storage time, but Morales-de la Peña et al.[17] also observed LOX reactivation during refrigerated storage in HIPEF or thermally treated fruit juice—soy milk beverages.

Color. Color parameters (L^* and $h°$) of the beverages during storage are represented in Figure 5. There were prominent differences in the color of both beverages as a function of their fat content, due to light scattering of fat globules and the white

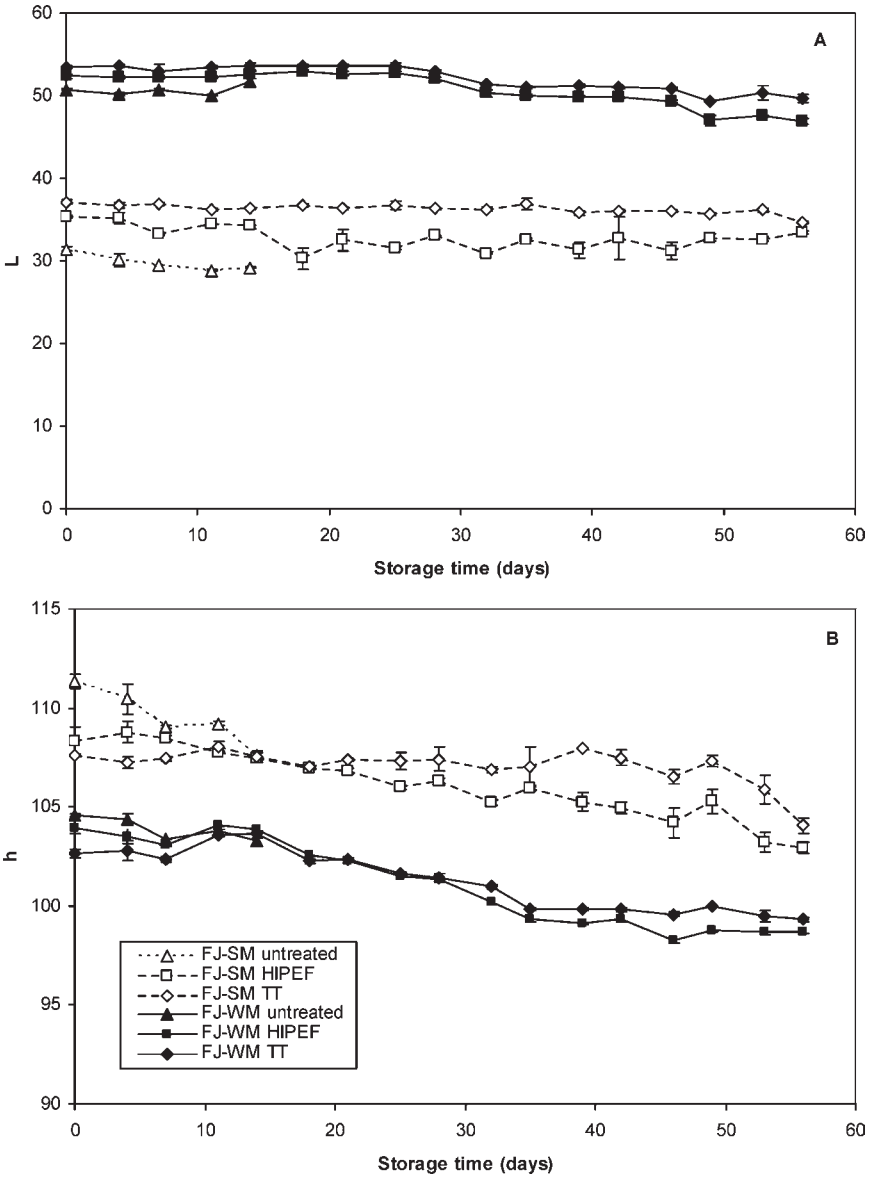

Figure 5. Color L^* (A) and $h°$ (B) values during storage at 4 °C in fruit juice−whole (FJ-WM) or −skim (FJ-SM) milk beverages treated by high-intensity pulsed electric fields (HIPEF) or heat (TT). Data shown are the mean ± standard deviation (n = 4).

color of casein micelles. HIPEF and thermally treated FJ-SM and FJ-WM beverages showed higher lightness values (L^*) immediately after processing and during storage at 4 °C in comparison with untreated beverages (Figure 5A). In agreement with our results, Morales-de la Peña et al.[17] observed higher L^* values in a fruit juice−soy milk beverage treated by HIPEF (800 or 1400 μs at 35 kV/cm) or heat (90 °C, 60 s) in comparison with a untreated beverage. In contrast, Sampedro et al.[55] reported a depletion in L^* values immediately after HIPEF (50 μs at 30 kV/cm) or heat (85 °C, 66 s) treatments applied to an orange juice−milk beverage. During storage, a slight decrease in luminosity values was observed in HIPEF-treated FJ-WM and FJ-SM beverages, probably due to nonenzymatic browning reactions.[56]

Just after processing, hue angle values ($h°$) of beverages significantly decreased regardless of the treatment applied (Figure 5B). During storage at 4 °C, $h°$ values depleted in all of the studied samples, showing higher values in thermally treated beverages. Consistently, Yeom et al.[9] also observed a significant drop of $h°$ values in HIPEF or thermally treated orange juice during refrigerated storage.

In summary, vitamin C was the main compound involved in changes of antioxidant capacity of beverages, and it was retained above 97% by applying a HIPEF treatment to FJ-SM or FJ-WM beverages, maintaining 50% of its initial concentration during 21 days at 4 °C. Nevertheless, beverages treated by heat showed higher vitamin C and antioxidant activity during refrigerated storage than those treated by HIPEF. The content of group B vitamins was not affected by HIPEF, and it remained constant during storage. However, thermally treated beverages presented lower riboflavin concentration than those HIPEF-treated. FJ-WM beverages showed a higher content of thiamin and riboflavin, as well as higher antioxidant capacity than those containing skim milk. HIPEF treatment better inactivated POD than LOX in both FJ-WM and FJ-SM beverages. On the other hand, thermal treatment was more effective than HIPEF on enzyme inactivation. The color of beverages was adequately preserved after HIPEF

processing and during storage. Consequently, HIPEF technology has been shown to be an appropriate technology to preserve water-soluble vitamins and maintain quality attributes of fruit juice−milk beverages.

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Funding Sources

This study was supported by the Ministerio de Ciencia e Innovación (Spain) through Project AGL2006-12758-C02-02. L.S.-T. and M.M.-P. thank the Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) (Catalonia, Spain) and the Consejo Nacional de Ciencia y Tecnologia (CONACYT) (Mexico) for predoctoral grants, respectively. O.M.-B. thanks the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia 2008 Award.

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