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Effects of pulsed electric fields on water-soluble vitamins and ACE inhibitory peptides added to a mixed orange juice and milk beverage

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Abstract

The effects of pulsed electric fields technology (15–40 kV/cm; 0–700 μs) and thermal processing (84 °C and 95 °C, 15–120 s) were studied on an orange juice and milk mixed beverage fortified with water-soluble vitamins (biotin, folic acid, pantothenic acid and riboflavin) and angiotensin-I-converting enzyme (ACE) inhibitory peptides. The evaluation of the technologies was carried out from two points of view: effect of treatments and effect of storage (4 °C, 81 days). The results confirmed the stability of the vitamins and the ACE inhibitory activity after the PEF treatment and during storage.

Keywords: ACE inhibitor peptides; Biotin; Folic acid; Orange juice–milk beverage; Pantothenic acid; Pasteurization; Pulsed electric fields; Riboflavin; Storage

1. Introduction

The increasing concern of consumers about their health and new lifestyles that are driving them away from healthy dietary habits has prompted the industry to become involved in the need for food products which contribute to the prevention of illness. The natural drinks (soy-based drinks or drinkable yogurts) that consumers consider healthy constitute one of the food industry sectors with highest growth worldwide (31% for soy-based drinks; and 19% for drinkable yogurts, in 2004) (Sloan, 2005). Reflecting a similar concern, as a result of the new Dietetic Guides for the Americans, published in 2005, the International Food Information Council has declared that now, and in the immediate future, food research and nutrition professionals must make the most of opportunities to develop functional foods that support and promote health (Davis & Reinhardt, 2005).

Among the most consumed functional foods are mixed fruit juice and milk beverages fortified with vitamins, biologically active peptides, minerals and fibre (Pszczola, 2005). In Spain, consumption of enriched juices of this kind, in 2005, represented 19.47% of the total *per capita* consumption of juices (http://www.mapa.es/es/alimentacion/pags/consumo/BD/resultado1.asp). Recently, milk beverages fortified with bioactive peptides and minerals with antihypertensive properties have been commercialized (Sloan, 2005).

One of the nutritive compounds to take into account is the group of water-soluble vitamins. B group vitamins are water-soluble vitamins that have many different fundamental biological properties, such as protection against cancer, heart disease, and birth defects (Lucock, 2004). The stability of B vitamins depends on each vitamin and on external factors, such as presence of oxygen, light and acids. Another cause of vitamin B group losses is thermal treat-

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ment, which is the classical technology generally applied to foods of this kind to preserve them. Heat-induced losses of vitamin content make it necessary to over-fortify foods to compensate for this degradation. The thermal stability of water-soluble vitamins depends on each vitamin, with riboflavin being the most thermostable and ascorbic acid the most thermosensitive under aerobic conditions. When milk is pasteurized, only 10% of the riboflavin is destroyed (Ford, Porter, Thompson, Toothill, & Edward-Webb, 1969; Haddad & Loewenstein, 1983; Lavigne, Zee, Simard, & Bellveau, 1989). Haddad and Loewenstein (1983) reported a thiamine destruction of 12% after mild heat treatment (72 °C for 16 s), and 25–50% after in-bottle pasteurization. In the case of ascorbic acid, thermal destruction in milk varies between 40% and 60%, depending on treatment intensity (Ford et al., 1969; Haddad & Loewenstein, 1983; Lavigne et al., 1989; Mottar & Naudts, 1979; Van Eeklen & Heijne, 1965).

Biologically active peptides are food-derived peptides that, in addition to their nutritional value, exert a physiological effect on the body. These bioactive peptides are inactive within the original protein but, once released, function as regulatory compounds with hormone-like activity, based on their inherent amino acid composition and sequence (Meisel, 1997; Tome, 1998). Numerous peptides exhibiting various activities have been reported, including opiate, mineral binding, immunomodulatory, angiotensin-I-converting enzyme (ACE) inhibitory, antithrombotic, and antimicrobial peptides (Clare & Swaisgood, 2000; Korhonen & Philanto-Leppälä, 2003; Meisel, 1998). Milk proteins are currently the main source of bioactive peptides (Dziuba, Minkiewicz, & Nalecz, 1999), and milk fermentation is a successful strategy for producing them (Gobetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000; Korhonen & Philanto-Leppälä, 2003; Silva & Malcata, 2005).

Hypertension is one of the most common chronic medical conditions in the developed world. It is estimated that about 20% of the world's adult population suffer from hypertension. The prevalence of high blood pressure increases with age, affecting approximately 65% of the population aged 65–74 in western nations (Alper, Calhoun, & Oparil, 2001; Duprez, Van Helshoecht, Van den Eynde, & Leeman, 2002). ACE plays an important role in the renninangiotensin system, which regulates arterial blood pressure as well as salt and water balance (Eriksson, Dzanilczyk, & Penninger, 2002; Riordan, 2003). Consequently, inhibition of ACE, by ACE inhibitory drugs and natural ACE inhibitory peptides, has been shown to result in an antihypertensive effect in hypertensive human subjects and animals (Cushman & Ondetti, 1999; Takano, 1998).

There are noteworthy antecedents proving that nonthermal preservation technologies, such as pulsed electric fields (PEF), are able to maintain the quality of certain fresh foods, improving their safety and shelf life, without changing their sensory and nutritive aspects as does thermal pasteurization (Barbosa-Canovas & Sepúlveda, 2005). Fruit and vegetable juices, liquid eggs, milk, and milk derivates are the most common foods to which PEF technology has been applied, mainly from a microbiological and enzymatic point of view (Elez-Martinez, Soliva-Fortuny, & Martin-Belloso, 2006; Rivas, Rodrigo, Martínez, Barbosa-Cánovas, & Rodrigo, 2006; Rodrigo, Barbosa-Cánovas, Martínez, & Rodrigo, 2003; Sobrino & Martín-Belloso, 2006). Recently, various studies have proved the validity of PEF technology for inactivating microorganisms in a more complex food, such as a mixed orange juice and milk beverage (Rivas, Sampedro, Rodrigo, Martínez, & Rodrigo, 2006; Sampedro, Rivas, Rodrigo, Martínez, & Rodrigo, 2006).

From a nutritive point of view, PEF studies have focussed on milk and fruit and vegetable juices. On the whole, vitamin C, carotenoids, flavonoids, and antioxidant activity are the nutritive compounds most studied (Bendicho, Espachs, Arántegui, & Martín, 2002; Cortés, Torregrosa, Esteve, & Frígola, 2006; Sánchez-Moreno, Pilar-Cano, et al., 2005; Sánchez-Moreno et al., 2005; Torregrosa, Cortés, Esteve, & Frígola, 2006; Torregrosa, Esteve, Frígola, & Cortés, 2006). However, no information has been published about the effectiveness of PEF treatment on water-soluble vitamins or ACE inhibitory peptides in a more complex product (a mixture of orange juice and milk). The aim of this study, therefore, was to evaluate the impact of PEF and thermal technologies on ACE inhibitory peptide activity and water-soluble vitamin (riboflavin, biotin, pantothenic acid, and folic acid) contents in a mixed orange juice and milk beverage, and their stability during storage.

2. Materials and methods

2.1. Beverage preparation

Frozen pasteurized squeezed orange juice (commercial source), UHT skimmed milk (commercial source), high methoxyl pectin (Unipectine, Degussa Texturant Systems France SAS, Boulogne, France), citric acid, distilled water and sugar (commercial source) were used to prepare the beverage. The beverage formulation was: orange juice (50% (v/v)), water (30% (v/v)), milk (20% (v/v)), sugar (7.5% (w/v)), citric acid (0.1% (w/v)), and pectin (0.3% (w/v)). The sugar, citric acid, and pectin were added to the water prior to the addition of the juice and milk. The electrical conductivity (Crison 525 conductimeter, Crison Instruments SA, Alella, Barcelona, Spain), pH (Crison 2001 pH-meter, Crison Instruments SA, Alella, Barcelona, Spain), viscosity (Haake Viscotester VT5, Thermo Electron Corporation, Sussex, UK), and soluble solids content (Atago RX-1000 digital refractometer, Atago Company Ltd., Tokyo, Japan) of the beverage were determined. The beverage was prepared immediately before PEF treatment.

The beverage had an electrical conductivity of 2.91 mS/cm and a pH of 4.05.

2.2. Vitamin fortification

After preparation, the beverage was enriched with hydrosoluble vitamins. Commercial water-soluble vitamins used in this study were: biotin (BioChemika), folic acid (Fluka), riboflavin (Sigma) and D-pantothenic acid hemicalcium salt (Sigma). The vitamins were added to water before preparing the beverage. The level of fortification was adjusted according to the recommended dietary allowances (RDAs) (Table 1), assuming that an intake of 250 ml/day of the beverage meets the RDA of the life stage group with highest demands.

2.3. ACE inhibitory peptide fortification

The effect of preservation technologies on ACE inhibitory peptides was studied using the beverage described above, but with the milk replaced by pasteurized fermented milk whey (75 °C 1 min) (provided by Leche Pascual). The whey was obtained by centrifugation (20,000*g*; 10 min), followed by filtration (0.45 μm) (Muguerza et al., 2006). The resulting beverage had an electrical conductivity of 4.32 mS/cm and a pH of 3.99.

2.4. Thermal treatment

The intensity of the thermal treatment (84 °C and 95 °C; 15–120 s) applied to the samples was similar to the treatment given by manufacturers of refrigerated juice (Chen, Shaw, & Parish, 1993; Jay, 1992). A water bath was used to treat the samples. The beverage was packaged in 150 ml sheet polyethylene bags. After filling, the bags were sealed up by a Multivac thermosealer (Multivac Export, Hünenberg, Switzerland) in a vacuum. Three bags were prepared for each temperature–time combination. Product temperature was monitored with a thermocouple submerged in the beverage and sealed to the bag. After heating, the bags were cooled in an ice/water bath. Duplicate heat inactivation tests were carried out.

2.5. PEF treatment system

The samples were PEF-processed using bench-scale equipment (OSU-4D, Ohio State University). Six co-field treatment chambers with a diameter of 0.23 cm and gap distance of 0.293 cm were connected in series. Two cooling coils were connected before and after each pair of chambers and submerged in a circulating refrigerated bath. Treatment temperature was maintained below 55 °C in the hottest chamber. Pulse waveform, voltage, and intensity in the treatment chambers were recorded with a digital oscilloscope (Tektronix TDS 210, Tektronix Inc., OR).

The flow rate was set at 60 ml/min with a peristaltic pump (Millipore Corporation, Bedford, MA). A square-wave bipolar pulse duration of 2.5 μs was selected. Treatment times ranged from 0 to 700 μs, inlet temperature was 32 °C, and the electric field strength was set at 15, 25, and 40 kV/cm. The experiments were performed in triplicate.

2.6. Sample packaging and storage

The treated beverage was packaged in clean, sterile polyethylene containers inside a laminar flux chamber, avoiding head-space. The closed containers were stored at 4 °C in darkness.

2.7. Microbial stability

For the microbial counts during storage, samples were serially diluted, plated in total count agar (PCA) for total flora counts, and in acidified potato dextrose agar (PDA) for mold and yeast counts. The plates were incubated at 30 °C for 48 h or 5 days for total flora, and molds and yeasts, respectively.

2.8. Water-soluble vitamins determination

The four hydrosoluble vitamins were analyzed at the same time, using an HPLC–MS/MS method previously adjusted and validated for the beverage in question at the AINIA Technological Center (Valencia-Spain).

A series of matrix-matched calibration standard curves, matrix blanks, and recovery samples were analyzed in order to determine method accuracy, linearity, precision, repeatability, and recovery. Recovery samples were spiked at three levels in order to validate the method.

Pantothenic acid, folic acid, biotin and riboflavin were purchased from Sigma–Aldrich Corp. Individual standard stock solutions (20 mg/l) were prepared by weighing 10 mg each of vitamins and dissolving. Calibration standards were made up to 10, 20, 50, 100, and 200 μg/l in matrix to quantify the samples. The samples were diluted and filtered through a syringe filter (0.45 μm) prior to injection by HPLC–MS/MS.

Table 1
Recommended dietary allowances and level of fortification in the beverage

Vitamin	RDA	Life stage group	Beverage	
			Before fortification (ppm)	After fortification (ppm)
Biotin	35 μg/d	Lactation	3.3	208.5
Folic acid	600 μg/d	Pregnancy	–	1283.5
Riboflavin	1.6 mg/d	Lactation	483	12345.5
Pantothenic acid	7 mg/d	Lactation	1179	44509.5

The HPLC–MS/MS system was a Waters Alliance 2695 HPLC coupled with a Waters Micromass Quattro micro API with electrospray mode and MassLynx 4.0 software (Micromass). In this study, the HPLC–MS/MS was set at multiple reaction monitoring (MRM) mode.

2.9. HPLC conditions

A 150 × 2.1 mm HyPURITY AQUASTAR reverse-phase C 18 column with a particle size of 0.3 μm was used. The mobile phase used was 0.1% formic acid (solvent A) and methanol (solvent B). The linear gradient was 0–5 min: 20% B; 5–15 min: 20–80% B; 15–15.1 min: 80–20% B; 15.1–25 min: 20% B. The flow rate was 0.2 ml/min. Injection volume was 20 μl.

2.10. MS–MS conditions

Capillary voltage: 3.5 kV (electrospray positive), 3.0 kV (electrospray negative); cone voltage: see multi-reaction monitoring (MRM) programme; (Table 2): extractor voltage: 5 V; RF lens voltage: 0 V; source temperature: 120 °C; desolvation temperature: 300 °C; cone gas flow: 60 l/h; desolvation gas flow: 600 l/h (nitrogen); LM 1 resolution: 13; HM 1 resolution: 13; ion energy: 1 V; entrance voltage: 1 V; collision voltage: see multi-reaction monitoring (MRM) programme; exit voltage: 2 V; collision cell pressure: 3.3×10^{-3} mbar (argon); LM 2 resolution: 14; HM 2 resolution: 14; ion energy: 3 V; multiplier voltage: 650 V.

2.11. ACE inhibitory peptide determination

The ACE enzyme activity was determined by a colorimetric assay based on Hayakari, Kondo, and Izumi (1978) and Pedroche et al. (2002). The inhibitory activity ($IACE = ACE/ACE_{100}$) of the beverage was obtained from the quotient of the enzyme activity without (ACE_{100}) and with (ACE) the inhibitor (beverage).

The assay for the angiotensin-converting enzyme activity was carried out in an incubation mixture containing potassium phosphate buffer (200 mM) with NaCl (600 mM) (pH 8.3), sodium chloride, HHL (Hippuryl-His-Leu), water, and the enzyme (30 μl). The reaction was initiated by the addition of the substrate, and the incubation mixture was incubated at 37 °C for 30 min. Reactions were blocked by adding 1500 μl of 200 mM phosphate buffer, pH 8.3, and 750 μl of 3% TT solution (cyanuric chloride in dioxane). The tubes were vigorously stirred and centrifuged at 10,000*g* for 10 min. The TT solution was added to the hippuric acid generated by the enzyme activity, forming a complex that was detected in the spectrophotometric reading at 382 nm. The control run was identical to the above procedure, but with the 3% TT added before the HHL.

The determination of the inhibitory activity of the sample was performed in the same way, but with the water in the incubation mixture replaced by 25 μl of beverage.

Table 2
Values of the mass spectrometry parameters used in vitamin quantification

Analyte	MRM transition (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
Pantothenic acid	218 > 88	25	13
	218 > 146		17
Folic acid	442 > 295	20	20
Biotin	245 > 97	25	20
	245 > 227		15
Riboflavin	377 > 172	40	40
	377 > 243		23

3. Results

3.1. Water-soluble vitamins

3.1.1. Effects of treatments on vitamin content

The aim of this research was to study the effect of PEF technology on a mixed orange juice and milk beverage fortified with biotin, folic acid, pantothenic acid and riboflavin. Consequently, a wide range of PEF conditions was selected ($E = 15$–40 kV/cm; $t = 40$–700 μs) (Table 3). To avoid a possible effect of thermal pasteurization due to the temperature reached by the PEF treatment, the maximum temperature reached was controlled and kept below 55 °C. These PEF conditions were similar to those used for *Escherichia coli* inactivation in this beverage (Rivas, Sampedro, et al., 2006). Several authors achieved significant inactivation levels on other microorganisms in this kind of beverage, e.g. *Lactobacillus plantarum* (Sampedro et al., 2006) or *Saccharomyces cerevisiae* (submitted) after similar treatments. The authors reported the protective effect of the product, due to its complexity, when compared with PEF inactivation of microorganisms in fruit juices or milk. PEF technology has also confirmed its ability to inactivate enzymes, with several studies reaching as much as 90% destruction of pectin methylesterase in orange and orange carrot juices (Elez-Martinez, Suarez-Recio, & Martin-Belloso, 2007; Min, Jin, Yeom, Min, & Zhang, 2003;

Table 3
Treatment conditions applied to study the behaviour of water-soluble vitamins and ACE inhibitory activity added to the mixed orange juice and milk beverage

Type of study	PEF technology		Thermal technology	
	E (kV/cm)	Treatment time (μs)	Temperature (°C)	Treatment time (s)
Treatment study	15	40, 130, 300 500, 700	84	15, 30, 60 90, 120
	25	40, 130, 200 230, 310	95	15, 30
	40	40, 60, 80 110, 130		45
Storage study	15	700	84	15, 60, 120
	40	130		

Rivas et al., 2006; Rodrigo et al., 2003; Yeom, Chism, & Zhang, 2002).

As can be seen in Table 4, no significant changes ($p \leqslant 0.05$) in vitamin content were observed after any PEF treatment combination (field strength or increasing treatment time). These results are confirmed by other studies that have reported the effectiveness of PEF technology for preserving nutritive compounds. Bendicho et al. (2002) also observed the PEF stability (PEF treatment of 18.3–27.1 kV/cm; 0–400 μs) of vitamin content (thiamine, riboflavin, ascorbic acid, cholecalciferol and tocopherol) in two different substrates (skim milk and simulated skim milk ultrafiltrate (SMUF)), except for ascorbic acid, retention of which follows a first-order kinetic model. Similarly, Torregrosa et al. (2006) confirmed PEF degradation of ascorbic acid in orange–carrot juice (from 15 to 40 kV/cm; from 30 to 340 μs), obtaining losses of 10% of initial content, following a first-order inactivation kinetics (k values ranged from 0.009 to 0.0220 μ^{-1}). Grahl and Maerkl (1996) reduced the ascorbic acid content of milk after PEF treatment, although vitamin A was not affected. No studies have been done, in relation to the effect of PEF technology, on biotin, folic and pantothenic acid contents.

The effect of temperature on vitamin degradation was studied at two temperatures, 84 and 95 °C, and over treatment times ranging from 15 to 120 s (Table 3). Fig. 1 shows the effect of thermal treatment on vitamin content at 84 and 95 °C. At 84 °C there was no statistically significant effect on any of the vitamin contents. At 95 °C, however, all vitamin content was reduced ($p \leqslant 0.05$), producing losses of 18–23% at 45 s. In general, the vitamins respond differently to thermal processing, water-soluble vitamins being more sensitive to heat-treatment than are fat-soluble ones (Bendicho et al., 2002; Rechciegl, 1982).

Several studies have reported the thermostability of riboflavin. Kwok, Shiu, Yeung, and Niranjan (1998) say that riboflavin is more heat-stable and less temperature-sensitive than is thiamin, and its thermostability is independent of heating methods (Ang, Chang, Frey, & Livingston, 1975). Only 10% destruction was determined when milk was sterilized (Ford et al., 1969; Haddad & Loewenstein, 1983; Lavigne et al., 1989). Heat treatment

Table 4
Effect of PEF treatment on water-soluble vitamin content expressed as retention (C/C_0) ± standard deviation and inhibitory activity of ACE (ACE/ACE_{100}) ± standard deviation

E (kV/cm)	Treatment time (μs)	Residual content (C/C_0)				Residual inhibitory activity ($IACE_T/IACE_B$)
		Riboflavin	Folic acid	Biotin	Pantothenic acid	
15	40	0.976 ± 0.031	1.00 ± 0.036	0.963 ± 0.033	0.977 ± 0.021	0.973 ± 0.051
	130	1.00 ± 0.024	0.985 ± 0.044	0.977 ± 0.016	0.946 ± 0.039	0.992 ± 0.032
	300	0.962 ± 0.065	0.957 ± 0.032	0.954 ± 0.019	0.979 ± 0.032	1.01 ± 0.072
	500	1.00 ± 0.045	0.934 ± 0.011	0.995 ± 0.034	0.953 ± 0.027	1.03 ± 0.043
	700	0.966 ± 0.034	0.971 ± 0.009	0.982 ± 0.040	0.971 ± 0.013	0.983 ± 0.034
25	40	0.993 ± 0.013	0.951 ± 0.041	1.00 ± 0.008	0.966 ± 0.016	0.985 ± 0.012
	130	1.01 ± 0.022	0.955 ± 0.038	1.00 ± 0.015	0.947 ± 0.028	0.969 ± 0.031
	200	1.01 ± 0.023	0.955 ± 0.047	1.00 ± 0.018	0.982 ± 0.044	0.998 ± 0.043
	230	0.982 ± 0.045	0.958 ± 0.032	1.00 ± 0.043	0.964 ± 0.063	1.01 ± 0.023
	310	0.964 ± 0.033	0.965 ± 0.027	1.00 ± 0.047	0.990 ± 0.013	1.00 ± 0.089
40	40	0.993 ± 0.003	0.975 ± 0.055	0.995 ± 0.023	0.947 ± 0.030	1.01 ± 0.087
	60	0.974 ± 0.045	0.951 ± 0.067	0.972 ± 0.011	0.981 ± 0.043	0.973 ± 0.047
	80	0.98 ± 0.066	0.924 ± 0.020	1.00 ± 0.013	0.971 ± 0.017	0.994 ± 0.039
	110	0.966 ± 0.030	0.974 ± 0.052	0.977 ± 0.026	0.991 ± 0.013	0.977 ± 0.075
	130	0.942 ± 0.021	0.942 ± 0.021	0.986 ± 0.036	0.981 ± 0.010	0.990 ± 0.101

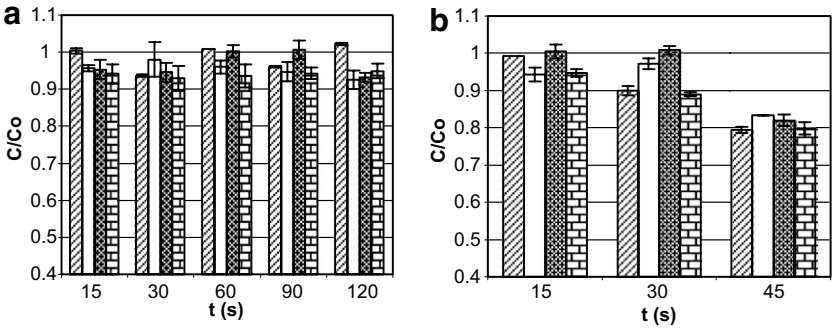

Fig. 1. Effect of heat treatment (a, 84 °C; b, 95 °C) on vitamin content (▨ pantothenic acid; □ biotin; ▩ folic acid; ▤ riboflavin). Bars express standard deviation.

(63, 80, 90, and 100 °C for 30 min, 73 °C for 15 min, and 90 °C for 10 min) caused a negligible reduction (0–7%) in the riboflavin content of camels' and cows' milk (Mehaia, 1994). The lower thermostability of riboflavin at 95 °C in the present study compared with other studies, such as by Mehaia (1994) or Sharma and Lal (1998), might be due to the presence of O_2 dissolved during the pasteurization process, as Dennison, Kirk, Bach, and Kokooczka (1977) observed that the stability of riboflavin was affected by oxygen.

Folates are sensitive to physical factors, such as temperature, pressure and exposure to light, and may therefore be affected during food processing. Another factor to take into account is the acidity of the food. Wilson and Chen (1979) found that folic acid and 5-formyltetrahydrofolic acid were stable when heated for 10 h at pH 4–12, and the stability decreased with decreasing pH below pH 4. They also observed that the methyl folate derivatives showed the highest thermal stability at pH 7, and a rapid decrease in stability was observed under alkaline or acid conditions. Those results agree with the data obtained in the present study, in which the folic content was affected by temperature and treatment time in an acid medium. Moreover, the stability of folates was affected by their nature. Nguyen, Indrawati, and Hendrickx (2003) reported that, during pasteurization (71.7 °C/15 s) of a buffer system, 10–15% of 5-methyltetrahydrofolic acid was lost, and it was totally degraded by sterilization (135 °C, 1 s), whereas folic acid was not affected by sterilization.

In general, biotin retention is relatively high during heat-treatment (80% in meat, 85–90% in milk pasteurization, 85–95% in legumes, 70% in preservation of fruits and vegetables). Cooking of tomatoes resulted in significant decreases of biotin contents, whereas cooking losses were less pronounced for potatoes (Macova & Krkoskova, 2003).

Pantothenic acid is the most stable vitamin during thermal processing in a pH range of 5–7. In milk, pantothenic acid is stable during pasteurization, as the normal pH of milk is in the optimal pH stability range (Fox, Lakritz, & Thayer, 1997). Bergström (1994) observed that pantothenic acid may be affected by the acidity of a marinade (pH 5.5).

Although our results show that PEF technology does not affect the stability of vitamins, we observed a loss of

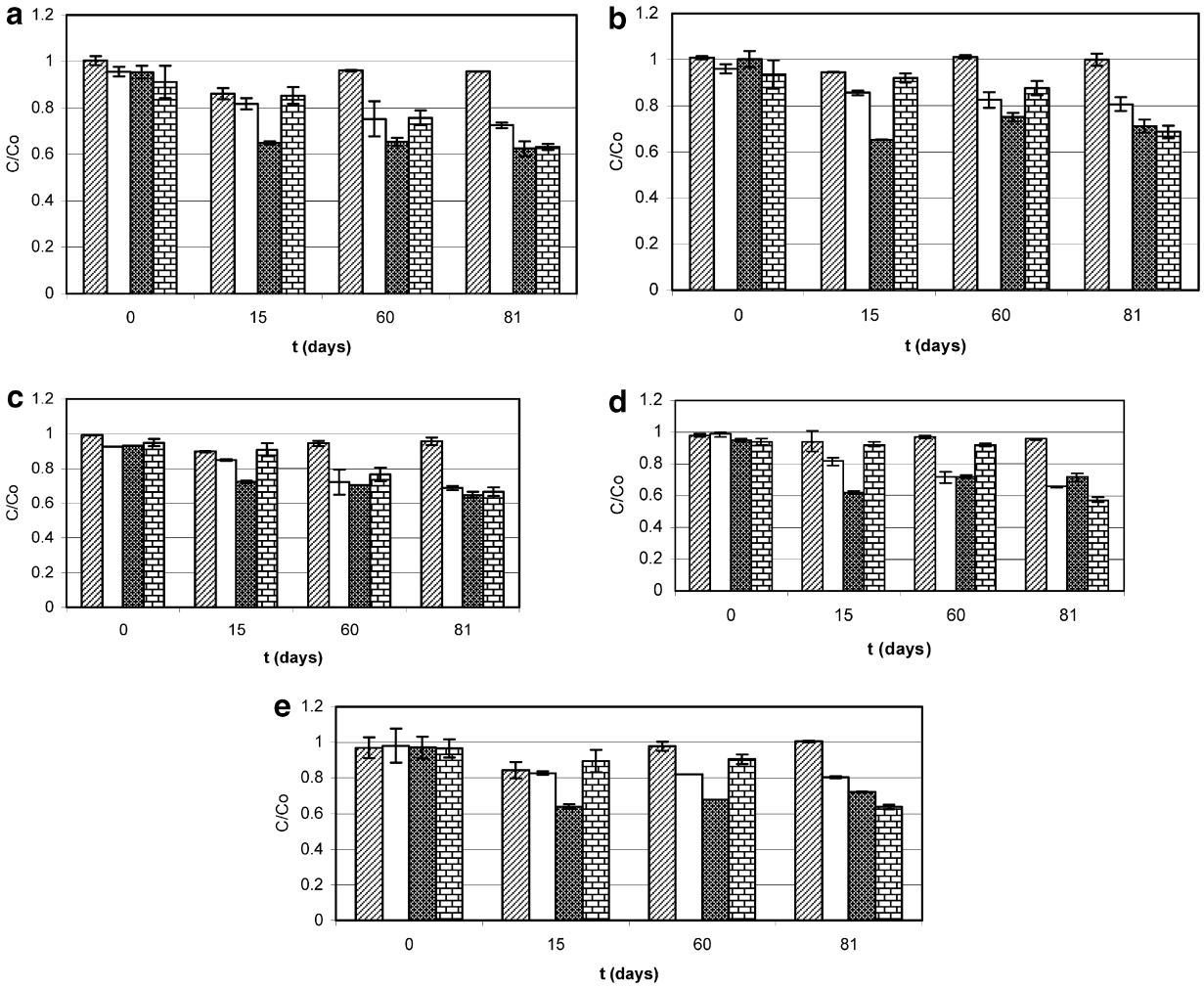

Fig. 2. Effect of storage on vitamin content (▨ pantothenic acid; □ biotin; ▩ folic acid; ▤ riboflavin) of a mixed orange juice and milk beverage treated by heat (a, 84 °C 15 s; b, 84 °C 60 s; c, 84 °C 120 s) or pulsed electric fields (d, 40 kV/cm 130 μs; e, 15 kV/cm 700 μs). Bars express standard deviation.

vitamins after high heat treatments, reaching 20% with the most intense treatment (95 °C, 45 s).

3.1.2. Effects of storage on stability of vitamins treated by PEF or heat

One point to take into account when studying the effect of a preservation technology on a particular target, such as microorganisms, enzymes, quality factors, or nutritive compounds, is the stability of the target after treatment. In the case of PEF technology, there are several studies comparing the stability of PEF- or heat-treated foods from the point of view of microorganisms, quality factors, or nutritive compounds (Elez-Martinez et al., 2006; Min et al., 2003; Min and Zhang, 2003; Rivas et al., 2006; Torregrosa et al., 2006). However, no stability studies have been done for beverages fortified with water-soluble vitamins treated by PEF, or for any target in this kind of beverage.

For the storage study, the selection of PEF treatments was based on the maximum achieved inactivation of *E. coli* suspended in the product (Rivas, Sampedro, et al., 2006); in the case of heat-treatment, we selected the treatments studied previously which did not cause a change in vitamin contents. A storage temperature of 4 °C was selected because it is the optimum temperature for preserving foods treated by HTST. During storage, total plate counts and mold and yeast flora counts were monitored, giving counts of less than 100 cfu/ml at 81 days.

As Fig. 2 shows, the stabilities of vitamins during storage were similar, irrespective of treatment and technology ($p > 0.05$). Pantothenic acid is the most stable vitamin, not being affected by storage time ($p \leqslant 0.05$). Although Bergström (1994) reported the possible influence of the low pH (pH 5.5) of a marinade on the stability of pantothenic acid, the data obtained showed that, at pH 4, there were no losses of pantothenic acid in the beverage in the range of treatments studied.

As just stated, the behaviour of biotin during storage was independent of the technology and intensity of treatment ($p \leqslant 0.05$). All samples showed the same tendency, with biotin content decreasing gradually during storage, reaching a degradation of 20–35% at 81 days of storage. The loss of biotin content might be due to the presence of O_2 dissolved in the beverage.

In the case of riboflavin, a difference of behaviour between samples treated by different technologies was observed (Fig. 2). While the loss of riboflavin content in the thermally treated samples during storage was gradual, reaching a retention of 63–68% after 81 days, the samples treated by PEF presented a higher stability after 15 and 60 days of storage (90% retention compared with 75% for the thermal samples). The loss of riboflavin content during storage can be explained by the presence of O_2, as the combination of heat and O_2 is more harmful for the stability of riboflavin. It is known that riboflavin stability is affected by oxygen, metal sulfates, amino acid chelates, and water activity (Choe, Huang, & Min, 2005). Dennison et al. (1977) observed that the presence of O_2 during storage increased the destruction rate dramatically. Also, Gaylord, Warthesen, and Smith (1986) observed a lower riboflavin destruction rate in whole milk than in skim milk. Consequently, the combination of O_2 and skim milk used in this beverage might explain the loss of riboflavin content during storage.

As can be seen in Fig. 2, folic acid is the most sensitive water-soluble vitamin during storage at 4 °C, irrespective of the technology applied. Vitamin destruction ranged from 28% to 36% after 15 days, remaining constant for the rest of the storage period. Although Eberhard, Buetikofer, and Sieber (2003) observed no losses of folic acid concentration during storage (4 weeks at 5 °C) of HTST-pasteurized milk (direct process 125 °C or indirect process 115 °C), its stability can be affected by the presence of O_2 (0.1 ppm $O_2 = 0\%$ losses after 60 days; 1–2 ppm = 5%; 8 ppm = 100%) (Rossi, Gobbetti, Buzzini, & Corsetti, 1995), with dissolved O_2 being the cause of loss of folic acid in the beverage during storage.

3.2. ACE inhibitory activity

3.2.1. Effect of PEF or thermal technology on ACE inhibitory activity

During the introduction of a new product preservation technology, it is essential to consider the effects of the technology from various viewpoints, such as food safety and quality and nutritional aspects. Cow's milk is a unique source of nutrients and bioactive components. The protein component of milk contains a variety of bioactive amino acids and peptides with different functional characteristics, ACE inhibitory peptides being the components that the scientific community is making most effort to study (Fitzgerald & Murray, 2006). Numerous studies have focussed on the production and isolation of ACE inhibitory peptides (Pihlanto-Leppälä, 2001; Yamamoto, 1997; Yamamoto, Ejiri, & Mizumo, 2003), but there are no studies on the stability of bioactive peptides incorporated in a complex food (a mixture of orange juice and milk) when processed by high intensity electric pulses or heat.

Table 4 shows the residual ACE inhibitory ability values of the beverage ($IACE_T/IACE_B$) after it had been subjected to high intensity electric pulses, $IACE_T$ and $IACE_B$ being the ACE inhibition values of the treated and the untreated beverage. As the Table shows, when the field intensity (15–40 kV/cm) or treatment time (0–700 μs) varies, the ACE inhibitory activity is hardly affected ($p \leqslant 0.05$). With regard to heat treatment, Fig. 3 shows the stability of the peptide when subjected to treatments of various intensities (84–95 °C) ($p \leqslant 0.05$).

For a study of the stability of a factor in relation to a technology, the immediate effect of the technology and the behaviour of the factor during storage after treatment are equally important. Fig. 4 shows the evolution of the ACE inhibitory activity of the beverage treated by the two technologies during storage at 4 °C. As can be seen,

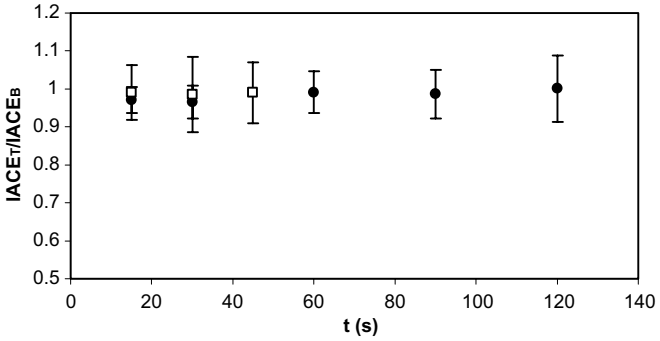

Fig. 3. Effect of heat treatment on ACE inhibitory activity (● 84 °C; □ 95 °C). Bars express standard deviation.

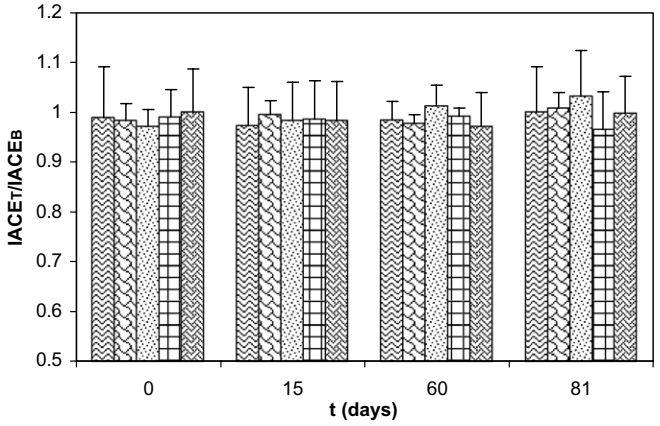

Fig. 4. Effect of storage on ACE inhibitory activity (▨ 40 kV/cm, 130 μs; ▨ 15 kV/cm, 700 μs; ▨ 84 °C 15 s; ▨ 84 °C 60 s, ▨ 84 °C 120 s) of a mixed orange juice and milk beverage treated by pulsed electric fields or pasteurization. Bars express standard deviation.

there was no variation in activity in any of the samples during storage, whether treated by PEF or by heat. These results show the validity of PEF in maintaining the ACE inhibitory activity of the beverage. The choice between PEF and heat as the means of preserving a beverage fortified with ACE inhibitory peptides does not depend, therefore, on the stability of the peptides with regard to the technologies used but on other factors (microbiological, nutritional or quality factors).

4. Conclusions

The most important step when studying the implementation of a preservation technology for a high quality food, such as a mixed orange juice and milk beverage fortified with water-soluble vitamins or ACE inhibitory peptides, is the achievement of safe foods, with minimal loss of fresh quality and nutritive compounds, both in the preservation treatment and during storage. This study proves the validity of PEF technology in a complex food, such as this kind of beverage, because, in PEF treatment conditions, other studies have achieved acceptable microbial and enzyme destruction rates in the same or similar foods, and vitamin content and ACE inhibitory capacity were not affected. If one compares PEF and thermal vitamin stability, PEF technology affects water-soluble vitamin content (biotin, folic acid, pantothenic acid) at least in the same way as mild heat treatments (84 °C) and better than high intensity thermal treatments (such as 95 °C and 45 s). In the case of riboflavin, PEF treatments preserve its stability better than do thermal treatments when stored at 4 °C for 60 days.

However, there are no differences in the ACE inhibitory ability of the beverage when treated by PEF or by heat. This *in vitro* study should be complemented by *in vivo* studies of the bioavailability of antihypertensive peptides in the beverage when treated by high intensity pulsed electric fields or by heat.

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