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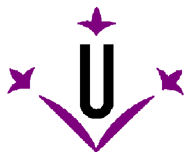

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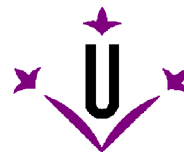
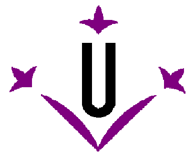


Aplicación de pulsos eléctricos de alta intensidad de campo en combinación con sustancias antimicrobianas para garantizar la calidad e inocuidad microbiológica de zumos de frutas

Jonathan Mosqueda-Melgar

Tesis Doctoral

Octubre 2007



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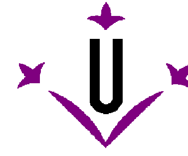

Aplicación de pulsos eléctricos de alta intensidad de campo en combinación con sustancias antimicrobianas para garantizar la calidad e inocuidad microbiológica de zumos de frutas

Memoria presentada por:
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para optar al grado de Doctor

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España, Lleida, Octubre de 2007

Resumen

La aplicación de pulsos eléctricos de alta intensidad de campo (PEAIC) es una nueva e innovadora tecnología no térmica de procesado mínimo que es usada como un proceso de preservación alternativa para zumos de frutas. El tratamiento de PEAIC es capaz de inactivar microorganismos y prolongar la vida útil de estos productos sin los efectos indeseables de calor. Partiendo de esta premisa, los objetivos se centraron en evaluar y optimizar los efectos del tiempo de tratamiento (hasta 2000 μs) y frecuencia del pulso (100-250 Hz), como parámetros de procesamiento de PEAIC, sobre poblaciones de *Escherichia coli* O157:H7, *Salmonella* Enteritidis y/o *Listeria monocytogenes* inoculadas en los zumos de melón, sandía, manzana, pera, naranja, fresa y tomate. También se evaluó el efecto de PEAIC en combinación con ácido cítrico (hasta 2,0%) o aceite esencial de canela (hasta 0,3%), como sustancias antimicrobianas, sobre estos microorganismos patógenos inoculados en los zumos de frutas. El efecto de PEAIC con o sin antimicrobianos sobre la microflora nativa presente en cada zumo de fruta y su impacto sobre los atributos sensoriales fueron evaluados igualmente. El tiempo de tratamiento tuvo una mayor influencia que la frecuencia de pulsos sobre las poblaciones microbianas, siendo mayor la inactivación microbiana cuando se aplicaron tiempos más largos de tratamiento. Se estimaron los valores óptimos de tiempo de tratamiento y frecuencia de pulso para reducir la cantidad máxima de los patógenos en cada zumo de fruta usando un análisis de respuesta múltiple. El zumo de naranja fue pasteurizado completamente por PEAIC, ya que se lograron más de 5 reducciones logarítmicas de las bacterias patógenas objetivos. *L. monocytogenes* mostró ser más resistente a tratamientos de PEAIC seguido por *S.* Enteritidis y luego *E. coli* O157:H7. Sin embargo, cuando PEAIC se combinó con ácido cítrico o aceite esencial de canela, se observó una mayor resistencia de *E. coli* O157:H7 al tratamiento, independientemente del zumo de fruta utilizado. Por otra parte, cuando PEAIC y antimicrobianos se combinaron se logró reducir a los microorganismos patogénicos por más de 5-$\log_{10}$, inactivar la flora nativa y extender la vida útil microbiológica de todos los zumos de frutas por más de 91 días. Aunque, la calidad e inocuidad microbiológica de los diferentes zumos de frutas estudiados fueron aseguradas al combinar PEAIC y antimicrobianos, se detectaron cambios perceptibles sobre algunos atributos sensoriales, tales como sabor, olor y acidez.

Resum

L'aplicació de polsos elèctrics d'alta intensitat de camp (PEAIC) és una nova i innovadora tecnologia no tèrmica de processat mínim que és utilitzada com un procés de preservació alternativa per a sucs de fruites. El tractament de PEAIC és capaç d'inactivar microorganismes i allargar la vida útil d'aquests productes sense els efectes indesitjables de calor. Partint d'aquesta premissa, els objectius es van centrar en avaluar i optimitzar els efectes dels temps de tractament (fins 2000 μs) i freqüència de pols (100-250 Hz), com a paràmetres de processament de PEAIC, sobre poblacions de *Escherichia coli* O157:H7, *Salmonella* Enteritidis i/o *Listeria monocytogenes* inoculades en els sucs de meló, síndria, poma, pera, taronja, maduixa i tomaquet. També s' avaluà l'efecte de PEAIC en combinació amb àcid cítric (fins a 2,0%) o oli essencial de canyella (fins a 0,3%), com a substàncies antimicrobianes, sobre aquests microorganismes patògens inoculats en els sucs de fruites. L'efecte de PEAIC amb o sense antimicrobians sobre la microflora nativa present en cada suc de fruita i el seu impacte sobre els atributs sensorials foren avaluats igualment. El temps de tractament va tenir una major influència que la freqüència de pols sobre les poblacions microbianes, sent major la inactivació microbiana quan es van aplicar temps més llargs de tractament. Es van estimar els valors òptims de temps de tractament i freqüència de pols per a disminuir la quantitat màxima dels patògens de cada suc de fruita utilitzant una anàlisi de resposta múltiple. El suc de taronja fou pasteuritzat completament per PEAIC, ja que s'aconseguiren més de 5 reduccions logarítmiques de les bactèries patògenes objectius. *L. monocytogenes* va mostrar ser més resistent a tractaments de PEAIC seguit per *S.* Enteritidis i després *E. coli* O157:H7. Tanmateix, quan PEAIC va ser combinat amb àcid cítric o oli essencial de canyella, es va observar una major resistència de *E. coli* O157:H7 al tractament, independentment del suc de fruita utilitzat. D'altra banda, quan PEAIC i antimicrobians es van combinar es va aconseguir reduït els microorganismes patògenes per més de 5-$\log_{10}$, inactivar la flora nativa i l'allargament de la vida útil microbiològica en tots els sucs de fruites per més de 91 dies. Tot i que la qualitat i seguretat microbiològica dels diferents sucs de fruites estudiats van ser garantits per combinar PEAIC i antimicrobians, es van detectar canvis perceptibles sobre alguns atributs sensorials, tals com sabor, olor i acidesa.

Summary

The application of high-intensity pulsed electric fields (HIPEF) process is a novel and innovative non-thermal minimal processing technology that is used as an alternative preservation process for fruit juices, because it is able to inactivate microorganisms and to increase the shelf-life of these products without undesirable heat effects. Based on these premises, the objectives were focused to evaluate and optimize the effects of treatment time (up to 2000 μs) and pulse frequency (100-250 Hz), as processing parameters of HIPEF, on populations of *Escherichia coli* O157:H7, *Salmonella* Enteritidis and/or *Listeria monocytogenes* inoculated in melon, watermelon, apple, pear, orange, strawberry and tomato juices. The HIPEF combination with citric acid (up to 2.0%) or cinnamon oil (up to 0.3%), as antimicrobial substances, against those pathogenic microorganisms in fruit juices was also evaluated. The effect of HIPEF with or without antimicrobial on the naturally occurring microorganisms in each fruit juice as well as their impacts on the sensory attributes were also studied. Treatment time was more influential than pulse frequency on the microbial populations, being greater the microbial inactivation when longer treatment times were applied. Optimum treatment time and pulse frequency values to reduce the highest levels of the pathogenic microorganisms in each fruit juice using a multiple response analysis were estimated. Only, orange juice could be pasteurized by HIPEF, since, more than 5 log reductions of those target pathogenic microorganisms were achieved. *L. monocytogenes* showed to be more HIPEF-resistant than *S.* Enteritidis and *E. coli* O157:H7. However, when HIPEF treatment was combined with citric acid or cinnamon oil, a higher resistant of *E. coli* O157:H7 to the treatment was found, irrespectively the kind of juice used. On the other hand, more than 5-$\log_{10}$ reductions of pathogenic microorganisms, inactivation of spoilage microorganisms, and an extension of the microbiological shelf-life by more than 91 days in all fruit juices were achieved when HIPEF treatment and antimicrobials were combined. Although, the microbiological safety and quality of the fruit juices were ensured by combining HIPEF and antimicrobials, noticeable changes on some sensory attributes such as taste, odor and sourness were detected.

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Introducción

Effects of pulsed electric fields on pathogenic microorganisms of major concern in fluid foods. A review

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Critical Reviews in Food Science and Nutrition
En prensa

ABSTRACT

Pathogenic microorganisms such as *Escherichia coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Yersinia enterocolitica* and *Campylobacter jejuni* have been implicated in foodborne diseases and outbreaks worldwide. These bacteria have been associated to the consumption of fresh fruit juices, milk and dairy products, which are foodstuff, highly demanded by consumers in retails and supermarkets. Nowadays, consumers require high quality, fresh-like and safe foods. Pulsed electric field (PEF) is a non-thermal preservation method, able to inactivate pathogenic microorganisms without significant loss of the organoleptic and nutritional properties of food. The PEF treatment effectiveness to destroy bacteria such as *Listeria innocua, E. coli, Salmonella* Typhimurium, *E. coli* O157:H7 and *E. coli* 8739 at pasteurization levels ($\geq$ 5.0 $\log_{10}$ cycles) in some fluid foods was reported. However, data on the inactivation of some microorganisms such as *Bacillus cereus*, *Staphylococcus aureus*, *Yersinia enterocolitica* and *Campylobacter jejuni* in fluid foods by PEF processing is very limited. Therefore, future works should be focused toward the inactivation of these pathogenic bacteria in real foods.

Keywords*: Pulsed electric fields, outbreaks, pathogenic microorganisms, fruit juice, milk*

INTRODUCTION

The majority of foods are harbor from several types of microorganisms. Some of them have desirable roles in the food industry, such as in the production of fermented foods, whereas others cause food spoilage and human diseases. Bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, *Bacillus cereus*, *Yersinia enterocolitica* and *Campylobacter jejuni*, may be present in foods and generate public health problems.

The outbreaks associated to the consumption of fresh products in special fruit juices, milk and dairy products have increased in the last years, due to their health-promoting image with slogans as "fresh products maintain its nutritional properties". However, those "fresh" products, which omit any effective microbial elimination step, result in foods that naturally would carry some microorganisms, including pathogenic bacteria[1]. Thus, a minimal processing of fresh products to decrease or eliminate bacteria without affecting significantly their nourishing attributes is required. For this reason, the consumer's today is demanding high quality, fresh-like and microbially safe foods [2, 3].

Conventionally, heat is the most popular preservation technology for the elimination of microbial contamination of foods, in particular pathogens. Although this process guarantees the safety of foods, their organoleptic, nutritional and physicochemical properties are extensively damaged [4, 5]. Thus, the food industry is exploring alternative preservation methods to counteract negative effects that occur during usual heat treatments [6].

Pulsed electric fields (PEF) treatment is a non-thermal technology able to inactivate microorganisms in foods without significant loss of flavor, color and nutrients [7, 8, 9 10]. Thus, this processing method may offer to consumer safe, fresh-like and nutritious food products. PEF treatment involves the application of short pulses (1 to 10 μs) with high intensity electric field (typically 20 to 80 kV/cm) to fluid foods placed between two electrodes in batch or continuous flow treatments. Nevertheless, PEF application is restricted to fluid foods that can tolerate high electric fields strength (E), have low electrical conductivity (σ), and do not contain or form bubbles. The particle size of the food is also a limitation [11]. However, others factors such as frequency (f), treatment time (t), type and shape of the pulse, in addition to the self-microorganism characteristics could influence on the efficacy of the PEF processing [12].

The Electroporation's Theory of Coster and Zimmermann [13] elucidate the microbial inactivation mechanism, when pulses of electric fields are applied. This Theory explains the formation of pores in the cellular membrane, which is able to generate cellular lysis with subsequent leakage out of intracellular compounds due to the induced electric field [14]. However, this phenomenon has demonstrated to be reversible or irreversible, depending on the level of electric fields intensity [15] and/or the membrane organizational changes [16, 17]. Mild electric fields intensity conditions form reversible pores in the cellular membrane, whereas drastic electric field intensity conditions lead to the irreversibility of this phenomenon, which results in cellular death [15, 17, 18].

The PEF application as a non-thermal method to assure the elimination of pathogenic microorganisms frequently responsible of outbreaks by consumption of fluid foods was the main motivation for this review.

Escherichia coli O157:H7

Enterohemorrhagic *E. coli* O157:H7 is a Gram-negative rod, which possesses several characteristics uncommon to most others *E. coli* such as inability to grow properly at 44.5ºC and produce β-glucuronidase [19]. Enterohemorrhagic *E.coli* O157:H7 is recognized as an important foodborne pathogen [20], since it has been implicated in several outbreaks involving fluid foods, such as juices [21, 22], and dairy products [23, 24, 25, 26] (*Table 1*). The Center for Disease Control and Prevention (CDC) [21] reported an outbreak in the United States due to *Escherichia coli* O157:H7 in unpasteurized apple juice. In that case, the pathogenic microorganism affected to 71 people, among them 14 suffered hemolytic uremic syndrome (HUS) and one died. The contamination may occurred by using poor quality and/or dropped apples and the localization of apples orchard near cattle/deer. Most of microbial contaminations in juices reported in the literature are due to fruits inadequately washed, brushed and pressed, the use of contaminated rinse water and poor manufacture practice applied during the processing. However nowadays, there are several methods for controlling the microorganisms' presence in these fluid foods, such as PEF treatment.

Evrendilek *et al.* [27] inactivated pathogenic microorganisms such as *E. coli* O157:H7 and *E. coli* 8739 at pasteurization levels in apple juice treated by PEF. They reached 5.0 $\log_{10}$ and 5.4 $\log_{10}$ reductions of *E. coli* O157:H7 and *E.coli* 8739, respectively, applying 29kV/cm electric field during 172μs

Table 1.–Most significant outbreaks of pathogenic microorganisms in fluid foods reported world wide

Causal agent	Years	Source	Country	N^r Cases	Reference
C. jejuni	2002	Unpasteurized milk	USA	46	CDC, 2002
C. jejuni	2001	Unpasteurized milk	USA	75	Harrington *et al.*, 2002
C. jejuni	2000	Unpasteurized milk	Austria	38	Lehner *et al.*, 2000
C. jejuni	1992	Raw milk	USA	50	CDC, 1992
C. jejuni	1985	Raw milk	USA	23	CDC, 1985
E. coli O157:H7	2005	Raw milk	USA	18	Anonymous, 2005b
E. coli O157:H7	1998	Milk	USA	28	Meng *et al.*, 2001
E. coli O157:H7	1996	Apple juice	USA	71(1[a],14[b])	CDC, 1996
E. coli O104:H21	1994	Pasteurized milk	USA	18	CDC, 1995
E. coli O157:H7	1994	Pasteurized milk	Scotland	100 (9[b])	Upton and Coia, 1997
E. coli O157:H7	1991	Apple cider	USA	23	Besser *et al.*, 1993
L. monocytogenes	1994	Chocolate milk	USA	56	Swaminathan, 2001
L. monocytogenes	1986	Raw milk	Austria	28	Lundén *et al.*, 2004
L. monocytogenes	1983	Pasteurized milk	USA	49 (14[a])	Swaminathan, 2001
S. Typhimurium	2005	Orange juice	USA	31	Anonymous, 2005a
S. Typhimurium	2002	Unpasteurized milk	USA	107	CDC, 2002
S. Enteriditis	2000	Orange juice	USA	74	D'Aoust *et al.*, 2001
S. Muenchen	1999	Orange juice	USA & Canada	220	Boase *et al.*, 1999
S. Typhimurium	1999	Orange juice	Australia	427	D'Aoust *et al.*, 2001
S. Enteriditis	1991	Orange juice	Germany	600	D'Aoust *et al.*, 2001
S. Typhimurium	1985	Pasteurized milk	USA	16,284 (7[a])	D'Aoust *et al.*, 2001
S. Dublin	1983	Raw milk	USA	123	CDC, 1983
S. Typhimurium	1981	Raw milk	Scotland	654 (2[a])	D'Aoust *et al.*, 2001
S. Typhimurium	1976	Raw milk	Australia	500	D'Aoust *et al.*, 2001
Bacillus cereus	1988	Milk	Canada	36	CDC, 2002
Bacillus cereus	1989	Milk	Canada	74	CDC, 2002
S. aureus	1985	Chocolate milk	USA	860	Everson *et al.*, 1988
Y. enterocolitica	1995	Pasteurized milk	USA	10	Robins-Browne, 2001
Y. enterocolitica	1980	Milk	Japan	1,051	Robins-Browne, 2001
Y. enterocolitica	1976	Chocolate milk	USA	38	Robins-Browne, 2001

([a]) Numbers of people death
([b]) Numbers of people with Hemolytic Uremic Syndrome (HUS)

in bipolar mode and continuous flow treatment (*Table 3*). Likewise, Iu *et al.* [28] achieved 5.35 $\log_{10}$ and 5.91 $\log_{10}$ reductions of *E. coli* O157:H7 in apple cider by PEF treatment when used 80 kV/cm and 90 kV/cm during 60 μs and 20 μs of treatment time, respectively. They kept the treatment temperature at 42ºC, since this pathogenic microorganism is likely to be sensitive to heat above 42ºC. In addition, when they combined PEF treatment (80 kV/cm and 60 μs) with antimicrobial agents such as cinnamon or nisin at 2.0 %, 6.23 $\log_{10}$ and 8.78 $\log_{10}$ reductions were reached, respectively.

On the other hand, Evrendilek and Zhang [29] studied the effect of pulse polarity and pulse delaying time on *E. coli* O157:H7 in apple juice (σ: 2.3 mS/cm; pH 3.70 ± 0.24) and skim milk (σ: 6.2 ± 3.4 mS/cm; pH 6.7 ± 0.65). Both apple juice and skim milk were processed at 20 μs pulse delaying time, 700 Hz of frequency and 30ºC outlet temperature in a continuous flow system that discharge square wave pulses. They found reductions up to 2.6 $\log_{10}$ cycles without significant differences among bipolar and monopolar pulses for the inactivation of the microorganism in apple juice when used 31 kV/cm by 202 μs treatment time and 4.0 μs pulse length. However, in skim milk, the polarity exerted a significant influence on the effectiveness of the PEF treatment, using 24 kV/cm by 141 μs treatment time and 2.8 μs pulse length. They reported a reduction of 1.27 $\log_{10}$ and 1.88 $\log_{10}$ cycles in skim milk using monopolar and bipolar pulses, respectively. Generally, bipolar pulses are slightly more efficient than monopolar pulses on the destruction of microorganisms [30, 31, 32, 33]. The PEF applications cause movement of charged molecules in the cell membranes of microorganisms, thus a reversal in the orientation o polarity of the electric field causes a change in the direction of movement of the charged molecules. The alternating changes in the direction of the movement of the charged molecules may cause a stress in the cell membrane that enhances electric breakdown of the cell membrane and microbial inactivation [4, 6, 29]. Moreover, the bipolar pulses offer the advantage of reducing the solid deposits on the electrode surface by more than 80 % and minimum energy consumption in comparison with monopolar pulses.

McDonald *et al.* [34] and Martín-Belloso *et al.* [35] reported from 5.0 to 6.0 $\log_{10}$ reductions of nonpathogenic *E. coli* strains, which showed behavior and characteristic very similar to the pathogenic strain under PEF treatment, in orange juice and liquid egg respectively. McDonald *et al.* [34] applied six pulses per unit of volume of 2.0 μs pulse width at 30 kV/cm and 54ºC outlet temperature to orange juice inoculated with the microorganism.

Whereas, Martín-Belloso *et al.* [35] applied 100 pulses of 4.0 μs pulse width at 26 kV/cm electric field and a maximum 37.2ºC temperature to liquid egg also inoculated with *E. coli* (*Figure 1*). However, in the study made by McDonald *et al.* [34], the high inactivation rate might be mainly attributed to thermal effects, since *E. coli* is sensible to heat above 46ºC. Likewise, Martín-Belloso *et al.* [35] working with a much higher number of pulses and lower temperature (37.2ºC maximum), reached the same inactivation of *E. coli*, ensuring that the inactivation of microorganism was achieved by PEF treatment and not by heat.

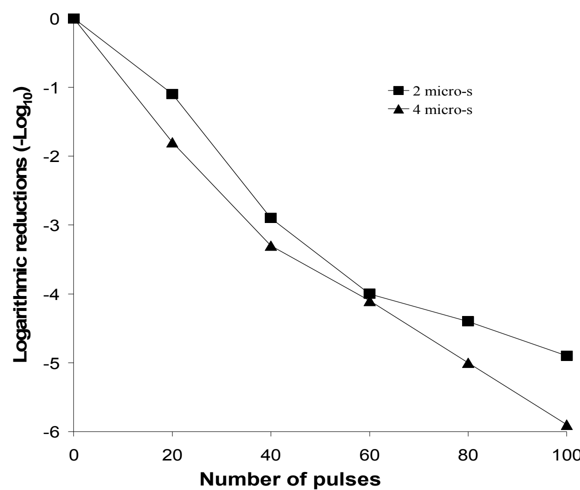

Figure 1.- *Inactivation of E. coli in liquid egg by PEF treatment at 26 kV/cm and 37ºC, using different number and width pulses (Adapted from Martín-Belloso et al., 1997).*

On the other hand, Malicki *et al.* [36] found a reduction of 4.7 $\log_{10}$ cycles of *E. coli* in liquid whole egg by PEF, using 180 pulses for 30 μs at 20ºC (*Table 3*). The inactivation found by those researchers is lower than the reported by Martín-Belloso *et al.* [35], due probably to the lower treatment temperature. Hence, the effect of the medium temperature could significantly influence over the membrane fluidity properties, because at

Table 2.-General characteristics of pathogenic microorganisms

Characteristics	*E. coli* O157:H7	*Listeria monocytogenes*	*Salmonella* spp.	*S. aureus*	*Bacillus cereus*	*C. jejuni*	*Yersinia enterocolitica*
Shape	Rod	Short rod	Rod	Spherical	Rod	Spiral rod	Rod
Diameter (µm)	0.9 to 1.5	0.5 to 0.8	1.0	0.5 to 1.0	1.0 to 2.0	0.2 to 0.9	0.5 to 1.0
Length (µm)	2.0 to 6.0	1.0 to 2.0	4.0	-	3.0 to 5.0	0.5 to 5.0	1.0 to 2.0
Type	Gram-negative	Gram-positive	Gram-negative	Gram-positive	Gram-positive	Gram-negative	Gram-negative
Temperature [1] (ºC)	7 to 46 37*	-1.5 to 45 37*	2 to 50 37*	7 to 48 37*	4 to 55 35*	30.5 to 45 42*	4 to 44 29*
Oxygen conditions	Aerobic or facultatively anaerobic	Aerobic or facultatively anaerobic	Aerobic or facultatively anaerobic	Aerobic or facultatively anaerobic	Aerobic or facultatively anaerobic	Facultatively anaerobic	Aerobic or facultatively anaerobic
pH [2]	4.0 to 9.0 7.0*	4.3 to 9.6 7.0*	3.6 to 9.6 7.0*	4.2 to 9.5 7.0*	4.3 to 9.3 7.0*	4.9 to 9.0 7.0*	4 to 10 7.3*
Water activity[3]	0.95	0.92	0.93	0.86	0.912	0.987	0.95
Spore-forming	Not	Not	Not	Not	Yes	Not	Not

[1] Growth temperature range
[2] Growth pH range
[3] Minimal water activity of growth
* Optimum values of growth

low temperature (10 to 20ºC) the phospholipids of the lipidic bilayer are closely packed into a rigid gel structure, while at higher temperature (30 to 40ºC) the phospholipids are less ordered and the cellular membrane has a liquid-crystalline structure [3]. Therefore, choosing the PEF treatment temperature is very important, given that a high temperature (up 30ºC) increases the susceptibility of the cellular membrane to the electroporation and the antimicrobial efficiency of PEF treatment. In the same direction,Bazhal *et al.* [37] achieved up to 4.0 $\log_{10}$ reductions of *E. coli* O157:H7 in liquid whole egg by PEF treatment using 40 pulses subject to 11 kV/cm and 60ºC outlet temperature (*Table 3*). In spite of the high temperature applied, the PEF treatment was not enough for inactivating the microorganism at pasteurization levels. It could be ascribed to the low electric field value applied during the treatment, since higher electric field values generate larger microbial inactivation. In addition, the thermal treatment (60ºC) applied to *E. coli* O157:H7 in liquid egg reduced 2.3 $\log_{10}$ cycles, given that this microorganism is sensible at temperatures above 46ºC. Therefore, the PEF treatment only inactivated 2.0 $\log_{10}$ cycles approximately of the pathogenic bacterium.

Table 3.-Process parameters used for the inactivation of pathogenic microorganisms in fluid foods by PEF treatment

Microorganisms	Fluid Food	E (kV/cm)	n[a]	τ[b] (μs)	t_t[c] (μs)	f (Hz)	T (°C)	Log_{10} reductions	References
L. innocua	Orange juice	30	6	2.0	12	-	54	6.0*	McDonald *et al*., 2000
L. monocytogenes	Whole milk	30	400	1.5	600	1700	50	4.0	Reina *et al*., 1998a
L. innocua	Skim milk	41	63	2.5	157.5	3	37	3.9	Dutruex *et al*., 2000
L. innocua	Liquid egg	50	32	2.0	64	3.5	36	3.4	Calderon-Miranda *et al*., 1999b
L. innocua	Whole milk	29	312	0.8	250	100	36	2.0	Picart *et al.,* 2002
L. innocua	Dairy cream	37.5	250	1.0	250	100	36	2.0	Picart *et al.,* 2002
L. monocytogenes	Skim milk	20	10	3.25	32.5	-	35	1.0	Fleischman *et al*., 2004
E. coli	Liquid egg	26	100	4.0	400	2.5	37	6.0*	Martín-Belloso *et al*., 1997
E. coli	Orange juice	30	6	2.0	12	-	54	6.0*	McDonald *et al*., 2000
E. coli O157:H7	Apple cider	90	10	2.0	20	-	42	5.91*	Iu *et al*., 2001
E. coli 8739	Apple juice	29	43	4.0	172	1000	42	5.4*	Evrendilek *et al*., 1999
E. coli O157:H7	Apple juice	29	43	4.0	172	1000	42	5.0*	Evrendilek *et al*., 1999
E. coli	Liquid egg	32.89	180	0.17	30	-	20	4.7	Malicki *et al*., 2004
E. coli O157:H7	Skim milk	41	63	2.5	157.5	3	37	4.0	Dutruex *et al*., 2000
E. coli O157:H7	Liquid egg	11	40	2.0	80	1	60	4.0	Bazhal *et al*., 2006
E. coli	Milk (1,5% fat)	23	20	-	-	-	45	4.0	Grahl and Märkl., 1996
Bacillus cereus	Skim milk	31	20		6.0		25	0.7	Sobrino *et al.* 2001
S. aureus	Raw milk	40	40	-	-	3.5	-	4.0	Raso *et al*, 1999
S. aureus	Skim milk	35	124	3.7	459	250	40	3.7	Evrendilek *et al*, 2004
S. aureus	Skim milk	31	35		6.0		25	3.0	Sobrino *et al.* 2001
S. aureus	Skim milk	35	600	4.0	2,400	100	25	1.0	Sobrino-López and Martín-Belloso, 2006
S. Typhimurium	Orange juice	90	50	2.0	100	-	55	5.9*	Liang *et al*., 2002
S. Dublin	Skim milk	35	164	1.0	164	2000	50	4.0	Sensoy *et al*., 1997
S. Enteriditis	Eggs white	35	8	-	-	900	-	3.5	Jeantet *et al*., 1999

[a] number of pulses
[b] pulse width
[c] treatment time (μs)
- No reported
* log_{10} reductions at pasteurization levels

Dutruex *et al.* [38] applied PEF to skim milk (σ: 4.8 mS/cm; pH: 6.8) and buffer phosphate (σ: 4.8 mS/cm; pH: 6.8) inoculated with *E. coli*. They found a reduction of 4.0 log_{10} and 4.6 log_{10} cycles for skim milk and buffer phosphate respectively, using 63 pulses of 2.5 μs length each (*Table 3*). The experiments were carried out using different media to test their effects on the reduction of the microbial load when PEF is applied. They concluded than the medium composition seems not to influence on inactivation of *E. coli* by PEF application. However, Grahl and Märk [39], Martín *et al.* [40] and Martín-Belloso *et al.* [35] encountered that the presence of fats and proteins in UHT-milk (1.5 and 3.5 % fat), skim milk and liquid egg constrained the effectiveness of PEF treatment. Grahl and Märk [39] subjected UHT-milk 1.5

or 3.5 % fat inoculated with *E. coli* to PEF. They observed that UHT-milk with 1.5 % fat had a lower inactivation constant (B_E) than UHT-milk with 3.5 % fat. This behavior indicates that the fat particles of milk seem to protect the bacteria against the induced electric field. However, they reported a critical electric field (E_C) value more elevated for UHT-milk with 1.5 % fat than for UHT-milk with 3.5 % fat. It appear to be contradictory to the showed B_E value, since an elevated E_C value indicates a higher resistance to cellular death. Thus, the E_C value in the UHT-milk with 1.5 % fat should be lower regarding UHT-milk 3.5 % fat based on the B_E value. On the other hand, Martín-Belloso *et al.* [35] and Martín *et al.* [40] mentioned that proteins decrease the lethal effect of PEF on microorganisms by absorbing free radicals and ions, which are active in the cell breakdown. Moreover, the inactivation of bacteria by PEF is a function of the solution resistivity, which is inversely proportional to ionic strength [35]. Thus, the microbial inactivation is more difficult in real foods than buffer solutions and model foods, due to the complex composition of food.

On the other hand, Martín-Belloso *et al.* [35] did not found significant differences between a continuous flow system and a stepwise flow treatment in the inactivation of *E. coli* in liquid eggs by PEF treatment. Similarly, Martín *et al.* [40] reported the same behavior in skim milk, but they achieved a reduction near to 3.0 $\log_{10}$ cycles of *E. coli*, using 64 pulses at 45 kV/cm, 15ºC and 1.8 μs duration pulse for stepwise treatment, and over 2.0 $\log_{10}$ cycles using 25 pulses at 25 kV/cm, 15ºC and 1.8 μs duration pulse for a continuous flow treatment.

Listeria monocytogenes

The *Listeria monocytogenes* specie is widely distributed in the environment and may survive and grow in temperature, pH and oxygen harsh conditions (*Table 2*), but it is little competitive within populations with different bacteria. This pathogenic microorganism is able to grow in various food products such as milk, dairy products, fruit and vegetable juices among others. Recently, listeriosis outbreaks have been associated with dairy products manufactured from raw milk (*Table 1*) in United States of America [41] and Europe [42] leading to a big concern in the dairy industry. Although outbreaks of listeriosis associated to consumption of fruit and vegetable juices have not been reported, Sado *et al*. [43] isolated *L. monocytogenes* from two samples of unpasteurized apple juices sold in a retail of United State of America. Thus, the ability of *Listeria* spp. to grow

at refrigeration temperatures and survive at a wide range of pH values, confirm the importance of the inactivation of this microorganism during food processing [44].

Reina *et al.* [45] studied the effect of the PEF treatment over *L. monocytogenes* inoculated in milk samples with different fat content (whole milk, 2 % milk and skim milk). They reached approximately 3.0 log_{10} reductions in each case, when treating the inoculated samples with 400 square wave pulses of 1.5 μs length each (*Table 3*) in a continuous flow treatment. They did not observe significant effects of the composition (fat content) of medium when the PEF treatment was applied. The same behavior was reported by Dutruex *et al.* [38] in skim milk and buffer phosphate inoculated with *L. innocua* when applied 63 pulses of 2.5 μs length each (*Table 3*). Nevertheless, Picart *et al.* [46] studied the influence of the fat content and frequency of pulsation on *L. innocua* in UHT sterilized whole milk (3.6 % fat), skim milk (0 % fat) and sterilized liquid dairy cream (20 % fat) by PEF treatment. They reported a higher inactivation rate at 100 Hz than at 1.1 Hz for whole (2.0 log_{10} cycles and 0.67 log_{10} cycles respectively) and skim milk (1.25 log_{10} cycles and 0.95 log_{10} cycles respectively), whereas they did not find significant difference on the inactivation rate of microorganism in dairy cream, since 2.0 log_{10} reductions was achieved at both frequencies 100 Hz and 1.1 Hz. Curiously, when whole and skim milk were treated at 100 Hz, a higher microbial inactivation in whole milk than in skim milk was observed (*Figure 2*) when 29 kV/cm electric field strength was applied. However, they observed a protective

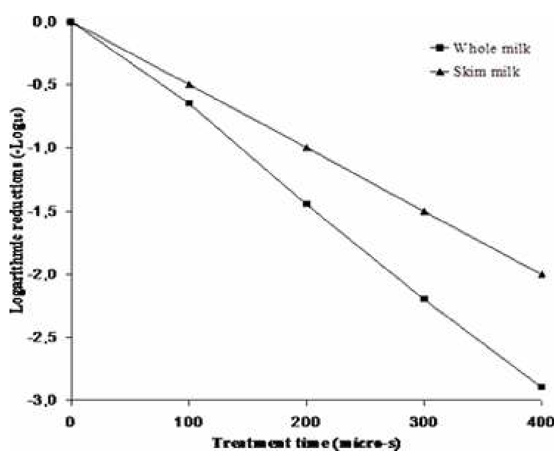

***Figure 2**.- Influence of the fat content on the inactivation of Listeria innocua as a function of treatment time at 29 kV/cm electric field and 0.86 μs pulse duration with 100 Hz pulse repeat frequency and 45ºC outlet temperature (Adapted from Picart et al., 2002).*

effect of the fat content on *L. innocua* inactivation in dairy cream when was treated by PEF at 38 kV/cm. In general, the protective effect of the fat content on *L. innocua* when it is submitted to PEF treatment is not very clear. Thus more studies about the influence of fat particles, as well as other components on the effectiveness of PEF to inactivate this pathogenic microorganism are necessary.

On the other hand, Reina *et al.* [45] found that increasing the treatment temperature from 25ºC to 50ºC in whole milk (*Table 3*), the inactivation of *L. monocytogenes* reached maximum values around 4.0 log_{10} reductions when 30 kV/cm for 600 µs treatment time were applied. Similarly, Fleischman *et al.* [47] observed that increasing the temperature from 35ºC to 55ºC in skim milk with gellam gum inoculated with *L. monocytogenes* gave a reduction of 1.0 log_{10} to 4.5 log_{10} cycles respectively, using 10 pulses of 3.25 µs pulse width (*Table 3*). However, they demonstrated that temperatures above at 50ºC were sufficient to inactivate *L. monocytogenes* up to 4.0 log_{10} cycles, since it is sensible at temperature up to 45ºC. Thus, this reduction was due to heat and not to PEF treatment. Finally, they concluded that *L. monocytogenes* seems not to be easily destroyed by PEF as a unique treatment; but a combination of the treatment with heat would be adequate for its inactivation.

There are others species included within the genus *Listeria,* such as *L. innocua, L. grayi, L. ivanovii, L. seeligeri* and *L. welshimeri* [48]. Nonetheless, *L. innocua* is often selected for inactivation studies because it is not a pathogenic microorganism and its behavior is closely related to *L. monocytogenes* [49].

Fernández-Molina *et al.* [50] reported a reduction of 2.7 log_{10} cycles of *L. innocua* in raw skim milk at 50 kV/cm for 60 µs treatment time, 2 µs pulse width, 4 Hz and 28ºC outlet temperature in a continuous flow system with exponential decay pulses. Calderón-Miranda *et al.* [44] observed the same behavior when treated skim milk inoculated with *L. innocua* by PEF. They achieved a reduction of 2.4 log_{10} cycles of the microorganism at 50 kV/cm, 32 pulses of 2 µs pulse duration, 34ºC and 3.5 Hz in a stepwise process with exponential decay pulse waveforms. In addition, when they exposed *L. innocua* to nisin after PEF application, a significant synergistic effect between PEF and nisin was observed, reaching 3.4 log_{10} and 3.8 log_{10} reductions at 10 IU/ml and 100 IU/ml respectively. In the same way, Calderón-Miranda *et al.* [51] achieved a reduction on the microbial population of 3.4 log_{10} cycles applying only PEF treatment in liquid whole egg when inoculated with *L. innocua* and processed in the same conditions of electric field strength, pulse duration, number of pulses and frequency (*Table 3*)

than skim milk, but at 36ºC. Moreover, they observed that the combination of PEF with nisin at 10 IU/ml and 100 IU/ml produced a higher microbial inactivation (4.1 log_{10} and 5.5 log_{10} reductions, respectively) than PEF alone. The difference in the inactivation levels between skim milk and liquid whole egg are attributed to electrical conductivity, ionic strength and composition of each medium. Likewise, Dutruex *et al.* [38] achieved a higher reduction of *L. innocua* (3.9 log_{10} cycles) by PEF treatment than the reported by Calderón-Miranda *et al.* [44], due to a higher pulse number applied and consequently higher treatment time. They discharged 63 pulses of 2.5 µs pulse length at 41 kV/cm (*Table 3*) in a continuous flow system to skim milk.

On the other hand, McDonald *et al.* [34] reported the maximum levels of *L. innocua* inactivation in fluid foods. They achieved reductions from 5.0 to 6.0 log_{10} cycles for *L. innocua* suspended in orange juice after PEF treatment with six pulses of 2.0 µs duration (*Table 3*) in a continuous flow system with exponential decay pulses and outlet temperature of 54ºC. However, this great inactivation may be attributed to the high treatment temperature applied during the processing and not only to PEF treatment, since *Listeria* spp. is sensible at temperature over 45ºC.

***Salmonella* spp**

The genus *Salmonella* groups two species, *enterica* and *bongori*, and currently encompasses 2,700 serovars approximately. These Gram-negative microorganisms can exhibit psycrotrophic properties by their ability to grow in foods stored between 2 and 4ºC [52], moreover they can survive and proliferate at low pH and water activity values (*Table 2*). Raw meats, eggs and dairy products are the most common source of human foodborne salmonellosis, but new products such as fruit and fresh juices have been incriminated in recent years as vehicles of human salmonellosis infections [53]. Some outbreaks of salmonellosis associated to consumption of pasteurized and raw milks and fruits juices [52, 54, 55, 56, 57] are showed in *Table 1*. In the United States of America and Germany [52], outbreaks of salmonellosis caused by *Salmonella* Enteritidis associated to unpasteurized orange juice affected 74 and 600 persons, respectively. Thus, studies on the inactivation of *Salmonella* spp. at pasteurization levels are very important to avoid these outbreaks.

Sensoy *et al.* [58] studied the effect of PEF processing on the inactivation of *Salmonella* Dublin suspended in skim milk. About 4.0 log_{10} reductions

were reached using an electric field of 35 kV/cm with 163.9 μs treatment time of 1 μs pulse duration and 2000 Hz (*Figure 3*). In addition, they observed that an enhance in microbial inactivation from 3.0 to 4.0 log_{10} reductions was obtained when increasing the treatment temperature from 25 to 50ºC.

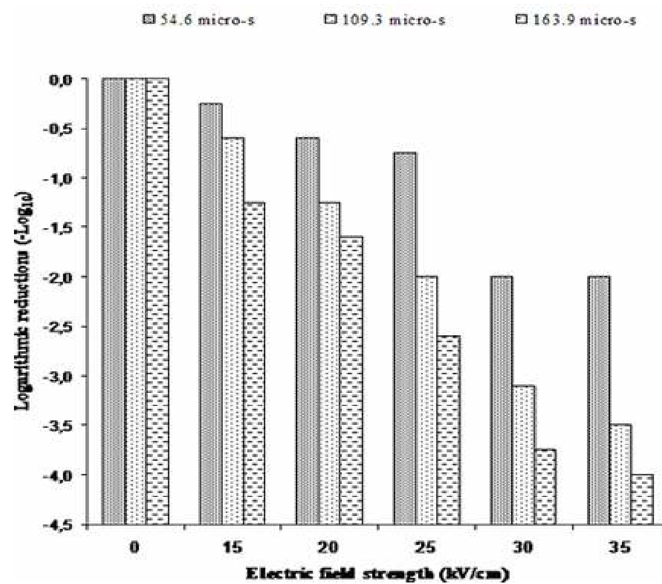

Figure 3.- *Effect of electric field strength on survival fraction (-log_{10}) of Salmonella serovar Dublin treated at different treatment times in skim milk, using 1 μs pulse length with 2,000 Hz of frequency in continuous flow mode (Adapted from Sensoy et al., 1997)*

Jeantet *et al.* [59] were able to inactivate *Salmonella* Enteritidis about 3.5 log_{10} cycles in diaultrafiltered egg white (σ = 2 mS/cm), when used an electric field strength of 35 kV/cm with 8 exponential decay pulses during 9 ± 1 μs decay time and 900 Hz frequency in a continuous flow system at 30ºC.

On the other hand, Liang *et al.* [60] reduced *Salmonella* Typhimurium at pasteurization levels (5.9 log_{10} cycles) in fresh squeezed orange juice (without pulp) when PEF was applied. They used 90 kV/cm and 50 pulses at 55°C treatment temperature. However, the high temperatures applied in this experiment could be the main cause of cellular death, since *Salmonella* spp. is heat sensible at temperature over 50ºC. Therefore, PEF in combination with moderate thermal treatment may lead to reach the pasteurization levels desirable in the food's industry.

Staphylococcus aureus

This Gram-positive microorganism of spherical shape is an important pathogenic microorganism that has been associated to food poisoning when found in the nourishment at concentrations that exceed 100,000 CFU/g [61]. At these levels, the bacterium is able to generate an enterotoxin highly heat-stable. However, *Staphylococcus aureus* is not regarded as a good competitor against other bacteria and therefore rarely causes food poisoning in raw products. In addition, this pathogen is very susceptible to destruction by thermal treatment. The fluid foods that have been frequently incriminated in staphylococcal food poisoning include milk, dairy products and egg derivates [62, 63].

Raso *et al*. [64] observed that *S. aureus* and coagulase negative *Staphylococus* spp. were inactivated in 4.0 and 2.0 log_{10} cycles, respectively, on raw milk when used 40 kV input voltage, 40 exponential decay pulses and 3.5 Hz. However, Evendrilek *et al.* [65] achieved 3.7 log_{10} reductions of *S. aureus* in skim milk at 35 kV/cm, 124 pulses and 250 Hz (*Table 3*) by both continuous flow and batch mode (*Figure 4*).

On the other hand, Sobrino *et al.* [66] reduced the survival fraction of *S. aureus* inoculated in skim milk up to 3.0 log_{10} cycles, using 35 exponential decay pulses at 31 kV/cm and room temperature. Likewise, Sobrino-López and Martín-Belloso [67] found a reduction of 1.0 log_{10} cycle of *S. aureus* in skim milk at its pH natural (6.8), using 600 square wave pulses of 4.0 μs length at 35 kV/cm (*Table 3*) in bipolar mode. However, when they combined PEF treatment with 20 ppm nisin, reached 6.0 log_{10} reductions of the pathogenic microorganism in the food.

Those results suggest that PEF application itself is not enough for inactivating *S. aureus* at pasteurization levels. Nonetheless, PEF treatment applied in combination with nisin have demonstrated to be effective in the *S. aureus* inactivation.

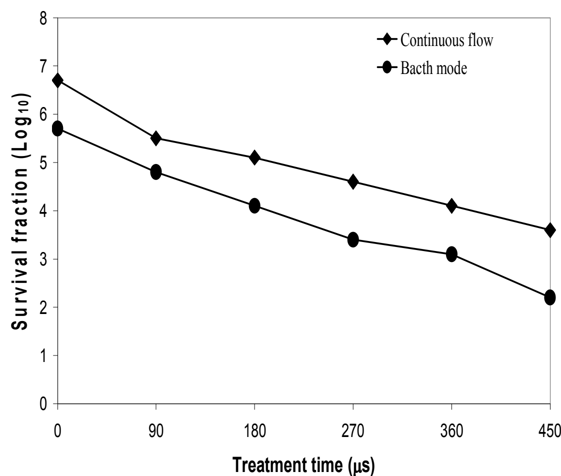

Figure 4.- *Inactivation of Staphylococcus aureus cells inoculated in skim milk by both continuous flow and batch mode. Treatment condition: 35 kV/cm electric field strength, 3.7 µs pulse width, 250 Hz, 40ºC outlet temperature, bipolar square wave pulses (Adapted from Evrendilek et al., 2004).*

Other pathogenic microorganisms

Pathogenic bacteria such as *Bacillus cereus, Yersinia enterocolitica* and *Campylobacter jejuni* have been associated to fluid foods such as milk and dairy products (*Table 1*). Different researches have been made about the effect of the PEF processing against these microorganisms in buffer and model solutions. However, studies applied directly on real fluid food are scarce.

Bacillus cereus is a spore forming bacterium that has a ubiquitous distribution in the environment and can be isolated from a variety of both raw and processed foods [68]. However, its presence in foods is not a significant hazard to consumer health unless be able to grow above 10^5 CFU/g and produce toxins. The stability and resistance of their spores and the increased number of psychrotolerant strains on foods has led to surveillance of this opportunistic foodborne pathogen, specifically in the dairy industry in recent years [69].

Sobrino *et al.* [66] achieved a reduction of 0.7 $\log_{10}$ cycles of *B. cereus* in skim milk by PEF, using 20 exponential decay pulses at 31 kV/cm and room temperature. However, when a thermal treatment of 10 min. at 75ºC was applied in combination with PEF technology, a maximum of 6.2 $\log_{10}$

reductions were obtained. In this case, the thermal treatment itself inactivated 5.8 log_{10} cycles, since *B. cereus* is susceptible to temperatures over 55ºC. Therefore, the combination of PEF with moderate heating may be useful to optimize milk processing to obtain a high quality product.

Yersinia enterocolitica is a Gram-negative psycrotrophic microorganism, which tolerates acidic conditions (*Table 2*). However, it may be quickly destroyed by thermal pasteurization. Several food-poisoning outbreaks of the pathogenic bacterium linked to the consumption of milk and dairy products [70] have occurred in recent years (*Table 1*). Nowadays, several works have been carried out in buffer solutions for evaluate the inactivation levels against this emerging pathogen by PEF treatment [71, 72]. However, none study has been made over real food systems.

Campylobacter jejuni is a pathogenic bacterium that causes human gastroenteritis, and is transmitted primarily through foods of animal origin. This pathogenic microorganism is fragile by nature and only could be a health problem if the foods are prepared using poor hygienic conditions or when the foods are consumed without be thoroughly cooked [73]. In addition, *C. jejuni* has a minimum growth temperature about 30ºC. On the other hand, this microorganim has been associated to the consumption of unpasteurized milk [55, 74, 75, 76, 77] (*Table 1*). Therefore, it is being considered an emerging pathogen of great importance for dairy industry. However, there is not data available on its inactivation by PEF treatment in neither fluid foods nor buffer or model solutions.

Comparison of the PEF effectiveness on pathogenic microorganisms

The numerous processing factors, variety of equipment and wide range of experimental conditions applied for the different researchers, limit the comparison about the effectiveness of PEF processing on pathogenic microorganisms in diverse fluid foods. However, some conclusions from studies carried out under the same experimental conditions can be made.

Dutruex *et al.* [38] observed 3.4 log_{10} reductions of *Escherichia coli* and 2.3 log_{10} reductions of *Listeria innocua* in skim milk when 35 pulses at 41 kV/cm were applied (*Table 4*). In this case, the different levels of microbial inactivation could be attributed to the type of microorganism and/or cellular diameter. *E. coli* and *L. innocua* are Gram-negative and Gram-positive microorganisms, respectively. Several authors have demonstrated that,

within bacteria, the Gram-negative are more susceptible to PEF treatment than those Gram-positive [38, 78, 79, 80, 81]. This fact might be attributed to the composition of their membranes, since the cellular membranes of the Gram-positive bacteria are more rigid and thicker than the Gram-negative, which could constitute an additional protection to PEF treatment [82]. On the other hand, *E. coli* have a higher cellular diameter than *L. innocua* (*Table 2*). Thus, *E. coli* is more sensible to PEF than *L. innocua,* since the induced voltage across the cell membrane is proportional to the geometric size [81]. Pothakamury *et al.* [80] also observed this behavior, when inactivated 4.0 $\log_{10}$ cycles of *Escherichia coli* and 3.0 $\log_{10}$ cycles of *Staphylococcus aureus* in simulate milk ultrafiltrate (SMUF), a model food. Both microorganisms were exposed to the same experimental conditions (*Table 4*).

Table 4.-Influence of cell size, shape and type of bacteria on microbial inactivation by PEF treatment

Microorganism	$\log_{10}$ reductions	Food	Φ	Cell shape	E	n	Type	T	Reference
E. coli	4.0	SMUF[a]	1.0	Rod	16	50	Gram negative	30	Pothakamury *et al*., 1995
S. aureus	3.0	SMUF[a]	0.9	Spherical	16	50	Gram negative	30	Pothakamury *et al.,* 1995
E. coli	3.5	SMUF[a]	1.0	Rod	60	10	Gram negative	40	Qin *et al.,* 1998
S. aureus	2.5	SMUF[a]	0.9	Spherical	36	10	Gram positive	40	Qin *et al.,* 1998
E. coli	3.4	Skim milk	1.0	Rod	41	35	Gram negative	37	Dutruex *et al.,*2000
L. innocua	2.3	Skim milk	0.8	Rod	41	35	Gram positive	37	Dutruex *et al.,*2000

E: electric field strength (kV/cm)
n: number of pulses
T: treatment temperature (ºC)
[a] SMUF : simulate milk ultrafiltrated
Φ : cellular diameter (μm)

On the other hand, *E. coli* O157:H7 was inactivated by 5.0 $\log_{10}$ [27] and 4.0 $\log_{10}$ reductions [38] in apple juice and skim milk, respectively (*Table 3*). Those fluid foods (juice and milk) are very different in composition and properties. Therefore, the degree of inactivation of this pathogenic microorganism by PEF treatment occurs unlikely in each food. Electrical

conductivity and pH are influential parameters in PEF effectiveness, simple to be measured and thus, always included in the characterization of fluid foods to be treated by PEF. Apple juice has lower conductivity (2.6 mS/cm) and pH (3.70) than skim milk (4.8 mS/cm and 6.80, respectively) and thereby, the microorganisms present in the juice are more easily inactivated by PEF than in milk. A low food conductivity increase the difference in conductivity between the microorganism cytoplasm and the medium, which causes an additional pressure on the microorganism membrane due to osmotic forces, and makes it more sensitive to the PEF treatment [58]. According to Dutruex *et al.* [38], the conductivity might be one of the most important parameters influencing the inactivation of microorganism by PEF. In addition, the pH of the medium also could affect the inactivation levels of the microorganism, since it is related to the ability of the microorganism to maintain the cytoplasm pH near the neutrality. Nevertheless, when an electric field is induced during the PEF treatment, the formation of pores on the cell membrane occurs and an osmotic imbalance around the cell is produced. Both acid and alkaline pH values induce additional stress to cells, and consequently increase their susceptibility to physical and chemical preservation treatments [3].

Describing the microbial inactivation of pathogens in fluid foods by PEF treatment using mathematical models

Mathematical models are important tools that can describe and predict the growth, survival and inactivation responses of foodborne microorganisms under specific environmental conditions. In food microbiology, these mathematical models usually are empirical. Nevertheless, the models should be based on reliable experiments and on the understanding of physiological mechanism of the microorganism inactivation. Likewise, is very important to have a consistent model that accurately express the behavior of the bacteria when are submitted at different environmental conditions [83]. In addition, the mathematical models should be validated in a continuous process and in real food systems [12].

Some models have been suggested to describe the microbial inactivation by PEF in fluid foods. Hülsheger and Niemann [84] and Hülsheger *et al.* [85] proposed various mathematical models for inactivation of *E. coli* K12 in aqueous suspensions using PEF treatment (*Equations 1, 2 and 3*). Those models are based on the dependence of the survival ratio, *S,*

defined as the microbial load after PEF treatment over the cell initial count before PEF processing (N/N_o), on the electric field intensity, *E*, treatment time, *t,* and the combination of both, *E* and *t,* according to the expressions *1, 2* and *3*:

$$Log(S) = - B_E (E - E_C) \quad (1)$$

$$Log(S) = - B_t (log (t / t_c) \quad (2)$$

$$S(E, t) = (t / t_c)^{- (E - Ec) / k} \quad (3)$$

where B_E and B_t are regression coefficients of the straight survival curves dependent of treatment time and electric field strength respectively, *E,* is the electric field strength (kV/cm) applied and, E_c, is the critical field strength value (kV/cm) which are threshold values where the inactivation occurs, *t,* is the treatment time (µs) and, t_c, is the critical treatment time value (µs). The E_c has been found to be a function of cell size, since is much lower for bigger cells due to the transmembrane potential experienced by the cell, which is proportional to the cell size [15, 39, 81]. Lower E_c values would indicate less resistance to the PEF treatment. The survival rate can be measured as a combined function of electric field strength (*Equation 1*) and treatment time (*Equation 2*) in a double-logarithmic relationship (*Equation 3*), where the symbol *k* represent an independent constant factor (cm/kV) for a specific microorganism. Therefore, a small value of the kinetic constant *k* indicates a wide span in the inactivation rate curve and lower sensitivity to PEF, whereas a large value implies a steep decline or higher susceptibility to PEF.

Equations 1, 2 and 3 fitted very well the experimental data of Grahl and Märkl [39], Martín *et al.* [40] and Martín-Belloso *et al.* [35] in UHT milk, liquid egg and skim milk, respectively, for PEF inactivation of *E. coli.*

Sensoy *et al*. [58] proposed a first-order kinetic model to describe the effect of the treatment temperature on *Salmonella* Dublin inactivation in skim milk (*Equation 4*)

$$S = exp^{- (kE)(t)} \quad (4)$$

where, *S,* is the survival ratio, k_E, a constant that first was evaluated as a function of electric field (μs^{-1}) and then as a function of the medium temperature (ºK) following the Arrhenius' model (*Equation 5*) and, *t*, the treatment time (µs).

$$k = (k_E)\ exp^{-(EA / (R)(T))} \qquad (5)$$

where, *k,* is survival fraction rate constant (μs^{-1}), E_A, is activation energy (J/Kg.mole), *R,* is universal gas constant (1.9872 J/Kg.mole.ºK) and, *T,* is medium temperature (ºK).

Peleg [86] also proposed a model (*Equation 6*) based on the Fermi's equation that describes the percentage (%) of survival microorganisms which reads as follows:

$$S\ (E,\ n) = 1 / (1 + exp^{E - Ec(n) / a(n)}) \qquad (6)$$

being, *S,* a function of the electric field strength *E* (kV/cm) and number of pulses *n*, E_c, is critical electric field strength value (kV/cm) where the survival level is 50%, and *a* parameter indicate the steepness of the survival curve around E_c. Both parameters, E_c and *a,* are exponentially related to the number of applied pulses, *n*. Sensoy *et al.* [58] used this model to describe the inactivation of *Salmonella* serovar Dublin by PEF in skim milk.

Other mathematical model based on the Weibull distribution (*Equation 7*) has been used to fit the survival curves, relating log_{10} of the microbial survival with treatment time [87, 88].

$$Log(S) = -(1/2.303)(t/b)^n \qquad (7)$$

where *t,* is the treatment time, *b* and *n* are scale and shape factors, respectively; *n*, factor interprets the shape of the survival curve, so that when $n < 1$ the survival curve is upward concave, $n > 1$ the survival curve is downward convex, and $n = 1$ indicate a linear survival curve on a log-scale. This model predicted accurately the inactivation of *Listeria monocytogenes* in apple juice [88]

Final Remarks

Human disease linked to the consumption of unpasteurized fruit juices, milk and dairy products due to pathogenic microorganisms is affecting various countries worldwide. Several publicized outbreaks of foodborne infections and intoxications in recent years have enhanced public awareness of this risk. For this reason, the consumer's today is demanding high

quality, minimally processed and microbially safe foods. So now, new processing technologies may counteract or destroy the presence of pathogenic bacteria in fluid foods. The PEF treatment is a non-thermal method that offers the advantages of maintaining the organoleptic and nutritional properties of foods, which results in a fresh-like product. Moreover, this technology has demonstrated to be able to inactivate some microorganisms such as *Listeria innocua*, *E. coli*, *E. coli* O157:H7, *E. coli* 8739 and *Salmonella* Typhimurium at pasteurization levels (over 5.0 $\log_{10}$ cycles) in some fluid foods. Nowadays, the PEF application at industrial level is a reality. Genesis Juice Corporation is a food industry that has initiated the application of PEF treatment to some juices and blends in the Portland-USA market demonstrating the feasibility of the industrial application of the technology [89].

However, more emphasis in inactivation of pathogenic microorganisms in fluid foods are required to standardize the experimental proceedures. The few studies on PEF inactivation of vegetative cells and spores of *Bacillus cereus* in fluid products have showed little effectiveness against them, but further research is needed to prove if this phenomenon occurrs generally. In addition, studies of inactivation by PEF on emerging pathogens such as *Yersinia enterocolitica* and *Campylobacter jejuni* should also be carried out to evaluate if their presence in foods may be avoided through a PEF treatment, since these bacteria have caused several problems of public health.

On the other hand, the relevant processing parameters used for the inactivation of microorganisms should be clearly highlighted to allow comparisons and scale up the technology at industrial level. Thus, processing conditions, intrinsic and extrinsic factors of both fluid foods and microorganisms should be reported to facilitate the optimization and standardization of the PEF treatment. Moreover, the knowledge and understanding of these factors would help to the development of new mathematical models that should adequately predict the microbial behavior under PEF and improve the food safety and quality.

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OBJETIVOS

Objetivo General

El objetivo principal de esta tesis fue estudiar el efecto de los pulsos eléctricos de alta intensidad de campo (PEAIC) solo o en combinación con sustancias antimicrobianas para reducir más de 5 unidades logarítmicas de los microorganismos patogénicos de mayor preocupación en la industria de alimentos (*Escherichia coli* O157:H7, *Listeria monocytogenes* y *Salmonella* spp.) inoculados en los diversos zumos de frutas (melón, sandía, manzana, pera, naranja, fresa y tomate), así como también evaluar sus efectos sobre la vida útil desde un punto de vista microbiológico. Con esta finalidad se plantearon los siguientes objetivos específicos:

Objetivos Específicos

1. Evaluar el efecto del tiempo de tratamiento (μs) y frecuencia de pulso (Hz), como factores de procesamiento de PEAIC, sobre poblaciones de *E. coli* O157:H7, *S.* Enteritidis y/o *L. monocytogenes* inoculados en zumos de melón, sandía, manzana, pera, naranja, fresa y tomate.

2. Encontrar los valores óptimos de tiempo de tratamiento (μs) y frecuencia de pulso (Hz) para obtener las máximas reducciones de estos microorganismos en cada zumo de fruta procesado por PEAIC.

3. Estudiar el efecto de PEAIC sobre poblaciones de *Escherichia coli* O157:H7, *Salmonella* Enteritidis y/o *Listeria monocytogenes* inoculados en los diversos zumos de frutas conteniendo diferentes concentraciones de sustancias antimicrobianas (ácido cítrico o aceite esencial de canela).

4. Estimar la vida útil microbiológica de los diversos zumos de frutas tratados con PEAIC solo o en combinación con ácido cítrico o aceite esencial de canela, usando como referencia los zumos de frutas sin tratamiento y tratado térmicamente.

5. Determinar la aceptabilidad sensorial de los diferentes zumos de fruta tratados con PEAIC y PEAIC con antimicrobianos.

Capítulo I

Influence of treatment time and pulse frequency on *Salmonella* Enteritidis, *Escherichia coli* and *Listeria monocytogenes* populations inoculated in melon and watermelon juices treated by pulsed electric fields

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ABSTRACT

Consumption of unpasteurized melon and watermelon juices has caused several disease outbreaks by pathogenic microorganisms worldwide. Pulsed electric field (PEF) has been recognized as a technology that may inactivate those bacteria present in fluid food products at low temperatures. Hence, PEF treatment at 35 kV/cm, 4 µs pulse duration in bipolar mode and square shape were applied on *S.* Enteritidis, *E. coli* and *L. monocytogenes* populations inoculated in melon and watermelon juices without exceeding 40 ºC outlet temperatures. Different levels of treatment time and pulse frequency were applied to evaluate their effects on these microorganisms. Treatment time was more influential than pulse frequency ($P \leq 0.05$) on the PEF microbial reduction levels for both melon and watermelon juices. Populations of *S.* Enteritidis, *E. coli* and *L. monocytogenes* were experimentally reduced and validated in a single process up to 3.71± 0.17, 3.7 ± 0.3 and 3.56 ± 0.26 $\log_{10}$ units, respectively, in melon juice when 1709 µs and 193 Hz were used; whereas reductions up to 3.56 ± 0.12, 3.6 ± 0.4 and 3.41 ± 0.13 $\log_{10}$ units of those microorganisms, respectively, were reached in watermelon juice treated for 1682 µs at 200 Hz. Although PEF treatment reduced the populations of the three microorganisms, *L. monocytogenes* was more resistant to PEF than *S.* Enteritidis and *E. coli* in both juices when treated at the same processing conditions.

***Keywords*:** PEF, treatment time, pulse frequency, *S.* Enteritidis*, E. coli, L. monocytogenes*, melon, watermelon, juice

INTRODUCTION

Melon and watermelon products are regarded as potentially hazardous foods by the Food and Drug Administration (FDA) (FDA, 2001) because they may favor the growth of pathogenic microorganisms due to their low acidity (pH 5.2 to 6.7) and high water activity (0.97 to 0.99). Outbreaks of *Salmonella* spp. and *E. coli* O157:H7 have been linked with the consumption of fresh-cut as well as juice of melon and watermelon (CDC, 1991, Mohle-Boetani et al., 1999, Powell and Luedtke, 2000, CDC, 2001, Meng et al., 2001, FDA, 2001 and CDC, 2002). The majority of outbreaks are linked to the presence of these pathogens on the fruit rind, presumably contaminated in the field by improperly composted fertilizer, irrigation with infected water or through infected workers (FDA, 2001). Hence, these pathogenic microorganisms can be transferred to the edible tissues and juices when melons and watermelons are cut during preparation (Ukuku and Sapers, 2001 and Sharma et al., 2005). Incidence, survival and growth of *Salmonella* spp., *E. coli* O157:H7 and *L. monocytogenes* on watermelon and melon slices and juices have been reported by several researchers (Fernandez-Escartin et al., 1989, Golden et al.,1993, Del Rosario and Beuchat, 1995, Penteado and Leitao, 2004 and Eswaranadan et al., 2004). Nowadays, fresh juices from those fruits are sold without pasteurization and, thus, they could be potential sources of pathogenic microorganisms such as *Salmonella, E. coli* and *Listeria* which at low doses (1-100 cells.ml^{-1}) may produce illness (D'Aoust et al., 2001, Meng et al., 2001, Swaminathan, 2001, Bell and Kyriakides, 2002a, 2002b and 2002c).

These pathogenic microorganisms can be easily eliminated through heat, but sensorial and nutritional attributes are extensively damaged (Jeyamkondan et al., 1999, Espachs-Barroso et al., 2003 and Elez-Matínez and Martín-Belloso, 2005). Nevertheless, significant efforts are leading to the development of novel non-thermal processes. One of them is the use of pulsed electric fields (PEF) as an alternative preservation process for fluid foods. The aim of this technology is to inactivate spoilage and pathogenic microorganisms and to decrease the activity of enzymes in order to extend the shelf life and safety of foods without undesirable heat and chemical effects (Cserhalmi, 2006). Moreover, the organoleptic and nutritional properties seem to be maintained after PEF treatment (Hodgins et al., 2002; Cserhalmi et al., 2006; Elez-Martínez et al., 2006a and 2006b). The effectiveness of PEF treatment for inactivating or reducing of some strains and serovars of *Salmonella* spp. and *E. coli* in some fruit juices has been studied (Evrendilek et al., 1999, Iu et al., 2001, Liang et al., 2002,

Evrendilek and Zhang, 2005, Zhong et al., 2005 and Mosqueda-Melgar et al., 2006). Evrendilek and Zhang (2005) have reported that bipolar pulses were more effective than monopolar pulses for reducing *E. coli* O157:H7 in apple juice. Iu et al. (2001) and Liang et al. (2002) obtained a higher inactivation of *Salmonella* Thyphimurium and *E. coli* O157:H7 populations in orange juice and apple cider, respectively, when higher number of pulses and electric field strength were applied. On the other hand, Evrendilek et al. (1999), Zhong et al. (2005) and Mosqueda-Melgar et al. (2006) reached higher microbial inactivation of *E. coli* O157:H7, *E. coli* and *Salmonella* Enteritidis in several fruit juices when treatment time was increased. However, studies on *L. monocytogenes* inactivation in fruit juices by PEF treatment were not found in the literature, although its incidence, survival and growth in fresh-cut as well as pulp of melon and watermelon has been reported (Penteado and Leitao, 2004 and Eswaranadan et al., 2004). Thus, the inactivation of *L. monocytogenes* by PEF in these fluid foods represents a new challenge to the fruit and derivatives industry.

The aims of this study were to evaluate the effect of the treatment time and pulse frequency, as variable parameters of PEF treatment, on *S.* Enteritidis, *E. coli* and *L. monocytogenes* populations inoculated in melon and watermelon juices, as well as to obtain optimized values of these processing factors for the standardization of the PEF treatment.

MATERIALS AND METHODS

Juice Preparation

Melon (*Cucumis melo* var. “Piel de sapo”) and watermelon (*Citrullus lanatus* var. “Seedless”) fruits at commercial ripeness were selected in a supermarket of Lleida, Spain. The fruits were washed, peeled and cut into pieces. Then juices were made through an Ufesa blender (Model BP 4512; Vitoria, Spain) and centrifuged at 12500 rpm for 15 min. at 4º C in an Avanti™ J-25 Centrifuge (Beckman Instrument, Inc.; USA). The supernatant juice was filtered, bottled and autoclaved in a Presoclave 75 (J.P. Selecta, S.A; Barcelona, Spain) at 121º C for 15 min. Finally, the samples were stored at refrigeration temperature (5º C) until inoculation and PEF treatment.

Physicochemical Analysis of the juices

Electric conductivity (Testo 240 conductivimeter; Testo GmBh & Co; Lenzkirch, Germany), pH (Crison 2001 pH-meter; Crison Instruments S.A; Barcelona, Spain) and soluble solid content (Atago RX-1000 refractometer; Atago Company Ltd., Japan) were measured. pH and soluble solid were carried out according to the B.O.E (1988) (*Table 1*).

Table 1.-Analytical parameters of melon and watermelon juices

Parameters (unit)	Values [1]	
	Melon juice	Watermelon juice
Electrical conductivity (mS/cm)	5.23 ± 0.03	3.66 ± 0.05
Soluble solids (%)	11.1 ± 0.0	6.5 ± 0.0
pH	5.82 ± 0.04	5.46 ± 0.11

[1]= Results are the mean ± standard deviation of three measurements

Microbial Culture Preparation

Pure cultures of *Salmonella* Enteritidis 1.82 (National Collection of Type Culture (NCTC) 9001, PHLS Central Public Health Laboratory; London, UK) and *Escherichia coli* 1.107 (Laboratoire de Répression des Fraudes (LRF); Montpellier, France) were grown in flasks containing tryptone soy broth (TSB) (Biokar Diagnostics; Beauvais, France), whereas, pure culture of *Listeria monocytogenes* 1.131 (Spanish Type Culture Collection (STCC) 932; University of Valencia, Valencia, Spain) was cultured in TSB plus 0.6 % of yeast extract (Biokar Diagnostics). *S.* Enteritidis and *E. coli* were incubated at 37º C with continuous agitation at 120 rpm for 15 hours to obtain cells in stationary growth phase, while *L. monocytogenes* was incubated at 35º C with continuous shaking at 200 rpm for 15 hours. The final concentration reached by the microorganisms in the growth media was approximately 10^9 - 10^{10}colony forming units/milliliter (CFU/ml).

Enumeration of Viable Cells

The injured cells were recovered (during 20 min) and serially diluted in tubes with 0.1% casein-meat peptone (Biokar Diagnostic) plus 0.85% NaCl (Scharlau Chemie, S.A. Barcelona, Spain). Then, the cells were

spread plated by duplicate in: a) Hektoen agar (Biokar Diagnostic) selective medium for *S.* Enteritidis count in accordance with ISO 6579:2002 (2002), b) membrane fecal coliforms (mFC) agar (Biokar Diagnostic) for *E. coli* count, and c) Palcam agar (Biokar Diagnostic) for *L. monocytogenes* count according to ISO 11290-2 method (1998). Counts were expressed as $\log_{10}$ CFU/ml.

PEF Equipment

A continuous flow bench-scale system OSU-4F (Ohio State University, Ohio, USA) which delivers square-wave pulses was used to treat the samples of fruit juices. The PEF system is composed of eight chambers accommodated in series and each one with two stainless steel electrodes separated by a gap of 0.292 cm. Each chamber has a diameter of 0.23 cm and a volume of 0.0121 cm^3 with a cross section of 0.0415 cm^2. The pulse waveform was monitored using a Tekscope THS 720 oscilloscope (Tektronix Inc., Oregon, USA) connected to the PEF apparatus. Pulse frequency and duration were controlled through of a Pulse Generator model 9410 (Quantum Composers, Inc., Bozeman MT, USA). The flow rate of the process was adjusted by a variable gear pump model 75210-25 (Cole Palmer Instrument Company; Illinois, USA). Finally, the product was refrigerated in an ice water bath with circulation pump (J.P. Selecta, S.A; Barcelona, Spain).

Juice Processing by PEF

Melon and watermelon juices were inoculated with *S.* Enteritidis, *L. monocytogenes* and *E. coli* all together to give a final concentration of 10^7 - 10^8 CFU/ml approximately of each one. Sterile fruit juices were first pumped through the PEF system as control, and then the inoculated juice was passed across the PEF system with a flow rate speed adjusted to 100 ml/min. In this study, a maximum electric field strength (35 kV/cm) and an optimum value of pulse duration (4 µs) of the PEF system were applied using square-wave pulses in bipolar mode; whereas treatment time (up to 2000 µs) and pulse frequency (100-250 Hz) were ranged within acceptable values to obtain the lowest heating effects. The outlet temperature did not exceed 39.1 ± 0.1º C and 30.3 ± 0.2º C for melon and watermelon juices, respectively. In addition, the electric energy density input (*Q,* J/cm^3) for melon and watermelon juices was calculated from *Equation 1* (Martín et al., 1994 and Elez-Martínez et al., 2006b):

$$Q = \frac{V \cdot I \cdot Tt}{v} \quad (1)$$

where V, is the peak voltage (V); I, is the intensity of current (A); T_t; is the treatment time (s); v, is the total volume of all treatment chambers (cm^3).

Experimental Design and Statistical Analysis

A multilevel factorial design was performed to evaluate the effects of treatment time and pulse frequency on the population reduction of *S.* Enteritidis, *L. monocytogenes* and *E. coli*. The experiments were performed twice, and microbial count was made in duplicate (n = 4). Means and standard deviations were calculated for each treatment. Analysis of Variance (ANOVA) was used to determine significant differences ($P \leq 0.05$) among the applied treatments and to obtain *F-Ratio* values (*Table 2*). Multiple range tests (MRT) was applied to those factors that were statistically meaningful ($P \leq 0.05$), to determine which levels of each factor were significantly different ($P \leq 0.05$) through a Fisher's least significant difference (LSD) procedure. In addition, single and multiple response analyses were employed to calculate the optimum values of the treatment time and pulse frequency to obtain maximum microbial reductions (*Equation 2*).

$$Y = k + A\cdot(Tt) + B\cdot(f) + C\cdot(Tt)^2 + D\cdot(f)^2 + E\cdot(Tt)\cdot(f) \quad (2)$$

where, Y is the maximum bacterial inactivation obtained after HIPEF treatment ($-\log_{10}$ CFU/ml), T_t is the treatment time (μs), f is the pulse frequency (Hz), k is a constant of the equation, A (μs^{-1}) and C (μs^{-2}) are regression coefficients depending on T_t, B (Hz^{-1}) and D (Hz^{-2}) are regression coefficients depending on f, and E ($\mu s^{-1}\cdot Hz^{-1}$) is a regression coefficient depending on T_t and f interaction.

After obtaining the optimal processing conditions in terms of treatment time and pulse frequency through a multiple response analysis, the PEF treatment conditions were experimentally checked to validate the calculations. All the statistical analyses were made with Statgraphics plus Centurion XV software Version 15.1.02.

RESULTS

***Salmonella* Enteritidis**

Maximum reductions of 3.75 (at 1250 µs and 175 Hz) and 4.27 (at 2000 µs and 100 Hz) $\log_{10}$ units of *S.* Enteritidis were achieved in melon and watermelon juices, respectively (*Figures 1A* and *2A*). The *S.* Enteritidis reduction was higher when treatment time increased in both juices. The ANOVA indicated significant differences ($P \leq 0.05$) among the *S.* Enteritidis counts by each treatment time applied in each juice. In melon juice, the MRT showed that microbial counts between 1250 and 2000 µs were not statistically different ($P > 0.05$) (*Figure 1A*), opposite to watermelon juice where a significantly higher microbial reduction was observed when the juice was treated for 2000 µs than 1250 µs (*Figure 2A*). On the other hand, *S.* Enteritidis reduction increased when pulse frequencies decreased in watermelon juice; however, significant differences among the microbial counts were not found when different levels of pulse frequencies were applied in melon juice (*Figure 1A*).

Escherichia coli

The application of PEF treatment to *E. coli* populations induced maximum reductions up to 3.91 (at 1250 µs) and 4.01 (at 2000 µs) $\log_{10}$ units in melon and watermelon juices, respectively, when 250 Hz was used (*Figures 1B* and *2B*). This microorganism was greatly reduced by increasing the treatment time applied in both melon and watermelon juices. The MRT in this case showed meaningful differences ($P \leq 0.05$) among the different PEF treatment time applied to each fruit juice (*Figures 1B* and *2B*). The pulse frequency only had significant effects on *E. coli* population inoculated in melon juice, being greatly reduced when higher pulse frequency was applied (*Figure 1B*).

Listeria monocytogenes

This pathogenic microorganism was reduced up to 4.27 (at 100 Hz) and 3.77 (at 250 Hz) $\log_{10}$ units in melon and watermelon juices, respectively, when 2000 µs treatment time was used (*Figures 1C* and *2C*). Data showed significant differences ($P \leq 0.05$) among the *L. monocytogenes* counts for each treatment time applied to each juice. In general, higher reduction of *L. monocytogenes* in melon and watermelon

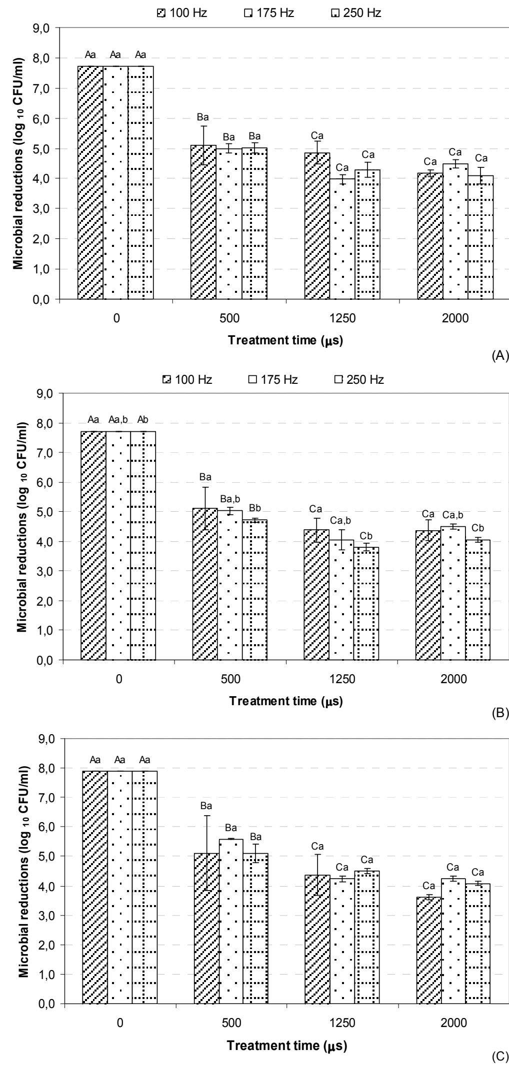

Figure 1.- *Reductions of S. Enteritidis (A), E. coli (B) and L. monocytogenes (C) populations inoculated in melon juice by PEF treatment at different treatment time (µs) and pulse frequency (Hz). The experiments were carried out at 35 kV/cm and 4 µs pulse duration of square shape, bipolar mode in continuous flow at 39.1ºC ± 0.1 maximum outlet temperature. The bars are means ± standard deviation of two trials in duplicate (n = 4). Different capital and small letters indicate significant differences (P ≤ 0.05) among treatment times and pulse frequencies, respectively.*

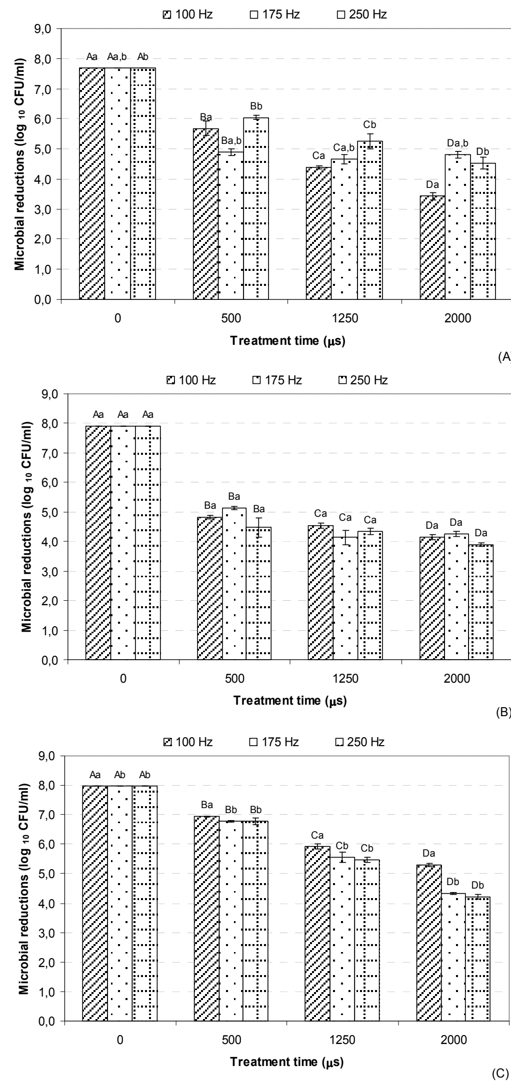

Figure 2.- *Reductions of S. Enteritidis (A), E. coli (B) and L. monocytogenes (C) populations inoculated in watermelon juice by PEF treatment at different treatment time (µs) and pulse frequency (Hz). The experiments were carried out at 35 kV/cm and 4 µs pulse duration of square shape, bipolar mode in continuous flow at 30.3°C ± 0.2 maximum outlet temperature. The bars are means ± standard deviation of two trials in duplicate (n = 4). Different capital and small letters indicate significant differences (P ≤ 0.05) among treatment times and pulse frequencies, respectively.*

was observed when treatment time increased (*Figures 1C* and *2C*). On the other hand, pulse frequency only showed significant effects ($P \leq 0.05$) on watermelon juice, where *L. monocytogenes* counts were slightly reduced at the highest pulse frequency (*Figure 2C*).

DISCUSSION

Populations of *S.* Enteritidis, *E. coli* and *L. monocytogenes* inoculated in melon or watermelon juices were reduced greatly when PEF treatment was applied. The degree of microbial reduction depended on the treatment time and the pulse frequency ($P \leq 0.05$), but treatment time was more influential than pulse frequency, since it had higher *F- Ratio* values (*Table 2*). In general, the higher the treatment time, the greater the microbial reduction in fluid foods and buffer solutions was demonstrated (Martín-Belloso et al., 1997, Jeyamkondam et al., 1999, Aronsson et al., 2001 and Martín-Belloso and Elez-Martínez, 2005). However, pulse frequency has received little attention, although being an influential factor in the PEF effectiveness.

Table 2.-Influence of treatment time and pulse frequency on the reduction levels of *S.* Enteritidis, *E. coli* and *L. monocytogenes* inoculated in melon or watermelon juices

Microorganism	*F* – Ratio[1]									
	Melon juice					Watermelon juice				
	Tt	*f*	*Tt·Tt*	*f·f*	*Tt·f*	*Tt*	*f*	*Tt·Tt*	*f·f*	*Tt·f*
S. Enteritidis	294.73 [b]	2.77 [a]	89.22 [b]	0.53 [a]	0.01 [a]	154.06 [b]	6.46 [b]	25.41 [b]	0.13 [a]	3.04 [a]
E. coli	340.92 [b]	9.01 [b]	136.21 [b]	0.03 [a]	0.03 [a]	126.54 [b]	0.56 [a]	45.54 [b]	0.17 [a]	0.05 [a]
L. monocytogenes	526.12 [b]	0.03 [a]	88.83 [b]	0.61 [a]	5.74 [b]	2528.35 [b]	55.36 [b]	27.26 [b]	8.92 [b]	44.43 [b]

[1]= values are the variance explained by a factor (mean square of the factor) to the unexplained variance (mean square error)
Tt = treatment time (µs)
f = pulse frequency (Hz)
[b] = statistically significant ($P \leq 0.05$)

Effect of treatment time on microbial populations

Melon and watermelon juices were submitted to PEF treatment and an increased reduction on *S.* Enteritidis, *E. coli* and *L. monocytogenes*

populations was observed when treatment time was extended. Sale and Hamilton (1967), Liu et al. (1997) and Martín et al. (1997) reported that the reduction of *E. coli* K12, *E. coli* O157:H7 and *E. coli* populations in buffer solutions and skim milk, respectively, decreased rapidly with the first pulses - which can be expressed as treatment time when pulse duration is fixed (Qin et al., 1994) - and then a minor effect on the microbial reduction was observed after the subsequent pulses. Our results are in accordance with these authors, since the first 500 µs of treatment time reduced quickly about 2.0 $\log_{10}$ cycles and afterwards one additional $\log_{10}$ cycle approximately was reduced when applying 1250 or 2000 µs treatment time (*Figures 1* and *2*). This behavior was generalized for the three microorganisms in each juice.

Mosqueda-Melgar et al. (2006) reported reductions up to 3.44 and 3.21 $\log_{10}$ units of *S.* Enteritidis and 3.48 and 4.02 $\log_{10}$ units of *E. coli*, respectively, in melon and watermelon juices, when 35 kV/cm, 4.0 µs pulse duration in bipolar mode at 2000 µs and 250 Hz were used. In this study, we achieved similar reductions on those microorganisms in melon and watermelon juices, but only 1250 µs were needed for reducing those in melon juice (*Figures 1A* and *1B*), whereas 2000 µs was the treatment time needed to inactivate those microorganisms in watermelon juice (*Figures 2A* and *2B*) as reported by Mosqueda-Melgar et al. (2006).

Others studies showing *S.* Enteritidis and *E. coli* inactivation by PEF in melon and watermelon juices have not been reported in the literature. However, there are others report related to these microorganisms in other fruit juices. Thus, Zhong et al. (2005) reached 3.8 $\log_{10}$ reductions of *E. coli* inoculated in carrot juice using 2173 µs treatment time and 10 Hz at 20 kV/cm and 1.5 µs pulses duration without exceeding 40ºC. We achieved reductions of *E. coli* very similar to Zhong et al. (2005), but using shorter treatment time and higher pulse frequency. The similarity of results in different media could be attributed to the close pH and electrical conductivity values that melon and watermelon juices have compared to carrot juice.

On the other hand, McDonald et al. (2000) and Liang et al. (2002) reported up to 5.0 $\log_{10}$ reductions of *E. coli* and *S.* Typhimurium, respectively, in orange juice. These researchers inactivated these microorganisms using electric fields of 30 and 90 kV/cm with outlet temperatures of 54 and 55º C, respectively. Those reductions could be a consequence of combining high acidity of juice, high intensity electric field and relatively high temperature. Aronsson and Rönner (2001) reported that PEF is more effective when the microorganisms are in an acidic

environment, since the acidity of the medium could induce an additional stress to cells due to osmotic imbalance produced around the cellular membrane when an electric field is induced during the PEF treatment. Thus, those microorganisms are more resistant to inactivation in melon and watermelon juices when PEF are applied than in orange juice, due to the lower pH value of the latter fruit. Furthermore, the high temperatures used by those authors could also contribute to the microbial death, since *E. coli* and *Salmonella* spp. are sensitive to heat above 46ºC and 50ºC, respectively. In contrast, we applied temperature below 40ºC in both juices, ensuring that the inactivation of the microorganisms was achieved only by PEF. In addition, the greater electric field intensity used by Liang et al. (2002) with regard to our produced a higher microbial inactivation, since at higher electric field strength greater microbial inactivation is caused (Qin et al., 1996, Martín et al., 1997, Martín-Belloso et al., 1997, Jeyamkondam et al., 1999, Aronsson et al., 2001 and Martín-Belloso and Elez-Martínez, 2005). This fact could be explained by the Electroporation's Theory of Coster and Zimmermann (1975), who investigated the microbial inactivation mechanism when pulses of electric fields were applied. This Theory explains the formation of pores in the cellular membrane due to the induced electric field, which could cause cellular lysis. The formation of pores has demonstrated to be reversible or irreversible, depending on the electric fields intensity (Barbosa-Cánovas et al., 1999) and/or the membrane organizational changes (Weaver et al., 1988 and Tsong, 1990). Low electric fields intensity form reversible pores in the cellular membrane, whereas severe electric field intensity leads to the irreversibility of this phenomenon, which results in cellular death (Tsong, 1990 and Ho and Mittal, 1996).

Reductions up to 4.27 and 3.77 $\log_{10}$ units of *L. monocytogenes* were reached in melon and watermelon juices using PEF technology (*Figures 1C* and *2C*). However, a comparison with previous studies is not possible, since any study has been reported on *L. monocytogenes* inactivation in fruit juice by PEF treatment. Thus, this study would represent the first report of inactivation of this microorganism by PEF in those kinds of products.

Effect of pulse frequency on microbial populations

In our study, very little influence of pulse frequency on the *S.* Enteritidis and *E. coli* inactivation in melon or watermelon juice by PEF treatment was observed.

So, populations of *S.* Enteritidis and *L. monocytogenes* in melon juice (*Figures 1A and 1C*), and *E. coli* population in watermelon juice (*Figures 2B*) showed not significant changes ($P > 0.05$) when different pulse frequencies (100 – 250 Hz) were applied. These results are in accordance with Hulsheger et al. (1981), who not found meaningful effect of pulse frequency on the *E. coli* K12 reduction in buffer solutions, when applied pulse frequency up to 5.0 Hz.

However, we also found that populations of *E. coli* and *L. monocytogenes* in melon and watermelon juices, respectively, were slightly reduced when the highest pulse frequency was applied (*Figures 1B and 2C*). The frequency of the pulse applied to the food plays an important role in the energy added to the medium, since an increase in pulse frequency keeping constant the rest of the processing factors, implicates higher power supply consumption of the PEF system, and consequently, an increase in the temperature of the food is observed (Jeyamkondan et al., 1999). Korolczuk et al. (2006) reported a positive linear relationship between the temperature and the amount of energy delivered to the food. Thus, the heat generated during PEF processing when high pulse frequency is used might produce a higher microbial inactivation. This heat generated could exert a significant influence over the membrane fluidity properties, because at low temperature (10 to 20º C) the phospholipids of the lipid bilayer are closely packed into a rigid gel structure, while at higher temperature (> 30º C) the phospholipids are less ordered and the cellular membrane has a liquid-crystalline structure (Jayaram et al., 1992, Qin et al., 1996 and Aronsson and Rönner, 2001). Hence, an increment in the temperature of the food (between 30ºC and 40ºC, in our case) may increase the sensitivity of the cellular membrane, and thus, the antimicrobial effect of PEF treatment.

In addition, populations of *S.* Enteritidis in watermelon juice showed a higher microbial reduction when the lower pulse frequency was used (*Figure 2A*). This result is according to Elez-Martínez et al. (2004 and 2005), who reported that a low pulses frequency (50 Hz) was more effective in the microbial inactivation of *Lactobacillus brevis* and *Saccharomyces cerevisae* populations in orange juice than high pulse frequency (350 Hz), when the electric field, treatment time and pulse duration were held constant.

After evaluating the little information found in the literature about the effects of pulse frequency in the microbial inactivation by PEF, and due to different results obtained in this study with regard to that factor, we can say that the effect of pulse frequency on the microorganisms is not fully

understood yet, since this factor would seem depend on the type of microorganism and media where it is.

Optimization of the processing factors

Single and multiple responses analyses were calculated from *Equation 2* and estimated values of regression coefficients (expressed in *Table 3*) were obtained from experimental design. Those regression coefficients depended of the influence of each treatment time and/or pulse frequency applied, being those related to treatment time the most determinant on microbial reduction in melon and watermelon juices.

Table 3.-Optimized values of treatment time and pulse frequency and estimated values of regression coefficients from the *Equation 2* for achieving maximum microbial reduction in melon and watermelon juices PEF treated.

Juice	Microorganism	*Tt*	*f*	R^2	*Regression coefficients*						*Y*
					K	*A*	*B*	*C*	*D*	*E*	
Melon	*S.* Enteritidis	1601	230	95.41	-0.6427	0.0050	0.0081	-1.63^{-6}	-1.81^{-5}	-1.42^{-7}	3.98
	E. coli	1571	250	96.38	-0.2605	0.0053	0.0014	-1.84^{-6}	4.15^{-6}	2.13^{-7}	4.19
	L. monocytogenes	1956	100	97.22	0.0548	0.0052	-0.0032	-1.44^{-6}	1.73^{-5}	-3.04^{-6}	3.96
Watermelon	*S.* Enteritidis	1658	100	91.59	-0.1400	0.0045	0.0044	-1.13^{-6}	-1.39^{-5}	-3.71^{-6}	3.92
	E. coli	1396	250	90.85	0.6188	0.0051	-0.0054	-1.78^{-6}	1.79^{-5}	5.65^{-7}	4.29
	L. monocytogenes	1991	250	99.37	-0.6423	0.0016	0.0095	-2.93^{-7}	-2.87^{-5}	3.62^{-6}	3.79

Tt = optimum treatment time (µs)
f = optimum pulse frequency (Hz)
k = constant of the *Equation 2*
A = regression coefficient of treatment time (μs^{-1})
B = regression coefficient of pulse frequency (Hz^{-1})
C = regression coefficient between treatment time interactions (μs^{-2})
D = regression coefficient between pulse frequency interactions (Hz^{-2})
E = regression coefficient between treatment time and pulse frequency interaction ($\mu s^{-1} \cdot Hz^{-1}$)
Y = calculated microbial reduction after PEF treatment ($\log_{10}$ CFU/ml)

Single response analysis of the data was performed to obtain optimum values of treatment time and pulse frequency with maximum microbial reduction for every microorganism in each juice (*Table 3*). However, for avoiding discrepancies or differences among the obtained values of treatment time and pulse frequency by each microorganism in melon and

watermelon juices, a multiple response analysis was employed (*Table 4*). This analysis was applied to know the minimum values needed of treatment time and pulse frequency for obtaining the highest *S.* Enteritidis, *L. monocytogenes* and *E. coli* reductions at the same time on melon or watermelon juice in a single process when PEF treated. These results were experimentally validated, and were not found to be significantly different ($P > 0.05$) between the microbial reductions calculated and observed. Hence, the obtained results could contribute to scientific knowledge of the PEF processing standardization.

Table 4.-Calculated and observed microbial reductions obtained after applying PEF optimum values of treatment time and pulse frequency on melon and watermelon juices.

Juices	Factors	Optimized values	*S.* Enteritidis (log_{10} CFU/ml)	*E. coli* (log_{10} CFU/ml)	*L. monocytogenes* (log_{10} CFU/ml)
Melon	*Tt* (µs)	1709	3.92 [a]	3.89 [a]	3.75 [a]
	f (Hz)	193	(3.71 ± 0.17) [b]	(3.7 ± 0.3) [b]	(3.56 ± 0.26) [b]
Watermelon	*Tt* (µs)	1682	3.33 [a]	4.05 [a]	3.21 [a]
	f (Hz)	200	(3.56 ± 0.12) [b]	(3.6 ± 0.4) [b]	(3.41 ± 0.13) [b]

Tt = optimum treatment time (µs)
f = optimum pulse frequency (Hz)
[a] Calculated maximum microbial reduction (log_{10} CFU/ml)
[b] Observed microbial reductions (log_{10} CFU/ml)
( ) = values are means of 3 determinations ± SD

The calculated and observed microbial reductions expressed in *Table 4* showed that *S.* Enteritidis and *E. coli* populations were more sensitive to PEF treatment than *L. monocytogenes* population in melon or watermelon juices, when the three microorganisms were submitted together to the treatment. This fact could be attributed to type of microorganism, since *E. coli* and *S.* Enteritidis are Gram-negative microorganisms, while *L. monocytogenes* is a Gram-positive bacterium. Some researchers have demonstrated that Gram-negative microorganisms are more susceptible to PEF treatment than those Gram-positive (Pothakamury et al., 1995, Qin et al., 1998 and Dutruex et al., 2000). The cellular membranes of the Gram-positive bacteria are more rigid and thicker than the Gram-negative, which might give an extra protection to PEF treatment (Aronsson et al., 2001). In addition, the cellular diameter could also be another influential factor during PEF application, since *E. coli* and *S.* Enteritidis have a higher

cellular diameter than *L. monocytogenes*. Qin et al. (1998) reported that the electric field induced across the cellular membrane is proportional to the geometric size, thus, *E. coli* and *S.* Enteritidis are more sensitive to PEF than *L. monocytogenes*.

On the other hand, *S.* Enteritidis, *E. coli* and *L. monocytogenes* populations were more sensitive to PEF treatment in melon juice than watermelon juice, possibly as a consequence of the higher energy density received by melon juice (7662.23 J/cm^3) than by watermelon juice (7541.18 J/cm^3) in the validation experiments. The difference in calculated energy densities (from *Equation 1*) for each fruit juice is due to treatment time value applied, being higher in melon juice than watermelon juice. These results are according to Korolczuk et al. (2006), who reported a greater microbial inactivation by PEF treatment when higher energy density is received by the food.

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Capítulo II

Inactivation of *Salmonella enterica* ser. Enteritidis in tomato juice by combining of high-intensity pulsed electric fields with natural antimicrobials

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ABSTRACT

The effect of high-intensity pulsed electric field (HIPEF) treatment (35kV/cm, 4 µs pulse length in bipolar mode without exceeding 38º C) as influenced by treatment time (200, 600 and 1000 µs) and pulse frequency (100, 150 and 200 Hz) for inactivating *Salmonella enterica* ser. Enteritidis inoculated in tomato juice was evaluated. Likewise, the effect of combining HIPEF treatment with citric acid (0.5, 1.0, 1.5 and 2.0 % wt/vol) or cinnamon bark oil (0.05, 0.10, 0.2 and 0.3 % vol/vol), as natural antimicrobials, against *S.* Enteritidis in tomato juice was also studied. Higher treatment time and lower pulse frequency produced the greater microbial inactivation. Maximum inactivation of *S.* Enteritidis (4.184 log_{10} units) in tomato juice by HIPEF was achieved when 1000 µs and 100 Hz of treatment time and pulse frequency, respectively, were applied. However, a greater microbial inactivation was found when *S.* Enteritidis was previously exposed to citric acid or cinnamon bark oil during one hour into tomato juice. Synergistic effects among HIPEF and natural antimicrobials were observed. Nevertheless, combinations of HIPEF treatment with 2.0 % of citric acid or 0.1 % of cinnamon bark oil were needed for inactivating *S.* Enteritidis by more than 5.0 log_{10} units (5.08 and 6.04 log_{10} reductions, respectively). Therefore, combinations of HIPEF with organic acids or essential oils seem to be a promising method to achieve the pasteurization in these kinds of products.

Keywords: High-intensity pulsed electric field; *Salmonella* Enteritidis; tomato juice; citric acid; cinnamon bark oil

INTRODUCTION

Tomato juice and others derivatives are believed to have health benefits due to the antioxidant capability of their bioactive compounds such as lycopene and vitamin C (Riso and others 2003). However, the consumption of these fresh products inadequately handled can be potentially hazardous to human health, because raw tomatoes have been recognized as vehicles of food-borne pathogenic microorganisms such as *Salmonella* spp. (Yuk and others 2005). Since 1990, outbreaks of *Salmonella* multi-ser. associated to consumption of raw tomatoes have been reported (Hedberg and others 1999; Cumming and others 2001; CDC 2002, 2005, 2007).

Zhuang and others (1995), Wei and others (1995) and Lin and Wei (1997) demonstrated that *Salmonella* ser. Montevideo can survive on the skin of tomatoes and grow on cut or sliced tomatoes held at room temperature. Likewise, Asplund and Nurmi (1991) reported that *Salmonella* ser. Enteritidis*,* Infantis and Typhimurium inoculated on tomatoes could grow from 1 to 5 or 6 log_{10} units at 22° C or 30º C, respectively, in 24 h. Therefore, the best approach to prevent the incidence of *Salmonella* spp. in tomatoes and outbreaks due to the consumption of contaminated tomatoes with that microorganism, would be minimizing the bacterial introduction by the application of good manufacturing practices (GMP) combined with minimal processing technologies; since, the washed of raw agricultural produce alone is not sufficient for eliminating completely the pathogens (Bari and others 2003).

Although, thermal treatment easily eliminates the pathogenic microorganisms, the organoleptic, nutritional and physicochemical properties of foods are extensively damaged (Jeyamkondan and others 1999; Espachs-Barroso and others 2003; Elez-Martínez and Martín-Belloso 2005). Hence, the development of novel technologies with low impact on the food and effective against pathogenic microorganisms is needed. One of them is the use of high intensity pulsed electric fields (HIPEF) treatment, as an alternative preservation method to the thermal treatment. This technology is able to inactivate both pathogenic and spoilage microorganisms in fluid foods without significant loss of nutrients, color and flavor (Yeom and others 2000; Hodgins and others 2002; Cserhalmi and others 2006; Elez-Martínez 2006a, 2006b). Thus, HIPEF may offer safe, fresh-like and nutritious foods to the consumers. This treatment involves the application of short pulses (1 to 10 μs) with high intensity

electric field (typically 20 to 80 kV/cm) to fluid foods placed between two electrodes in batch or continuous flow treatments at low temperatures.

Previous studies have demonstrated that HIPEF treatment is effective against *Salmonella* spp. in skim milk (Sensoy and others 1997), orange juice (Liang and others 2002), melon and watermelon juices (Mosqueda-Melgar and others 2007) and buffer solutions (Álvarez and others 2000; Raso and others 2000; Korolczuk and others 2006). However, information in the literature about the effects of HIPEF on *Salmonella* spp. in tomato juice was not found.

On the other hand, the use of natural antimicrobials such as organic acids and plant essential oils could enhance the antimicrobial effect of HIPEF treatment in fruit juices (Iu and others 2001; Raybaudi-Massilia and others 2006a). Nonetheless, the combined effects of citric acid or cinnamon bark oil and HIPEF against *Salmonella* spp. in tomato juice have not been studied. Citric acid and cinnamon bark oil are GRAS (Generally Recognized As Safe) substances permitted by the Food Additive Status List (USFDA 2006) and European Parliament and Council Directive Nr. 95/2/EC (1995). Despite the antimicrobial activity of these substances is well documented (Sharma 2000; Friedman and others 2004; Ceylan and others 2004; Burt 2004; Nazer and others 2005; Raybaudi-Massilia and others 2006b; Oussalah and others 2006, 2007) there are not studies reported in the literature about their effects against *Salmonella* spp. inoculated in tomato juice.

The purpose of this study was to evaluate the effect of HIPEF treatment on *S.* Enteritidis inoculated in tomato juice submitted to different levels of treatment time and pulse frequency, as well as to evaluate the effect of combining HIPEF treatment with citric acid or cinnamon bark oil on the *S.* Enteritidis inactivation in tomato juice.

MATERIALS AND METHODS

Tomato juice preparation

Tomatoes (*Solanum lycopersicum* var. Roma) at commercial ripeness were acquired in a supermarket of Lleida, Spain. Those tomatoes were washed, dried, peeled, cut into pieces and liquefied through an Ufesa blender (Model BP 4512; Vitoria, Spain) for obtaining juice. This juice was then centrifuged to 12500 rpm for 15 min at 4º C in an Avanti™ J-25 Centrifuge (Beckman Instrument, Inc.; USA). The supernatant juice was

filtered, bottled and autoclaved in a Presoclave 75 (J.P. Selecta, S.A; Barcelona, Spain) at 121º C for 15 min to obtain the free-microorganisms juice. Afterwards, the tomato juice at room temperature (22º C) was inoculated and subsequently processed by HIPEF.

Analytical characteristics of tomato juice

Soluble solid content (Atago RX-1000 refractometer; Atago Company Ltd., Japan), pH (Crison 2001 pH-meter; Crison Instruments S.A; Barcelona, Spain) and electric conductivity (Testo 240 conductivimeter; Testo GmBh & Co; Lenzkirch, Germany) were measured. Soluble solid content and pH were carried out in accordance with Spanish Regulation (B.O.E. 1988) (*Table 1*).

Table 1.-Analytical characteristics of tomato juice

Antimicrobial	Concentration (%)	Parameters [a]		
		pH	Electrical conductivity (mS/cm) [b]	Solid soluble content (%)
Control	0.0	4.30 ± 0.02	5.71 ± 0.01	4.4 ± 0.1
Citric acid	0.5	3.70 ± 0.02	5.83 ± 0.02	4.8 ± 0.1
	1.0	3.38 ± 0.01	5.96 ± 0.01	5.0 ± 0.1
	1.5	3.16 ± 0.01	6.11 ± 0.01	5.3 ± 0.2
	2.0	3.01 ± 0.03	6.21 ± 0.02	5.8 ± 0.1
Cinnamon bark oil	0.05	4.29 ± 0.02	5.71 ± 0.01	4.4 ± 0.1
	0.10	4.27 ± 0.03	5.73 ± 0.02	4.4 ± 0.1
	0.20	4.24 ± 0.02	5.82 ± 0.03	4.5 ± 0.1
	0.30	4.17 ± 0.02	6.01 ± 0.02	4.5 ± 0.1

[a] Values are the mean ± standard deviation of three determinations.
[b] Electrical conductivity measured at 22º C.

Preparation of microbial culture and inoculation on tomato juice

Strain of *Salmonella enterica* ser. Enteritidis 1.82 (National Collection of Type Culture (NCTC) 9001, PHLS Central Public Health Laboratory; London, UK) used in this study was grown in tryptone soy (TS) broth (Biokar Diagnostic; Beauvais, France). This microorganism was incubated

at 37º C with continuous shaking at 120 rpm for 15 hours to obtain cells in stationary growth phase. The final concentration reached by *S.* Enteritidis in the growth medium was approximately 10^9 colony forming units/milliliter (CFU/ml). Each sample of tomato juice (100 ml) to be processed by HIPEF was inoculated with 1 ml of that stock culture to reach a final concentration of 10^7 CFU/ml approx.

High-intensity pulsed electric fields equipment

A continuous flow bench-scale system OSU-4F (Ohio State University, Ohio, USA) which discharges square-wave pulses was used to treat tomato juice inoculated or not with *S.* Enteritidis. The HIPEF system is composed of eight chambers accommodated in series and each one with two stainless steel electrodes separated by a gap of 0.292 cm. Each chamber has a diameter of 0.23 cm and a volume of 0.0121 cm^3 with a cross section of 0.0415 cm^2. The pulse wave form was monitored using a Tekscope THS 720 oscilloscope (Tektronix Inc., Oregon, USA) connected to the PEF apparatus. The duration, frequency and polarity of pulse were controlled through of a Pulse Generator model 9410 (Quantum Composers, Inc., Bozeman MT, USA). The flow rate of the process was adjusted by a variable gear pump model 75210-25 (Cole Palmer Instrument Company; Illinois, USA). The circulating tomato juice during the HIPEF treatment was cooled through the heat exchangers connected to the chambers, which were immersed in an iced water bath (5º C) with circulation pump (J.P. Selecta, S.A; Barcelona, Spain).

Tomato juice processing

Prior to apply each HIPEF treatment, the system was disinfected as suggested by Elez-Martínez and others (2006b) and then sterile tomato juice was passed for 3 min to calibrate the system. Afterwards, the inoculated tomato juice with or without antimicrobial was pumped across the HIPEF unit for 2 min at a flow rate of 90 ml/min to displace the non-inoculated tomato juice. Once adjusted the flow rate and filled the HIPEF system with inoculated tomato juice, a continuous recirculation for 3 min before HIPEF applying was carried out. From that moment, the pulse generator and high voltage were turn on and then the cycle start was activated. The circulation time of the sample began after achieved the desirable voltage. Depending on the applied treatment, the inoculated tomato juices was pumped across the HIPEF unit several times in

continuous circulation mode (*Table 2*). The calculation of the number of passes (n_p) through the HIPEF unit (*Equation 1*) was settled according to Evrendilek and others (2004) using the following expression:

$$n_p = \frac{F_r \cdot t_c}{V_m} \qquad (1)$$

where F_r, is the flow rate (ml/min); t_c, is the circulation time (min) and V_m, is the volume of sample to be processed (ml).

During the HIPEF treatment, the outlet temperature of the tomato juice was maintained between 30 and 40º C using a cooling device that consisted of cooling coils and a water bath. The pre- and post-treatment temperatures in each pair of treatment chambers (T2-T1, T4-T3, T6-T5 and T8-T7) were monitored by thermocouples attached to exit of the chamber-pair. The recorded inlet (T2-T1) and outlet (T8-T7) temperatures during HIPEF processing were 21.8 ± 2.0 and 35.8 ± 1.8º C, respectively. Residence time of the tomato juice in each chamber calculated as volume of each chamber divided by flow rate was 8.09 ms. In this study, pulse length (4 μs) in bipolar mode (Aronsson and others 2001), total treatment time (up to 1000 μs) and pulse frequency (100-250 Hz) were used within acceptable values to obtain the lowest heating effects; whereas, maximum electric field strength (35 kV/cm) given by HIPEF equipment was applied.

In addition, the total energy density input (Q) of the tomato juice (Table 2) was calculated (*Equation 2*) according to Martín and others (1994) and Elez-Martínez and others (2006a) as follow:

$$Q(J/ml) = \frac{V \cdot I \cdot T_t}{v} \qquad (2)$$

where V, is the peak voltage (V); I, is the intensity of current (A); T_t; is the treatment time (s); v, is the total volume of all treatment chambers (ml).

Effect of treatment time and pulse frequency

A total of 9 samples of tomato juice were prepared in duplicate (n = 18) to evaluate the effect of different combinations of treatment time (200, 600, 1000 μs) and pulse frequency (100, 150, 200 Hz) on *S.* Enteritidis populations inoculated in tomato juice.

Effect of citric acid or cinnamon bark oil combined with high-intensity pulsed electric fields

The combination of treatment time and pulse frequency that better resulted for inactivating *S.* Enteritidis in tomato juice was subsequently used to evaluate the effect of different concentrations of citric acid (Scharlau Chemie, S.A., Barcelona, Spain) or cinnamon bark oil (Aceites Esenciales Dicana, Barcelona, Spain) on the pathogenic microorganism. Citric acid was added into the tomato juice samples at 0.5, 1.0, 1.5 and 2.0 % (wt/vol) based on preliminary experiments carried out in the laboratory (not shown data), whereas cinnamon bark oil was added at 0.05, 0.10, 0.20, and 0.30 % (vol/vol) according to previous studies (Raybaudi-Massilia and others 2006; Oussalah and others 2007). Prior to HIPEF processing, the effect of each antimicrobial at different concentration levels on *S.* Enteritidis in tomato juice was evaluated. Samples of tomato juice containing the antimicrobial were inoculated with the microorganism and exposed during one hour at room temperature (22º C) with continuous shaking through a magnetic stirrer to evaluate their effects on the microorganism (Raybaudi-Massilia and others 2006a). Once passed that time, HIPEF was immediately applied and the effect combined of each antimicrobial with HIPEF was evaluated.

Recovery and enumeration of viable cells

Once processed the tomato juice by HIPEF with or without antimicrobials, the injured cells were recovered during 20 min in buffered peptone water (Biokar Diagnostic) pH adjusted at 7.2 at room temperature, and then serially diluted in saline peptone water (0.1% casein-meat peptone plus 0.85% Sodium chloride supplied by Biokar Diagnostic and Scharlau Chemie, S.A., respectively). Finally, the viable cells of *S.* Enteritidis were pour plated in duplicate using a non-selective growth medium TS agar (Biokar Diagnostic) and incubated at 35º C by 24 - 48 hours. Plate counts were expressed as $\log_{10}$ (CFU/ml).

Statistical analysis

A multi-factor analysis of variance (ANOVA) was carried out to evaluate the effects of treatment time and pulse frequency, as well as citric acid or cinnamon bark oil alone or in combination with HIPEF treatment on the microbial inactivation of *S.* Enteritidis in tomato juice. Multiple range

tests (MRT) was applied to determine which levels of each factor were significantly different ($P \leq 0.05$). The MRT was performed using a Fisher's least significant difference (LSD) procedure. The experiments were done twice, and microbial counts were made in duplicate, therefore, means and standard deviations of four measurements were calculated for each treatment. All the statistical analyses were made with Statgraphics plus Centurion XV software Version 15.1.02.

RESULTS AND DISCUSSION

Characterization of tomato juice

Analytical characteristics such as pH, electrical conductivity and soluble solid content were measured to offer information detailed about tomato juice used in this study (*Table 1*). The pH value of control tomato juice (4.30) does not appear to be limiting on *Salmonella* spp. populations, since its survival and growth in tomatoes have been demonstrated (Asplund and Nurmi 1991; Zhuang and others 1995; Wei and others 1995; Lin and Wei 1997). A decrease of the tomato juice pH from 4.30 to 3.01 was observed when different concentrations of citric acid were added; however, negligible variations of this parameters were observed (from 4.29 to 4.17) when cinnamon bark oil was added. On the other hand, the value of electrical conductivity of the control tomato juice (5.71 mS/cm) is situated in the middle of the electrical conductivity range from 1 to 10 mS/cm that include those of the different products treated by HIPEF; thus, this tomato juice may support high electric field intensities without a great increase on the temperature of medium. Nonetheless, when citric acid is added to the control tomato juice a slight increase in electrical conductivity is observed (from 5.71 to 6.21 mS/cm) as a consequence of the dissociation of the acid molecule in ions charged positive- and negatively into the medium. Likewise, when cinnamon bark oil was added to the tomato juice a slight increase in electrical conductivity (from 5.71 to 6.01 mS/cm) was also detected. This fact could imply an increase in the temperature of the medium due to a higher number of particles in movement that transport the electric current when an electric field is applied. But, those slight increments in electrical conductivity could be considered as negligible on the effectiveness of HIPEF treatment and microbial reduction. Finally, the soluble solids content into sample juices increased (from 4.4 to 5.8%) when

citric acid was added; instead not significant variations were observed when cinnamon bark oil was added (from 4.4 to 4.5%), proving that the addition of organic acids contributes to the final soluble solids content of the product. Furthermore, the soluble solids levels reported in this study about tomato juice are within the range (5 - 8%) from those observed in commercial tomato juice (FAO 2001; Sánchez-Moreno and others 2006) where citric acid is normally added.

Effect of treatment time and pulse frequency

Maximum inactivation of 4.184 $\log_{10}$ units of *S.* Enteritidis in tomato juice was achieved when 1000 µs of treatment time and 100 Hz of pulse frequency were applied at 35 kV/cm electric field intensity and 4 µs pulse length of square-wave in bipolar mode without exceeding 35.8 ± 1.8º C. Since, *Salmonella* spp. is generally sensible at temperatures above 50º C (D'Aoust 2001); the treatment temperature was kept below 40º C for ensuring that the microbial destruction was reached only by HIPEF treatment and not by a combination of HIPEF and heat. Aronsson and Rönner (2001) reported that temperatures above 30º C should be needed for maximizing the microbial lethality by HIPEF treatment, since, the phospholipids of the lipid bi-layer are less ordered and the cell membrane has a liquid-crystalline structure (membrane fluidity properties); therefore, a greater sensibility to the cell electroporation by HIPEF could be observed.

The ANOVA showed significant effects ($P \leq 0.05$) of treatment time and pulse frequency on the *S.* Enteritidis inactivation in tomato juice processed by HIPEF treatment. The MRT indicated significant differences ($P \leq 0.05$) on the microbial counts when different treatment times (200, 600 and 1000 µs) were used, being greater the microbial inactivation when higher treatment time was applied (*Table 2*). Although, there are not studies in the literature about the inactivation of *Salmonella* spp. in tomato juice by HIPEF treatment, the results obtained in this study related to the effect of treatment time are according to those found by others authors using different serotypes of *Salmonella* and different media (Sensoy and others 1997; Álvarez and others 2000; Raso and others 2000; Liang and others 2002; Mosqueda-Melgar and others 2007).

The treatment time is the product of the number of pulses and their length; and an increase of any of these variables results in an increase in microbial inactivation (Barbosa-Cánovas and others 1999). However, the microbial inactivation observed in this study was non-lineal, since a rapid microbial reduction at the beginning of the treatment (up to 200 µs) and

then gradually with the next treatment times (600 and 1000 μs) were detected (*Table 2*). This non-lineal behavior could be attributed to the different HIPEF-resistances or -responses that may exist within the same microbial population, due to the biological variations or heterogeneity between cells of a population of the same microorganism (van Boekel 2002). The latter author, indicated that when a microorganism survive or die to a lethal event such as heat, high pressure, pulsed electric fields, etc., considering such events as probabilistic, is unlikely biologically that all cells behave in the same way under one single event. Thus, a HIPEF-resistance distribution of microorganisms of a same population could be observed. In addition, Álvarez and others (2003) indicated that this non-lineal behavior could be attributed to the accumulation of dead cells around of the remaining survivors, which could serve as protection of such survivor cells.

Table 2.-Influence of the total treatment time and pulse frequency on the microbial inactivation of *S.* Enteritidis, number of passes and total energy density input of the tomato juice treated by high-intensity pulsed electric fields.

Pulse frequency (Hz)	Total treatment time (μs)	Microbial inactivation [1] ($-\log_{10}$ CFU/ml)	Number of passes [2]	Total energy density input (J/ml)
	200	-1.99 ± 0.16^{Aa}	3.86	1401.1
100	600	-3.54 ± 0.12^{Ba}	11.59	4192.3
	1000	-4.184 ± 0.018^{Ca}	19.32	6994.5
	200	-2.19 ± 0.09^{Aa}	2.58	1401.1
150	600	-3.67 ± 0.08^{Ba}	7.73	4192.3
	1000	-3.94 ± 0.11^{Ca}	12.88	6.994.5
	200	-1.96 ± 0.10^{Ab}	1.93	1401.1
200	600	-3.28 ± 0.04^{Bb}	5.80	4192.3
	1000	$-3. 88 \pm 0.15^{Cb}$	9.66	6994.5

[1] Values are the mean ± standard deviation of four determinations; [2] Number of times that juice sample has been processed by HIPEF (from *Equation 1*). Different capital superscript letters (*A, B, C*) indicate significant differences ($P \leq 0.05$) among treatment times by each pulse frequency; Different lower-case superscript letters (*a, b*) indicate significant differences ($P \leq 0.05$) among pulse frequencies by each treatment time. Treatment conditions: 35 kV/cm and 4 μs pulse length in bipolar mode without exceeding 35.8 ± 1.8º C outlet temperature. Sample of juice was pumped through the HIPEF system at a flow rate of 90 ml/min.

On the other hand, significant differences in microbial counts by different pulse frequencies applied were observed when MRT was carried out. The obtained results indicated that 100 Hz was slightly more effective in *S.* Enteritidis reduces than 200 Hz under the same total energy density input (6994.5 J/ml) (*Table 2*). In general, a greater microbial inactivation was reached when lower pulse frequency was applied. This fact could be attributed to the number of passes of the tomato juice through the system when HIPEF are applied; since, a higher number of passes was required for lower pulse frequencies than high pulse frequency at the same total treatment time (*Table 2*). Hence, a greater cell stress could be induced as a consequence of higher exposure time to HIPEF treatment, which could end in greater microbial inactivation.

Elez-Martínez and others (2004) and Mosqueda-Melgar and others (2007) reported similar behaviors on the microbial reduction when different pulse frequencies were applied. These authors found a greater reduction of *Saccharomyces cerevisae* and *S.* Enteritidis in orange and watermelon juices, respectively, when the lowest pulse frequencies were applied in a range from 50 to 350 Hz (Elez-Martínez and others 2004) or 100 to 250 Hz (Mosqueda-Melgar and others 2007).

Effect of citric acid combined with high-intensity pulsed electric fields

The effect of citric acid added at different concentrations on the tomato juice inoculated with *S.* Enteritidis without HIPEF application was evaluated. In this case, negligible effects ($P \leq 0.05$) on microbial reductions were detected (*Figure 1*). However, when HIPEF treatment (35 kV/cm for 1000 µs at 100 Hz, 4 µs pulse length and 35.8 ± 1.8º C) was applied to tomato juice containing citric acid, a synergistic effect was observed from a concentration of 1.0 % (*Figure 1*). In contrast, when tomato juice with 0.5 % of citric acid was treated by HIPEF, only an additive effect was found.

Citric acid is a weak-acid not lipophilic (-0.172 partition coefficient), with a pKa of 3.14 and a molecular weight of 192.14 g/mol. Its low lipid solubility difficult the diffusion through the plasmatic membrane, for that reason, its individual antimicrobial activity is limited. Therefore, the antimicrobial effect of citric acid may be attributed to the combination of two mechanisms of action: a) acidification of the external medium to inhibit the microbial growth, and/or b) chelation of metal ions which are essentials for microbial growth (Gould and Jones 1989). However, when HIPEF is applied, the pore formation on the cell membrane of the

microorganism is observed, and a flow of citric acid molecules through the pores of cell membrane towards the cell inside may occur. For many acids, only the undissociated acid molecule is assumed to possess the antimicrobial activity (Stratford and Eklund 2003). Hence, depending of medium pH (< 3.14) the undissociated form of citric acid would pass rapidly to the cell inside when HIPEF is performed and a progressive decrease in intracellular pH take place, whereas at the near-neutral pH of the cell cytoplasm the acid molecules are forced to dissociate into charged anions. This decrease in intracellular pH may affect the cell signaling, active transport and genetic material, which cause cell inactivation (Stratford and Eklund 2003).

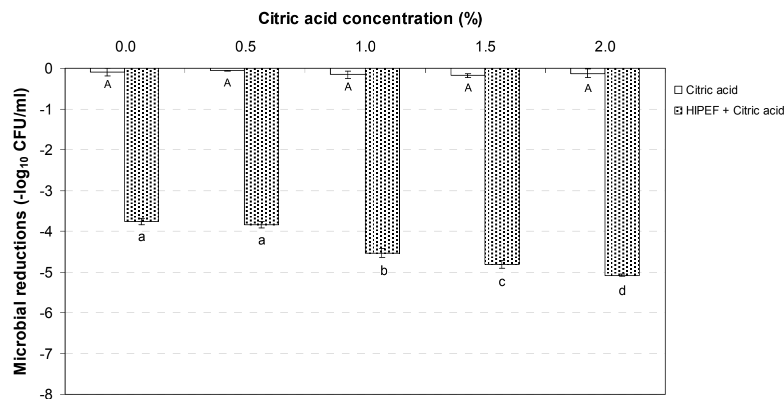

Figure 1.-*Inactivation of S. Enteritidis in tomato juice by combining of high-intensity pulsed electric fields with citric acid. Treatment conditions: 35 kV/cm for 1000 µs at 100 Hz, 4 µs pulse length and 35.8 ± 1.8º C. Bars (means ± SD of 4 determinations) with different capital and lower-case letters are significantly different (P ≤ 0.05).*

In this study, even though citric acid concentrations at 1.0, 1.5 and 2.0 % acted synergistically with HIPEF treatment, only combinations with 2.0 % of the acid were enough for inactivating *S.* Enteritidis above 5.0 $\log_{10}$ units (5.08 $\log_{10}$) (*Figure 1*). Fulfilling thus, the request of the USFDA (U.S. Food and Drug Administration) for pasteurization of juice products, which requires that the novel technologies or treatments can achieve at least 5.0 $\log_{10}$ reductions of the “target” microorganism on the juice product (USFDA 2002).

The greater effectiveness of citric acid when 2.0 % was added at tomato juice to inactivate *S.* Enteritidis under HIPEF treatment, may be attributed to the undissociated form of acid molecules, since, a pH value of 3.01 units was observed at this concentration (*Table 1*), which is below pKa value (3.14).

Although, there are not studies about the inactivation of *Salmonella* spp. in tomato juice by combining of HIPEF and citric acid, there is a study related to this microorganism and treatment, but in different medium and organic acid. Raybaudi-Massilia and others (2006b) inactivated *S.* Enteritidis in apple juice over 5.0 log_{10} units when HIPEF treatment (35 kV/cm for 1500 μs at 321 Hz and 4 μs pulse length in bipolar mode) was applied immediately or after one hour of exposure with 1.5 or 1.0 % of malic acid, respectively. The inactivation reached by those authors with a lower concentration of the organic acid than that used in the present study, could to be attributed to molecular size of the acid employed. Since, malic acid has smaller molecular weight (134.09 g/mol) than citric acid (192.14 g/mol), which may facilitate a higher penetration to cell inside and cause inactivation by depression of internal pH of the microbial cell and denaturalization of genetic material (Eswaranadan and others 2004).

Effect of cinnamon bark oil combined with high-intensity pulsed electric fields

Significant differences ($P \leq 0.05$) in *S.* Enteritidis counts were found when different concentrations of cinnamon bark oil were tested (*Figure 2*). Concentrations from 0.1 % of the essential oil in tomato juice inoculated with *S.* Enteritidis, showed antimicrobial effects by itself after of exposure during one hour against the microorganism. Microbial reductions up to 1.09, 2.07 and 4.11 log_{10} units were achieved when 0.1, 0.2 and 0.3 % of cinnamon bark oil, respectively were used (*Figure 2*). Similar results were reported by Raybaudi-Massilia and others (2006b), who totally eliminated at *S.* Enteritidis in apple, pear and melon juices when up to 0.2 % of cinnamon leaf oil was added. On the other hand, Oussalah and others (2007) and Friedman and others (2006) reported that up to 0.05 % of cinnamon bark oil were enough to inhibit at *S.* Typhimurium and *S.* Hadar in brain-heart infusion and wines, respectively.

Although is still uncertain the mechanism of action of cinnamon oil on the microbial cells, is believed that the antimicrobial action of this essential oil is due to the interaction of carbonyl group of the cinnamaldehyde, main compound of cinnamon oil from bark, on the cell proteins embedded in the

cytoplasmatic membrane to inhibit the action of the enzymes amino acid decarboxylases, necessary for the amino acids biosynthesis and biodegradation (Wendakoon and Sakaguchi 1995; Burt 2004). On the other hand, Oussalah and others (2006) reported a release of the cell constituents, a decrease of intracellular ATP concentration and a decrease in intracellular pH due to an increase in the permeability of cell membrane when cinnamon oil was applied up to 0.1 %. However, these authors did not observe an apparent change on the cell surface (by electron micrographs) when cinnamon oil was added.

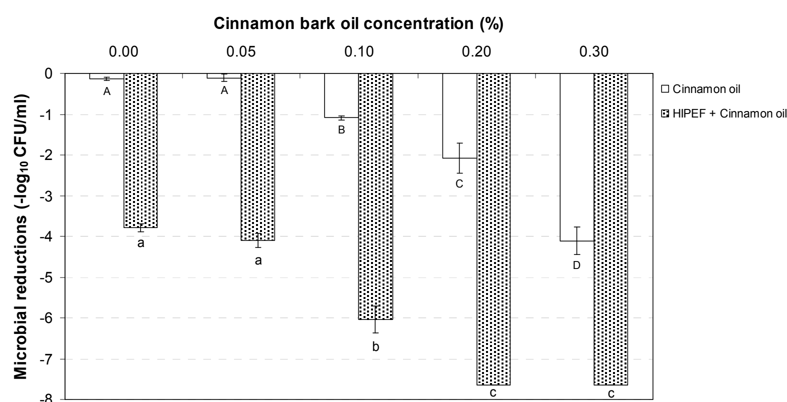

***Figure 2.**-Inactivation of S. Enteritidis in tomato juice by combining of high-intensity pulsed electric fields with cinnamon bark oil. Treatment conditions: 35 kV/cm for 1000 µs at 100 Hz, 4 µs pulse length and 35.8 ± 1.8º C. Bars (means ± SD of 4 determinations) with different capital and lower-case letters are significantly different (P ≤ 0.05).*

The antimicrobial action of cinnamon bark oil was enhanced when HIPEF treatment (35 kV/cm for 1000 µs at 100 Hz, 4 µs pulse length and 35.8 ± 1.8º C) was applied, since a synergistic effect from 0.1 % was observed (*Figure 2*). In this way, concentrations of 0.05 % of cinnamon bark oil did not show significant effect on the *S.* Enteritidis inactivation in tomato juice, and concentrations of 0.2 and 0.3 % of essential oil totally inactivated to the pathogenic microorganism of the juice (*Figure 2*). Nonetheless, combinations up to 0.1 % of cinnamon bark oil with HIPEF were enough to reduce *S.* Enteritidis upper 5.0 log_{10} units (6.04 log_{10}) in

tomato juice, achieving thus, the pasteurization required by the Regulatory Organizations (USFDA 2002).

The enhancing effect of this combination, as antimicrobial effective, could be due to the formation of pores on the cell membrane when HIPEF is applied, which may favor more easily the entrance of the oil to cell inside and cause damage in the cell functions.

Combinations of HIPEF treatment with essential oils to inactivate pathogenic microorganism in juices have not been found in the literature. As a consequence, results obtained in this research contribute to the scientific knowledge about the effect of combined treatments of HIPEF with essential oils to reduce the risk of foodborne illness caused by juices consumption.

CONCLUSION

The use of high-intensity pulsed electric fields in combination with citric acid or cinnamon bark oil resulted effective for the non-thermal pasteurization of tomato juice. Since, a synergistic effect by combinations of HIPEF and those natural antimicrobials was observed. Concentrations up to 2.0 % of citric acid or up to 0.1 % of cinnamon bark oil into tomato juice exposed during one hour on *S.* Enteritidis and then processed by HIPEF treatment were needed to achieve the pasteurization level (up to 5.0 $\log_{10}$ units) required by Regulatory Organizations. Nonetheless, studies about the sensory quality of tomato juice treated by HIPEF and combinations with these natural antimicrobials are required to evaluate the consumer acceptance.

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Capítulo III

Non-thermal pasteurization of fruit juices by combining high-intensity pulsed electric fields with natural antimicrobials

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ABSTRACT

The effect of high-intensity pulsed electric fields (HIPEF) on the *Salmonella* Enteritidis and *Escherichia coli* O157:H7 populations inoculated in apple, pear, orange and strawberry juices as influenced by treatment time and pulse frequency was investigated. Combinations of HIPEF (35kV/cm, 4µs pulse length in bipolar mode without exceeding 40ºC) with citric acid or cinnamon bark oil against these pathogenic microorganisms in fruit juices were also evaluated. Treatment time was the more influential factor on the microbial reduction in all the fruit juices analyzed. *S.* Enteritidis and *E. coli* O157:H7 were reduced by more than 5.0 log_{10} units in orange juice treated by only HIPEF; whereas strawberry, apple and pear juices were pasteurized when HIPEF was combined with citric acid at 0.5, 1.5, 1.5%, respectively, or cinnamon bark oil at 0.05, 0.1 and 0.1%, respectively. Synergistic and additive killing effects against *S.* Enteritidis and *E. coli* O157:H7 in fruit juices by combining treatments were observed.

Industrial relevance: The use of high-intensity pulsed electric fields treatment as a non-thermal pasteurization method in combination with organic acids or essential oils is an effective process for eliminating *S.* Enteritidis and *E. coli* O157:H7 populations in fruit juices upper 5.0 log_{10} reductions. Therefore, combinations of those treatments may help to ensure the microbiological safety in juice products, and to reduce the risk of food-borne illness caused by the consumption of these kinds of foods.

***Keywords*:** HIPEF; citric acid; cinnamon bark oil; *S.* Enteritidis; *E. coli* O157:H7; apple; pear; orange; strawberry

1.-INTRODUCTION

In the past, fruit juices were considered as safe foods because of their low pH caused by naturally occurring organic acids, which may generally prevent the growth of pathogenic bacteria (Parish, 1997). However, changes occurred in the diet of the consumers have produced new problems of public health associated to the presence of pathogenic microorganisms (Balla & Farkas, 2006). One of those changes has pointed out toward the consumption of fresh fruit juice, due to their high nutritional and sensory qualities as well as to their health benefits. However, those kinds of products can be harbor of several pathogenic microorganisms, among them *Salmonella* spp. and *E. coli* O157:H7 as emerging pathogenic microorganisms in fruit juices. Since, several salmonellosis and enterohemorraghic *E. coli* outbreaks associated with unpasteurized juices have been reported during the last three decades. Foodborne outbreaks of diseases by *Salmonella* serovars Enteritidis, Muenchen, Typhimurium, Hartford, Gaminara and Rubislaw, and enterohemorraghic *E. coli* O157:H7 have been identified as causative agents in orange juice and apple cider (Besser et al., 1993; CDC, 1996a; 1996b; Parish, 1997; Boase, Lipsky & Simani 1999; Powell & Luedtke, 2000; D'Aoust Maurer & Bailey, 2001; CDC, 2007). In addition, an outbreak by *S.* Enteritidis was involved in consumption of citrus fruit juices such as orange, grapefruit and lemonade (Powell & Luedtke, 2000). Likewise, a foodborne outbreak of *S.* Group B and *E. coli* O157:H7 in strawberry and pear, respectively, were reported as possible causative agents (CDC, 2007).

Although, thermal pasteurization is the most popular technology used for eliminating pathogenic microorganisms and spoilage bacteria in fruit juices, it may extensively damage the organoleptic, nutritional and physicochemical properties of food (Jeyamkondan, Jayas & Holley, 1999; Espach-Barroso, Barbosa-Cánovas & Martín-Belloso, 2003; Elez-Martínez & Martín-Belloso, 2005). For that reason, the today's consumers are demanding high quality, fresh-like and microbiologically safe foods (Mittal and Griffiths, 2005). Therefore, a great interest in the development of novel technologies that offers the advantages of using low processing temperatures, low energy consumption, and retention of nutritional and sensory attributes, while inactivating pathogenic microorganisms to levels that do not cause a public health risk, are being tested (Smith, Mittal & Griffiths, 2002). One of them is the use of high-intensity pulsed electric fields (HIPEF) treatment, which is able to inactivate both pathogenic and spoilage microorganisms in foods without significant loss of flavor, color

and nutrients (Yeom, Streaker, Zhang & Min, 2000; Hodgins, Mittal & and Griffiths, 2002; Cserhalmi, Sass-Kiss, Tóth-Markus & Lechner, 2006, Elez-Matínez, Soliva-Fortuny & Martín-Belloso 2006). Nonetheless, this innovative technology appears to be more effective for food safety and preservation when is combined with others methods such as organic acid, enzymes, bacteriocins, and spices (Liu, Yousef & Chism, 1997; Iu, Mittal & Griffiths, 2001; Smith et al., 2002, Liang, Mittal & Griffiths, 2002; Sobrino-López & Martín-Belloso, 2006; Raybaudi-Massilia, Mosqueda-Melgar & Martín-Belloso, 2006a).

HIPEF treatment involves the application of short pulses (1 to 10 μs) of high-intensity electric field (typically 20 to 80 kV/cm) to fluid foods placed between two electrodes in batch or continuous flow treatments. The effectiveness of HIPEF treatment to inactivate or reduce some strains and serovars of *Salmonella* spp. and *E. coli* in orange, apple, carrot, melon and watermelon juices has been reported (Evrendilek, Zhang & Richter, 1999; McDonald, Lloyd, Vitale, Petersson & Inning, 2000; Iu et al., 2001; Liang et al., 2002; Gupta, Masterton & Magee, 2003; Zhong et al., 2005; Mosqueda-Melgar, Raybaudi-Massilia & Martín-Belloso, 2007); however, studies about the *Salmonella* spp. and *E. coli* O157:H7 inactivation in pear and strawberry juices by HIPEF treatment have not been found in the literature. Hence, further studies on the inactivation of target pathogenic microorganisms in juice products by HIPEF treatment are needed to meet commercial pasteurization standards (Mertens & Knorr, 1992; Liang et al., 2002). Since, the U.S. Food and Drug Administration (USFDA) have published regulations about the necessity of achieving at least 5.0 $\log_{10}$ reductions of the target microorganism on the juice product when novel technologies or treatments are applied (USFDA 2002).

Currently, the HIPEF treatment application has received USFDA approval and its use at commercial scale is being applied for some few juices (Clark, 2006). As for, citric acid and cinnamon bark oil are GRAS (Generally Recognized As Safe) substances permitted by the Food Additive Status List (USFDA 2006) and European Parliament and Council Directive Nr. 95/2/EC (1995). Despite the antimicrobial activity of these substances is well documented (Sharma, 2000; Friedman, Henika, Levin & Mandrell, 2004; Ceylan, Fung & Sabah, 2004; Burt, 2004; Nazer, Kobilinsky, Tholozan & Dubois-Brissonnet, 2005; Raybaudi-Massilia, Mosqueda-Melgar & Martín-Belloso 2006b; Oussalah, Caillet & Lacroix 2006; Oussalah, Caillet, Saucier & Lacroix, 2007), there are no studies reported in the literature about the effects of HIPEF combined with citric acid or

cinnamon bark oil against *Salmonella* spp. and *E. coli* O157:H7 inoculated in fruit juices.

The objectives of this study were to evaluate the effect of HIPEF treatment on *Salmonella* ser. Enteritidis and *E. coli* O157:H7 inoculated in apple, pear, orange and strawberry juices as influenced by treatment time and pulse frequency, as well as to find the optimum values of these processing factors for inactivating the highest levels of those microorganisms using HIPEF treatment. The effect of combining HIPEF treatment with citric acid or cinnamon bark oil on the *S.* Enteritidis and *E. coli* O157:H7 inactivation in fruit juices was also evaluated.

2.-MATERIALS AND METHODS

2.1.-Fruit juices obtaining

Apple (*Malus domestica* Borkh var. “Fuji”), pear (*Pyrus communis* L. var. “Flor de invierno”), orange (*Citrus sinensis* L. var. “Valencia”) and strawberry (*Fragaria* spp.) fruit at commercial ripeness were chosen in a supermarket of Lleida, Spain. Apple, pear and strawberry fruit were washed, dried, peeled, cut into pieces, and liquefied through an Ufesa blender (Model BP 4512; Vitoria, Spain) for obtaining the juices; while oranges were washed, cut in two pieces and then squeezed with a Presse-Citron model (ALJUAN S.L., Alicante, Spain) squeezer to obtain the juice. All fruit juices were centrifuged at 12,500 rpm for 15 min at 4ºC in an Avanti™ J-25 Centrifuge (Beckman Instrument, Inc.; USA). The supernatant juice was filtered, bottled and autoclaved in a Presoclave 75 (J.P. Selecta, S.A; Barcelona, Spain) at 121º C for 15 min. Afterwards, the sterilized fruit juices were cooled at room temperature (22º C) to be inoculated and subsequently processed by HIPEF as described in 2.3 and 2.4.

2.2.-Physicochemical analyses of fruit juices

Electrical conductivity (Testo 240 conductivimeter; Testo GmBh & Co; Lenzkirch, Germany), pH (Crison 2001 pH-meter; Crison Instruments S.A; Barcelona, Spain) and soluble solid content (Atago RX-1000 refractometer; Atago Company Ltd., Japan) were measured in each sterilized fruit juice according to the Spanish Regulation (BOE, 1988) (Table 1).

Table 1.-Physicochemical characteristics of fruit juices

Fruit juice	Concentration (%)		Parameters [1]					
			pH		Electrical conductivity [c]		Soluble solid (%)	
	CA [a]	CBO [b]	CA	CBO	CA	CBO	CA	CBO
Apple	0.0		4.46 ± 0.03		2.18 ± 0.03		13.7 ± 0.1	
	0.5	0.05	3.71 ± 0.02	4.45 ± 0.02	2.31 ± 0.01	2.22 ± 0.01	13.8 ± 0.2	13.8 ± 0.1
	1.0	0.10	3.31 ± 0.01	4.46 ± 0.01	2.42 ± 0.01	2.24 ± 0.03	14.0 ± 0.1	13.9 ± 0.1
	1.5	0.20	3.13 ± 0.02	4.46 ± 0.01	2.54 ± 0.01	2.27 ± 0.02	14.1 ± 0.1	13.9 ± 0.1
	2.0	0.30	2.92 ± 0.02	4.45 ± 0.02	2.66 ± 0.02	2.32 ± 0.01	14.2 ± 0.1	14.1 ± 0.1
Pear	0.0		4.40 ± 0.03		2.99 ± 0.01		13.8 ± 0.1	
	0.5	0.05	3.45 ± 0.02	4.41 ± 0.02	3.15 ± 0.01	3.04 ± 0.01	14.3 ± 0.1	13.8 ± 0.1
	1.0	0.10	3.17 ± 0.01	4.39 ± 0.01	3.33 ± 0.01	3.08 ± 0.02	14.5 ± 0.1	13.9 ± 0.2
	1.5	0.20	2.88 ± 0.03	4.40 ± 0.01	3.46 ± 0.01	3.10 ± 0.02	14.9 ± 0.1	14.0 ± 0.1
	2.0	0.30	2.72 ± 0.01	4.40 ± 0.02	3.56 ± 0.02	3.17 ± 0.02	15.2 ± 0.1	14.0 ± 0.1
Orange	0.0		3.44 ± 0.03		5.40 ± 0.03		10.8 ± 0.1	
	0.5	0.05	3.12 ± 0.02	3.44 ± 0.03	5.48 ± 0.01	5.44 ± 0.01	11.5 ± 0.1	10.9 ± 0.2
	1.0	0.10	2.94 ± 0.01	3.45 ± 0.02	5.57 ± 0.02	5.49 ± 0.02	12.3 ± 0.1	10.9 ± 0.1
	1.5	0.20	2.82 ± 0.03	3.44 ± 0.01	5.64 ± 0.02	5.52 ± 0.01	12.9 ± 0.1	11.0 ± 0.1
	2.0	0.30	2.71 ± 0.02	3.43 ± 0.03	5.73 ± 0.01	5.54 ± 0.02	13.3 ± 0.2	11.2 ± 0.2
Strawberry	0.0		3.16 ± 0.01		5.13 ± 0.02		7.2 ± 0.1	
	0.5	0.05	2.94 ± 0.02	3.15 ± 0.03	5.25 ± 0.03	5.17 ± 0.02	7.5 ± 0.1	7.3 ± 0.1
	1.0	0.10	2.80 ± 0.02	3.16 ± 0.01	5.37 ± 0.01	5.20 ± 0.02	7.8 ± 0.2	7.5 ± 0.1
	1.5	0.20	2.65 ± 0.01	3.16 ± 0.02	5.50 ± 0.01	5.24 ± 0.01	8.3 ± 0.1	7.5 ± 0.1
	2.0	0.30	2.53 ± 0.03	3.16 ± 0.02	5.58 ± 0.02	5.28 ± 0.02	8.7 ± 0.2	7.7 ± 0.1

[1] Results are mean ± SD of three measurements; [a] Citric acid (CA); [b] Cinnamon bark oil (CBO); [c] Electrical conductivity measured at 22º C (mS/cm).

2.3.-Microbial strains preparation and inoculation in fruit juices

Pure cultures of *Salmonella enterica* serovar Enteritidis 1.82 (National Collection of Type Culture (NCTC) 9001, PHLS Central Public Health Laboratory; London, UK) and *Escherichia coli* O157:H7 (Colección Española de Cultivos Tipo (CECT); University of Valencia, Valencia, Spain) were grown in tryptone soy broth (TSB) and TSB + 0.6 % Yeast Extract, respectively. These culture media were provided by Biokar Diagnostic (Beauvais, France). *S.* Enteritidis and *E. coli* O157:H7 were incubated at 37º C with continuous agitation at 120 rpm for 15 hours to obtain cells in stationary growth phase. The final concentration reached by

the microorganisms in the growth media was approx. 10^9 - 10^{10} colony forming units/milliliter (CFU/ml). One hundred milliliters of apple, pear, orange and strawberry juices were individually inoculated with an aliquot of 1 ml of *S.* Enteritidis and *E. coli* O157:H7, to obtain a final concentration of 10^7 - 10^8 CFU/ml approx. of each one.

2.4.-HIPEF equipment and fruit juice processing

A continuous flow HIPEF system, model bench-scale OSU-4F (Ohio State University, Ohio, USA) which discharges square-wave pulses was used to process the samples of fruit juices. The HIPEF system is composed of eight collinear field chambers connected in series and each one with two stainless steel electrodes separated by a distance of 0.292 cm. Each chamber has a diameter of 0.23 cm and a volume of 0.0121 cm^3 with a cross section of 0.0415 cm^2. The pulse waveform, peak voltage and intensity of current were monitored using a Tekscope THS 720 oscilloscope (Tektronix Inc., Oregon, USA) connected to the HIPEF apparatus. Pulse frequency and duration were controlled through a Pulse Generator model 9410 (Quantum Composers, Inc., Bozeman MT, USA). The flow rate of the process was adjusted by a variable gear pump model 75210-25 (Cole Palmer Instrument Company; Illinois, USA). Finally, the product was refrigerated in an iced water bath (5ºC) with circulation pump (J.P. Selecta, S.A; Barcelona, Spain).

Prior to each HIPEF processing the system was disinfected as suggested by Elez-Martínez et al. (2006). Afterwards, each sterile fruit juice (non-inoculated) was first pumped through the HIPEF system as negative control, and subsequently the inoculated fruit juices with or without antimicrobial were pushed across the HIPEF system at flow rate adjusted to 80-110 ml/min (Table 2). Depending on the applied treatment, inoculated fruit juices were pumped through the HIPEF unit several times in continuous circulation mode (Table 3). The calculation of the number of passes (n_p) across the HIPEF unit (Equation 1) was performed according to Evrendilek, Zhang & Richter (2004) using the following expression:

$$n_p = \frac{F_r \cdot t_c}{V_m} \qquad \textbf{(1)}$$

where F_r, is the flow rate (ml/min); t_c, is the circulation time (min) and V_m, is the volume of sample to be processed (ml).

The residence time of the circulating juice in each chamber ranged from 0.0066 to 0.0091 s. Maximum electric field strength (35 kV/cm) given by HIPEF equipment and 4 μs pulse length in bipolar mode were used. The peak voltage, intensity of current and ohmic resistance of the treatment chambers for each fruit juice during HIPEF processing is showed in the Table 2.

Table 2.-Values of flow rate, peak voltage, intensity of current and ohmic resistance of fruit juices during HIPEF processing

Fruit juice	Flow rate (ml/min)	Peak voltage (V)	Intensity of current (A)	Ohmic resistance (Ω)
Apple	90	10220	25.36	402.99
Pear	90	10220	34.78	293.82
Orange	110	10220	62.82	162.69
Strawberry	80	10220	59.68	171.25

Treatment time and pulse frequency were ranged within acceptable values to obtain the lowest heating effects. The pre- and post-treatment temperatures in each pair of treatment chambers (T2-T1, T4-T3, T6-T5 and T8-T7) were monitored by thermocouples attached to exit of the chamber-pair. Temperature of each fruit juice during HIPEF processing was maintained below 40º C using a cooling device that consisted of cooling coils and a water bath. In addition, total energy density input (*Q*, J/ml) (Equation 2) and electric power (*P*, W) (Equation 3) used by each fruit juice during HIPEF processing (Table 3) were calculated according to Martín, Zhang, Castro, Barbosa-Cánovas & Swanson (1994) and Zhang, Barbosa-Cánovas & Swanson. (1995), respectively, as follow:

$$Q = \frac{V \cdot I \cdot T_t}{v} \quad (2)$$

$$P = V \cdot I \cdot \tau \cdot f \quad (3)$$

where V, is the peak voltage (V); I, is the intensity of current (A); T_t; is the treatment time (s); v, is the total volume of all treatment chambers (ml); τ, is the pulse duration; and f, is the pulse frequency.

2.5.-Effect of treatment time and pulse frequency

A total of 9 samples by each fruit juice (apple, pear, orange and strawberry) were prepared in duplicate (n = 18) to evaluate combinations of 3 treatment times different (500, 1250, 2000 μs) at 3 pulse frequencies different (100, 175, 250 Hz). The order of combinations was randomly carried out to provide protection against the effects of lurking variables.

2.6.-Effect of citric acid or cinnamon bark oil combined with HIPEF treatment

The optimized treatment time and pulse frequency values used to evaluate the effect of different concentrations of citric acid (Scharlau Chemie, S.A., Barcelona, Spain) or cinnamon bark oil (Aceites Esenciales Dicana, Barcelona, Spain) on *S.* Enteritidis and *E. coli* O157:H7 in each fruit juice by HIPEF treatment are showed in the Table 6. Citric acid was added into each sample of fruit juice at 0.0, 0.5, 1.0, 1.5 and 2.0 % (wt/vol), whereas cinnamon bark oil was added at 0.00, 0.05, 0.10, 0.20, and 0.30 % (vol/vol). Prior to HIPEF processing, the effect of each antimicrobial at different concentrations on *S.* Enteritidis and *E. coli* O157:H7 in each fruit juice was evaluated. Samples of fruit juices with or without antimicrobials were inoculated with a cocktail of both pathogenic microorganisms and exposed during one hour at room temperature (22º C) with continuous shaking through a magnetic stirrer to evaluate their effects on the microorganisms (Raybaudi-Massilia et al., 2006a). Once passed that time, HIPEF was immediately applied, and the combined effect of each antimicrobial with HIPEF was evaluated.

2.7.-Recovery and viable cells counts treated by HIPEF and antimicrobials

The injured cells of *S.* Enteritidis and *E. coli* O157:H7 from 25 ml of fruit juices treated by HIPEF with or without natural antimicrobials were recovered during 20 min in 225 ml of buffered peptone water (Biokar Diagnostic) pH adjusted at 7.2 at room temperature, and then serially diluted in saline peptone water (0.1% casein-meat peptone plus 0.85% Sodium chloride supplied by Biokar Diagnostic and Scharlau Chemie, S.A., respectively). Afterwards, the viable cells of *S.* Enteritidis and *E. coli* O157:H7 were spread plate in selective media of Hektoen (Biokar Diagnostic) and McConkey-Sorbitol (Biokar Diagnostic) agars,

respectively. The plates were incubated at 35º C for 24 hours and counts were expressed as $\log_{10}$ CFU/ml.

2.8.-Experimental design and statistical analysis

A multilevel factorial design was used to evaluate the effects of treatment time and pulse frequency on the bacterial reductions of *S.* Enteritidis and *E. coli* O157:H7 populations inoculated in different fruit juices treated by HIPEF. The experiment was performed twice and microbial counts were done in duplicate (n = 4). Means and standard deviations (SD) were calculated for each treatment. Analysis of variance (ANOVA) was used to determine significant differences ($P \leq 0.05$) among the applied treatments. Multiple range tests (MRT) was applied to determine which levels of each factor were significantly different ($P \leq 0.05$). The MRT was performed using a Fisher's least significant difference (LSD) procedure. *F-Ratio* values were also reported (Table 4) to evaluate the influence of those factors on the bacterial reductions. In addition, a model (Equation 4) given by the applied experimental design was fitted to the experimental data.

$$Y = k + (A \times t) + (B \times f) + (C \times t)^2 + (D \times f)^2 + (E \times t \times f) \qquad (4)$$

where, Y is the maximum bacterial inactivation obtained after HIPEF treatment ($-\log_{10}$ CFU/ml), t is the treatment time (µs), f is the pulse frequency (Hz), k is a constant of the equation, A (μs^{-1}) and C (μs^{-2}) are regression coefficients depending on t, B (Hz^{-1}) and D (Hz^{-2}) are regression coefficients depending on f, and E ($\mu s^{-1} \cdot Hz^{-1}$) is a regression coefficient depending on t and f interaction.

Maximum reductions of *S.* Enteritidis and *E. coli* O157:H7 were calculated from Equation (4) using both single and multiple responses analyses. The single response analysis of the data was performed in a first step to obtain optimum values of treatment time and pulse frequency with maximal inactivation of each microorganism in each juice (Table 5). However, for avoiding discrepancies between the different treatment times and pulse frequencies obtained for each microorganism in each fruit juice, a multiple response analysis was employed as a second step (Table 6). The latter analysis was applied to know the minimum values needed of treatment time and pulse frequency for obtaining the highest *S.* Enteritidis and *E. coli* O157:H7 reductions on each fruit juice using HIPEF tretment.

Finally, after obtaining the optimal processing conditions in terms of treatment time and pulse frequency through a multiple response analysis, the HIPEF treatment conditions were experimentally checked to validate the calculations. All the statistical analyses were done with Statgraphics plus Centurion XV software Version 15.1.02.

3.-RESULTS AND DISCUSSION

3.1.-Effect of treatment time on the microbial inactivation

Significant differences ($P \leq 0.05$) among the different treatment times used (500, 1250, 2000 μs) on microbial counts were found in apple, pear, orange and strawberry juices. The microbial reductions of *S.* Enteritidis and *E. coli* O157:H7 inoculated in the different fruit juices increased when treatment time was higher. However, this microbial reduction did not show a lineal tendency, but a logarithmic behavior (Figure 1); that is, a rapid microbial reduction at the beginning of the treatment (up to 500 μs) and then a gradual microbial reduction or long tailing when higher treatment times (up to 1250 and/or 2000 μs) were applied (Figure 1). These results are in accordance with Liu et al. (1997), Martín, Qin, Chang, Barbosa-Cánovas & Swanson (1997), Evrendilek et al. (1999) and Mosqueda-Melgar et al. (2007) who observed similar tendencies, using different *E. coli* strains, media, HIPEF equipment and treatment conditions.

Although, the occurrence of tailing in survival curves is not clearly understood, some assumptions have been proposed. One possible explanation may be that there are heterogeneous sensitivities to HIPEF treatment within the bacterial populations caused by different physiological states within the population (Chen, 2007); therefore, a part of the population might create a higher HIPEF-resistant than the rest of the population. van Boekel (2002) also attributes this behavior to biological variations or heterogeneity between cells of a population of the same microorganism. This author indicated that when microorganisms survive or die to a lethal event such as heat, high pressure, pulsed electric fields, etc., considering such events as probabilistic, it is unlikely biologically that all cells behave in the same way under one single event. Thus, a HIPEF-resistance distribution of microorganisms of a same population could be observed. On the other hand, Álvarez, Virto, Raso & Condón(2003) indicated that the occurrence of tailing could be due to the accumulation

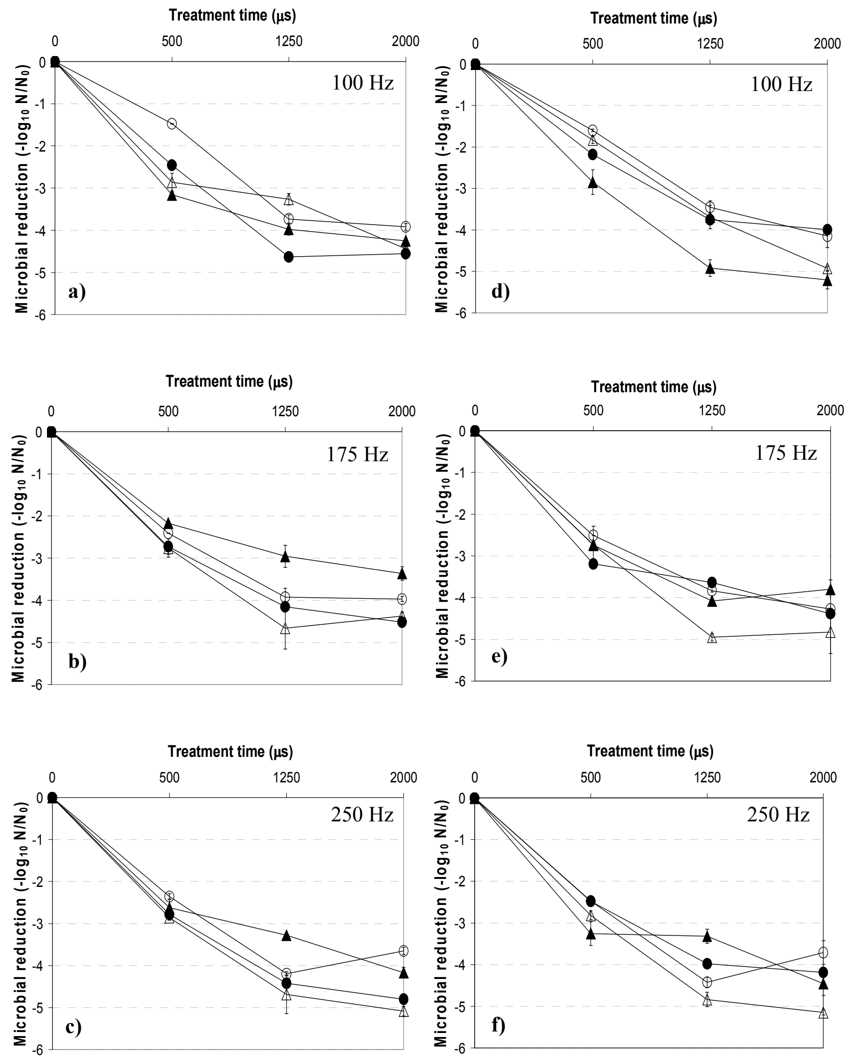

Figure 1.-*Effect of treatment time and pulse frequency on the microbial reductions of S. Enteritidis (a, b, c) and E. coli O157:H7 (d, e, f) inoculated in fruit juices treated by HIPEF. Symbols of apple (○), pear (●), orange (Δ) and strawberry (▲) juices are the mean of four determinations ± SD. Treatment conditions: 35 kV/cm and 4 µs pulse length in bipolar mode without exceeding 40º C.*

of dead cells around of the remaining survivors, which may serve as shield of such survivor cells. In our study, this tailing could be related to the circulation mode fluid handling system, which was continuous and in multiple passes; therefore, after each pass there was possibility that dead cells could circulate again and protect to the healthy or more HIPEF-resistant cells. In general, the occurrence of tailing in survival curves indicates that is not desirable to prolong the treatment time by much more time to increase the microbial inactivation by HIPEF, since, few reductions are achieved under this phenomenon.

On the other hand, a relation among treatment time, microbial reduction and energy density input was observed. An increase in the treatment time produced a higher microbial reduction and energy density input delivered (Table 3). Although, the relation between treatment time and energy density input is directly proportional, the existing relation between microbial reduction and energy density input is not lineal due to the occurrence of tailing as previously mentioned. In contrast, Korolczuck, McKeag, Fernandez, Baron, Grosset & Jeantet (2006) reported that the relation between the amount of energy density input received by the product and the decimal reduction number can be considered as lineal. These found differences could be due to circulation mode of the samples, because, these authors used the stepwise circulation mode and in this study the continuous circulation mode was employed.

The effect of the energy density input on the microbial reductions in fruit juices could be attributed to the increase in temperature of the medium, which may influence on the membrane fluidity properties. Since, at temperature below 30º C the phospholipids of the lipid bi-layer are closely packed into a rigid gel structure, whereas, at temperature upper 30º C the phospholipids of the lipid bi-layer are less ordered and the cellular membrane has a liquid-crystalline structure (Aronsson & Rönner, 2001), which cause greater sensibility to the cell electroporation by HIPEF treatment. Nonetheless, in this study the treatment temperature should not to be more than 40º C because cell damage by heat could be induced, and the effect of HIPEF treatment on the microbial inactivation would be caused by a combination of both treatments; since, E. coli O157:H7 populations are generally sensitive at temperatures above 46º C. Therefore, to observe the effect of only HIPEF treatment on the microbial inactivation in fluid foods, the temperatures applied during HIPEF processing should be ranged between 30 and 40º C for maximizing the microbial lethality by HIPEF without undesirable heat effects (Mosqueda-Melgar et al., 2007).

Table 3.-Influence of pulse frequency (*f*) and treatment time (T_t) on the number of passes (n_p), electric power (P) and total energy density input (*Q*) of HIPEF-processed fruit juices

Fruit juice	*f* (Hz)	T_t (µs)	n_P [1]	P (W)	*Q* (J/ml)
Apple	100	500	9.66	207.35	1335.3
		1250	24.15	207.35	3338.1
		2000	38.64	207.35	5341.0
	175	500	5.52	362.86	1335.3
		1250	13.80	362.86	3338.1
		2000	22.08	362.86	5341.0
	250	500	3.86	518.37	1335.3
		1250	9.66	518.37	3338.1
		2000	15.45	518.37	5341.0
Pear	100	500	9.66	284.39	1831.4
		1250	24.15	284.39	4578.4
		2000	38.64	284.39	7.325.5
	175	500	5.52	497.68	1831.4
		1250	13.80	497.68	4578.4
		2000	22.08	497.68	7.325.5
	250	500	3.86	710.98	1831.4
		1250	9.66	710.98	4578.4
		2000	15.45	710.98	7.325.5
Orange	100	500	11.81	513.62	3307.5
		1250	29.51	513.62	8268.8
		2000	47.22	513.62	13230.0
	175	500	6.75	898.83	3307.5
		1250	16.87	898.83	8268.8
		2000	26.99	898.83	13230.0
	250	500	4.72	1284.04	3307.5
		1250	11.81	1284.04	8268.8
		2000	18.89	1284.04	13230.0
Strawberry	100	500	8.59	487.93	3142.1
		1250	21.46	487.93	7855.3
		2000	34.34	487.93	12569.0
	175	500	4.91	853.84	3142.1
		1250	12.27	853.84	7855.3
		2000	19.63	853.84	12569.0
	250	500	3.43	1219.84	3142.1
		1250	8.59	1219.84	7855.3
		2000	13.74	1219.84	12569.0

[1] Number of times that juice sample has been processed by HIPEF (from *Equation 1*)

3.2.-Effect of pulse frequency on the microbial inactivation

Significant effect ($P \leq 0.05$) of pulse frequency on microbial counts was only observed in orange juice. In this case, significant differences ($P \leq 0.05$) in *S.* Enteritidis and *E. coli* O157:H7 counts among 100 and 250 Hz were found (Figure 1); being greater the microbial reduction when higher pulse frequency was applied. This effect was also reported by Mosqueda-Melgar et al. (2007), who found a greater reduction of *E. coli* and *L. monocytogenes* in melon and watermelon juices, respectively, when higher pulse frequencies were applied. Although in our study, an increase in both treatment time and pulse frequency resulted in a greater microbial inactivation in orange juice when HIPEF was applied, a higher influence of treatment time than pulse frequency was observed; since, lower *F*-ratio values of latter factor were found (Table 4).

Table 4.-Influence of treatment time, pulse frequency and their interactions on the reductions of *S.* Enteritidis and *E. coli* O157:H7 in fruit juices using HIPEF

Fruit juice	*F* – Ratio[1]									
	***S.* Enteritidis**					***E. coli* O157:H7**				
	t	*f*	*t·t*	*f·f*	*t·f*	*t*	*f*	*t·t*	*f·f*	*t·f*
Apple	741.86 [b]	3.69 [a]	165.14 [b]	2.97 [a]	1.76 [a]	456.61 [b]	3.92 [a]	85.16 [b]	1.98 [a]	1.16 [a]
Pear	1778.94 [b]	0.87 [a]	335.57 [b]	1.54 [a]	0.04 [a]	408.23 [b]	1.02 [a]	77.51 [b]	2.18 [a]	0.07 [a]
Orange	328.36 [b]	5.83 [b]	59.89 [b]	0.07 [a]	2.74 [a]	646.47 [b]	10.83 [b]	79.07 [b]	2.01 [a]	0.10 [a]
Strawberry	209.29 [b]	1.99 [a]	40.25 [b]	7.76 [b]	0.01 [a]	222.99 [b]	4.05 [a]	48.31 [b]	2.32 [a]	3.51 [a]

[1] = values are the variance explained by a factor (mean square of the factor) to the unexplained variance (mean square error); ***t***, treatment time; ***f***, pulse frequency; [b] significantly different (*P ≤ 0.05*); [a] not significant (*P > 0.05*).

Chang (1989) and Evrendilek & Zhang (2005) revealed that the sudden alternating changes of charged molecules on the cell membrane when pulses in bipolar mode are applied under a given electric field could produce a structural fatigue of the cell membrane, and thus, enhancing its electric breakdown by HIPEF treatment. In addition, an increase in pulse frequency, which plays an important role in the energy added to the medium, would implicate a higher electric power consumption of the HIPEF system, and consequently, a raise in the temperature of the medium is observed (Jeyamkondan et al., 1999; Wouter, Alvarez & Raso, 2001). The increase of the electric power when higher pulse frequencies are

applied is showed in the Table 3. Therefore, the generated heat during HIPEF processing could significantly influence the membrane fluidity properties.

On the other hand, populations of *S*. Enteritidis and *E. coli* O157:H7 inoculated in apple, pear and strawberry juices were not significantly affected by the different pulse frequencies applied under our experimental conditions (Figure 1). These results were similar to those found by Hulsheger, Pottel & Niemann (1981), who did not obtain significant effect of pulse frequency on the *E. coli* K12 reduction in buffer solution, when pulse frequency up to 5.0 Hz was applied. Likewise, Mosqueda-Melgar et al. (2007) did not report significant effects of pulse frequency against *S.* Enteritidis and *L. monocytogenes* in melon juice, and *E. coli* in watermelon juice in a range from 100 to 250 Hz.

Thereby, the effect of pulse frequency on the *S.* Enteritidis and *E. coli* O157:H7 counts inoculated in different fruit juices by HIPEF treatment is still unclear. Therefore, further studies are needed to understand the effect of this processing factor on these microorganisms in different media and experimental conditions.

3.3.-Optimization of HIPEF treatment

The needed minimum values of treatment time and pulse frequency, as processing factors of HIPEF treatment, for obtaining the highest *S.* Enteritidis and/or *E. coli* O157:H7 reductions on each fruit juice calculated from a single response analysis were similar to those found by multiple response analysis (Tables 5 and 6). The regression coefficients obtained (Table 5) from Equation (4) were depended on the treatment time and/or pulse frequency, being those related to treatment time (A and C) the most influential on the microbial inactivation in apple, pear, orange and strawberry juices.

Hence, based on the calculated maximal reductions of *S.* Enteritidis and *E. coli* O157:H7 by HIPEF treatment using a multiple response analysis (Table 6), the calculations on microbial reduction predicted that *E. coli* O157:H7 was more sensible to HIPEF than *S.* Enteritidis in orange and strawberry juices, but more resistant in apple and pear juices. However, when experimental validations of those calculations were made, the results indicated that *E. coli* O157:H7 was always more sensible to HIPEF treatment than *S.* Enteritidis (Table 6). Therefore, *S.* Enteritidis could be better considered as target microorganism than *E. coli* O157:H7 due to its higher HIPEF-resistance under acidic conditions. Thereby, based on the

Table 5.-Estimated values of regression coefficients and calculated maximum reduction of *S.* Enteritidis and *E. coli* O157:H7 in fruit juices obtained from optimized values of treatment time and pulse frequency using a single response analysis

Microorganism	Juice	*t*	*f*	R^2	*Regression coefficients* [a]						*Y*
					K	***A***	***B***	***C***	***D***	***E***	
S. Enteritidis	Apple	1592	172	98.14	-1.523	0.0055	0.0155	-1.62^{-6}	-3.50^{-5}	-1.54^{-6}	-4.34
	Pear	1576	250	99.19	0.500	0.0057	-0.0062	-1.74^{-6}	-1.90^{-5}	-1.68^{-7}	-4.87
	Orange	1676	250	95.81	-0.160	0.0050	0.0035	-1.67^{-6}	-9.08^{-6}	3.29^{-6}	-5.22
	Strawberry	1569	100	93.65	3.245	0.0047	-0.0369	-1.41^{-6}	9.98^{-5}	-1.98^{-7}	-4.43
E. coli O157:H7	Apple	1558	192	96.94	-1.665	0.0053	0.0171	-1.53^{-6}	-3.76^{-5}	-1.64^{-6}	-4.29
	Pear	1641	180	96.58	-1.101	0.0050	0.0153	-1.53^{-6}	-4.13^{-5}	-4.24^{-7}	-4.53
	Orange	1720	235	97.71	-1.636	0.0054	0.0172	-1.53^{-6}	-3.92^{-5}	4.88^{-7}	-5.16
	Strawberry	1869	100	94.19	1.692	0.0063	-0.0206	-1.73^{-6}	-6.12^{-5}	4.30^{-6}	-5.56

[a] estimated values by the model of Equation 1; ***t***, optimum treatment time (μs); ***f***, optimum pulse frequency (Hz); $\mathbf{R^2}$, determination coefficient (%); ***k***, constant of the Equation 1; ***A***, regression coefficient depending on *t* (μs^{-1}); ***B***, regression coefficient depending on *f* (Hz^{-1}); ***C***, regression coefficient depending on the interaction between *t* (μs^{-2}); ***D***, regression coefficient depending on the interaction between *f* (Hz^{-2}); ***E***, regression coefficient depending on the interaction between *t* and *f* ($\mu s^{-1} \cdot Hz^{-1}$); ***Y***, calculated bacterial inactivation after PEF treatment ($-\log_{10} N/N_0$).

Table 6.-Calculated and observed maximum microbial inactivation obtained after applying optimum values of treatment time and pulse frequency on HIPEF-processed fruit juices in a unique process using a multiple response analysis

Juices	**Optimized values**		**Microbial reductions ($-\log_{10}$ N/N$_0$)**		**Total energy density input (J/ml)**
	t (μs)	*f* (Hz)	***S.* Enteritidis**	***E. coli* O157:H7**	
Apple	1575	180	-4.34 [a] (-4.01 ± 0.12) [b]	-4.28 (-4.17 ± 0.15)	4206.0
Pear	1600	215	-4.77 (-4.31 ± 0.10)	-4.51 (-4.42 ± 0.23)	5860.4
Orange	1700	235	-5.15 (-5.01 ± 0.07)	-5.15 (-5.09 ± 0.10)	11246.0
Strawberry	1700	100	-4.43 (-4.56 ± 0.12)	-5.46 (-5.14 ± 0.12)	10683.0

t, treatment time; ***f***, pulse frequency; [a]Calculated maximum microbial inactivation; [b]Observed microbial inactivation, which are means of 3 determinations ± SD. HIPEF treatment conditions: 35 kV/cm and 4μs pulse duration in bipolar mode without exceeding 40º C outlet temperature.

microbial inactivation experimentally achieved (observed values), the higher HIPEF-tolerance of *S.* Enteritidis with regard to *E. coli* O157:H7 could be assumed as the cause of this different behavior, since both microorganisms share similar characteristics such as cell size and membrane composition (Gram-negative), which are important factors when HIPEF is applied (Mosqueda-Melgar et al., 2007).

The microbial sensibility of *E. coli* O157:H7 treated by HIPEF mainly depended on the medium acidity, being more sensible to HIPEF when it was inoculated in highest acidity juices (strawberry > orange > pear > apple); whereas, *S.* Enteritidis was more sensible in orange juice than strawberry juice when HIPEF was applied (orange > strawberry > pear > apple). Similar results were reported by García, Gómez, Raso & Pagán (2005), who obtained a higher reduction of *Salmonella* Seftenberg and *E. coli* by HIPEF in citrate-phosphate buffer of pH 4.0 than pH 7.0, but observed a lower HIPEF-resistance of *E. coli* at pH 4.0 than *S.* Seftenberg. Therefore, the ability of each microorganism for recovering after being submitted to HIPEF treatment could be the cause of the higher HIPEF-resistant of *S.* Enteritidis than *E. coli* O157:H7.

On the other hand, the microbial reductions of *S.* Enteritidis and *E. coli* O157:H7 populations in apple, pear, orange and strawberry juices could also be related to the total energy density input (Q) received by the juices; since, orange juice showed higher Q and microbial reductions followed by strawberry, pear and apple juices (Table 6). This fact could be attributed to the electric energy conversion into heat, which would produce an increase in the temperature of the medium and would enhance the efficiency of HIPEF treatment as previously mentioned.

In this study, only orange juice was pasteurized when HIPEF treatment was applied; since, both *S.* Enteritidis and *E. coli* O157:H7 were reduced more than 5.0 $\log_{10}$ units in the juice. Likewise, McDonald et al. (2000) also reached up to 5.0 $\log_{10}$ reductions of *E. coli* in orange juice, but using higher outlet temperatures (54º C) when 30 kV/cm was applied. The high temperatures used by those authors could contribute to the microbial death, since *E. coli* in general is sensible to heat above 46º C; in contrast, we applied temperature below 40º C ensuring that the inactivation of the microorganisms was achieved only by HIPEF.

In the same way, Liang et al. (2002) reported more than 5.0 $\log_{10}$ reductions of *Salmonella* Typhimurium in fresh squeezed orange juice (without pulp) under HIPEF treatment. Nevertheless, they used higher electric field intensity (90 kV/cm) and outlet temperature (55° C). A greater electric field strength produce a higher microbial inactivation due to

induced electric field through of the cell membrane, which may form pores and inactivates the microorganism by leakage of the cytoplasm content, depending on the electric fields intensity and/or the membrane organizational changes (Coster & Zimmermann, 1975; Tsong, 1990; Barbosa-Cánovas, Pothakamury, Góngora-Nieto & Swanson, 1999; Elez-Martínez & Martín-Belloso, 2005). Moderate electric fields intensity cause reversible pores in the cell membrane, whereas extreme electric field intensity leads to the irreversibility of this phenomenon, which results in cell death (Tsong, 1990; Ho & Mittal, 1996). In addition, the high temperatures used by Liang et al. (2002) could also contribute to a higher microbial reduction, since *Salmonella* spp. are generally sensible to heat above 50º C.

On the other hand, only *E. coli* O157:H7 population in strawberry juice was reduced by more than 5.0 $\log_{10}$ units when HIPEF was applied; whereas, *S.* Enteritidis population was inactivated up to 4.56 $\log_{10}$ units in the same juice. Likewise, we found reductions upper 4.0 $\log_{10}$ units of *S.* Enteritidis and *E. coli* O157:H7 in apple and pear juices when HIPEF was applied (Table 6). Evrendilek, Jin, Ruhlman, Qiu, Zhang & Ritcher (2000) achieved up to 4.5 $\log_{10}$ reductions of *E. coli* O157:H7 in apple juice (pH 3.70) when 34 kV/cm by 166 μs at 800 Hz and 4 μs pulse length in bipolar mode were used. In contrast, Evrendilek et al. (1999) and Gupta et al. (2003) reported reductions over 5.0 $\log_{10}$ units of *E. coli* O157:H7 and *E. coli* K12 in apple juice, respectively, when HIPEF was performed. In the same way, Iu et al. (2001) achieved 5.35 $\log_{10}$ and 5.91 $\log_{10}$ reductions of *E. coli* O157:H7 in apple cider by HIPEF treatment when used 80 kV/cm and 90 kV/cm during 60 μs and 20 μs, respectively, without exceeding 42º C.

Although, there are several studies on the *E. coli* inactivation by HIPEF in apple juice, reports about it in strawberry and pear juices have not been found in the literature. Likewise, studies on the *Salmonella* spp. inactivation by HIPEF in strawberry, apple and pear juices neither have been reported in the literature. Thus, this study contributes to the scientific knowledge over their inactivation by HIPEF in this kind of product and to the standardization of HIPEF treatment for inactivating these pathogenic microorganisms.

3.4.-Effect of HIPEF treatment combined with citric acid

The effect of citric acid added at different concentrations on each fruit juice inoculated with a cocktail of *S.* Enteritidis and *E. coli* O157:H7

without HIPEF application was evaluated. Little effects of citric acid on microbial reductions in all fruit juices were detected (Figures 2-5). In general, *S.* Enteritidis was slightly more sensible to citric acid than *E. coli* O157:H7 in all fruit juices studied. This fact could attributed to the higher acid-resistant developed of the former bacterium, due probably to the buffering capacity of its cytoplasm, low proton permeability, and the extrusion of protons from the cytoplasm by a membrane-bound proton pump (Benjamin & Data, 1995). Nonetheless, when HIPEF treatment (Table 6) was applied to the fruit juices inoculated with both microorganisms containing citric acid, additive and synergistic effects were observed, except for apple and pear juices, whose synergistic effects were not detected (Figures 2 and 3). Hence, synergistic effects from a combination of HIPEF with 1.0 and 1.5 % of citric acid in orange and strawberry juices, respectively, were observed to eliminate *S.* Enteritidis; whereas synergistic effects by combining HIPEF with that organic acid from 1.5 and 2.0 % in orange and strawberry juices, respectively, were found for inactivating *E. coli* O157:H7.

However, combinations of HIPEF treatment with 1.5 % of citric acid in apple and pear juices, and with 0.5 % of that same acid in strawberry juices were enough for inactivating *S.* Enteritidis and *E. coli* O157:H7 above 5.0 $\log_{10}$ units (Figures 2, 3 and 5); since in orange juice HIPEF treatment alone was able to eliminate both pathogenic microorganisms at pasteurization levels (Table 6), fulfilling thus, the request of the USFDA (U.S. Food and Drug Administration) for pasteurization of juice products (USFDA 2002).

The minimal concentrations of citric acid to inactivate *S.* Enteritidis and *E. coli* O157:H7 above 5.0 $\log_{10}$ units in all fruit juices treated by HIPEF could be related to the undissociated form of the acid molecule, which is assumed to possess the antimicrobial activity (Stratford & Eklund 2003). The citric acid molecules remain in undissociated form when the pH of the medium is below the pKa value (3.14) of the acid. In our case, those pH values were achieved from 1.5 % of citric acid in apple (3.13) and pear (2.88) juices, and from 0.5 % in orange (3.12) and strawberry juices (2.94) (Table 1).

Even though, citric acid is a weak-acid not lipophilic (-0.172 partition coefficient), that is, low lipid solubility, when HIPEF treatment is applied, the pore formation on the cell membrane of the microorganism take place (Coster & Zimmerman, 1975) and a flow of undissociated citric acid molecules towards the cell inside may occur. Once inside, the acid molecules are forced to dissociate into charged anions due to the near-neutral pH of the cell cytoplasm, and a gradual decrease in intracellular pH

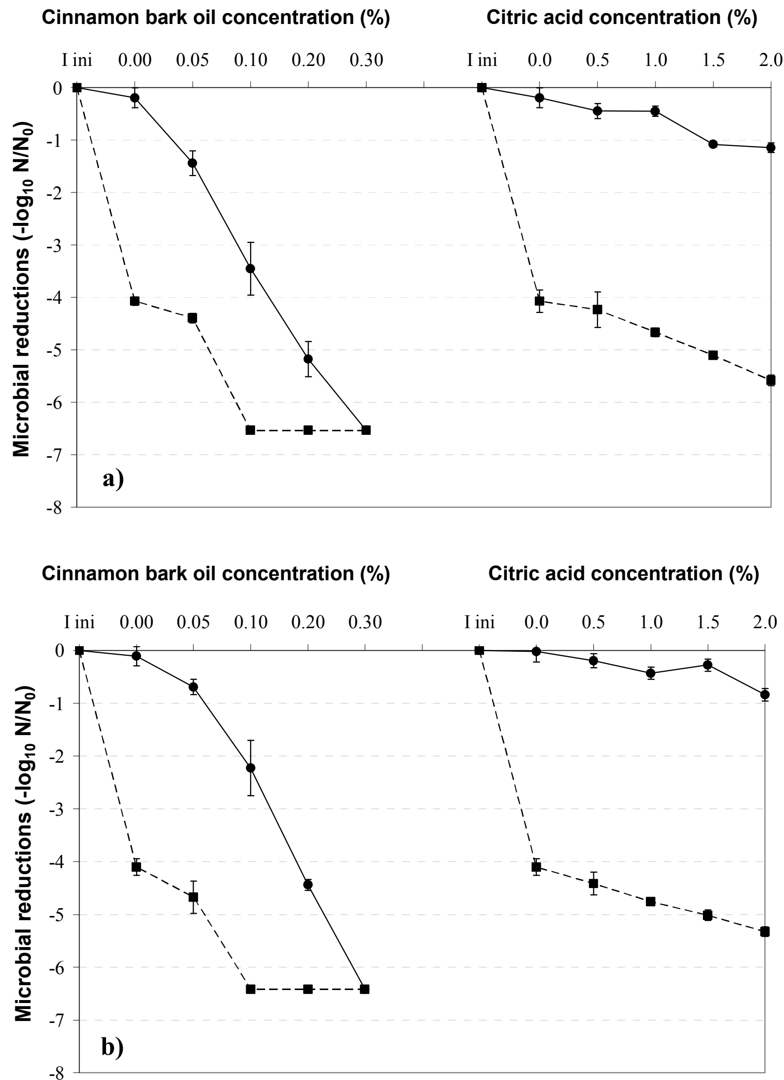

Figure 2.-*Microbial reductions of S. Enteritidis (a) and E. coli O157:H7 (b) in apple juice treated by antimicrobials (●) or by HIPEF and antimicrobials (-■-). Treatment conditions: 35 kV/cm and 4 µs pulse length in bipolar mode without exceeding 35º C. Symbols are means ± SD of two trials in duplicate (n = 4). I ini = Initial inoculums' level.*

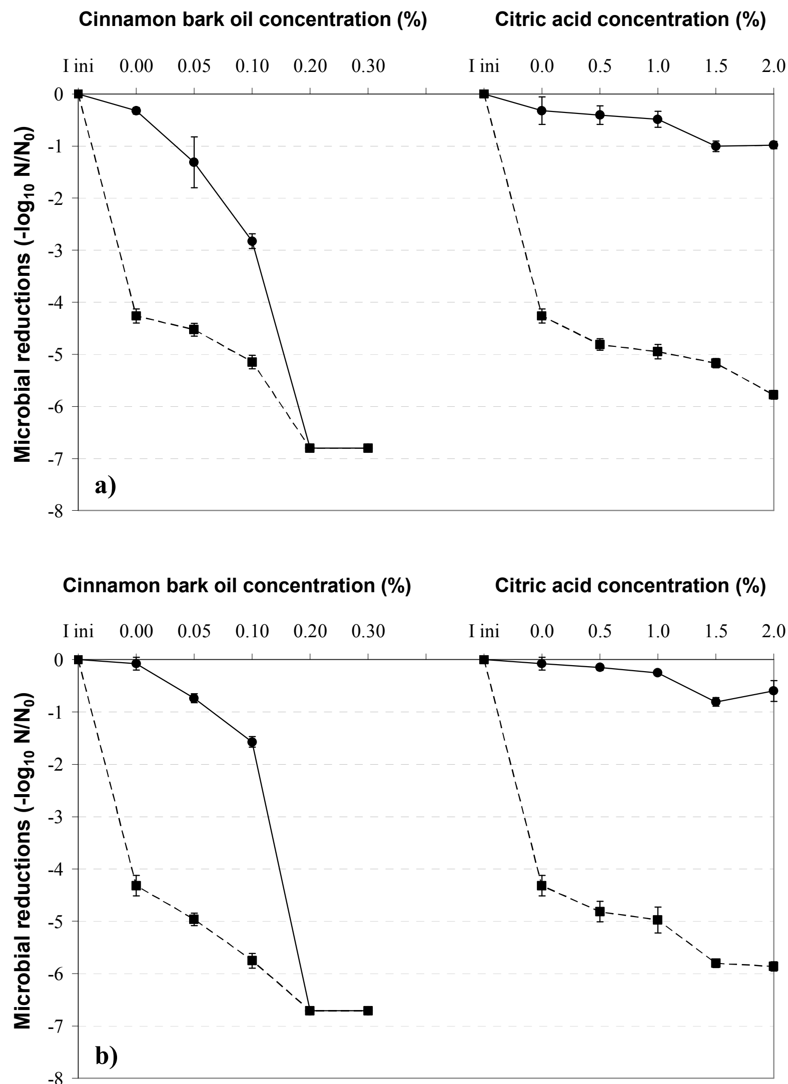

Figure 3.-*Microbial reductions of S. Enteritidis (a) and E. coli O157:H7 (b) in pear juice treated by antimicrobials (—●—) or by HIPEF and antimicrobials (- -■- -). Treatment conditions: 35 kV/cm and 4 µs pulse length in bipolar mode without exceeding 37º C. Symbols are means ± SD of two trials in duplicate (n = 4). I ini = Initial inoculums' level*

take place which may cause cell inactivation by damage in cell signaling, active transport and genetic material (Stratford & Eklund 2003).

Raybaudi-Massilia et al. (2006b) inactivated *S.* Enteritidis and *E. coli* O157:H7 by more than 5.0 $\log_{10}$ units in apple juice when HIPEF treatment (35 kV/cm for 1500 µs at 321 Hz and 4 µs pulse length in bipolar mode) was applied immediately or after one hour of exposure to 1.5 or 1.0 % of malic acid, respectively. The inactivation reached by those authors with a lower concentration of the organic acid than those used in the present study, could be attributed to the molecular size of the acid employed. Since, malic acid has smaller molecular weight (134.09 g/mol) than citric acid (192.14 g/mol), which may facilitate a higher penetration to cell inside and cause inactivation by decrease of internal pH of the microbial cell and denaturalization of genetic material (Eswaranadan, Hettiarachchy & Johnson, 2004). In addition, malic acid has a higher pKa value (3.40) than citric acid; thus, a minor concentration of organic acid is required to find it in undissociated form and to exert the antimicrobial activity. In the same way, Liu et al. (1997) inactivated *E. coli* O157:H7 up to 5.6 and 4.2 $\log_{10}$ units in 10 % glycerol of pH 3.4 when HIPEF (12.5 kV/cm, 5 pulses) was combined with 0.1 % of benzoic (pKa, 4.19) or sorbic acid (pKa, 4.76), respectively.

3.5.-Effect of HIPEF treatment combined with cinnamon bark oil

Significant effects ($P \leq 0.05$) on *S.* Enteritidis and *E. coli* O157:H7 populations inoculated in apple, pear, orange and strawberry juices were found when different concentrations of cinnamon bark oil were tested without HIPEF treatment (Figures 2-5). Populations of *S.* Enteritidis showed a higher susceptibility to the essential oil than *E. coli* O157:H7 in all fruit juice studied. That sensitivity was similar to the reported by Helander et al. (1998), who found a higher susceptibility of *Salmonella* Thyphimurium to thymol and carvacrol (essential oil compounds) than *E. coli* O157:H7. On the other hand, when HIPEF treatment was applied to the fruit juices (Table 6) containing cinnamon bark oil, a greater antimicrobial effect against *S*. Enteritidis and *E. coli* O157:H7 was observed. Additive effects between HIPEF and essential oil of cinnamon were detected from 0.1 % of that oil in apple, pear and orange juices, and from 0.05 % in strawberry juice for *S.* Enteritidis and from 0.05 % of cinnamon bark oil in all fruit juices for *E. coli* O157:H7. In contrast, synergistic effects by combining these treatments were not found in this work.

Nonetheless, to inactivate *S.* Enteritidis and *E. coli* O157:H7 populations by more than 5.0 $\log_{10}$ units in fruit juices, as proposed by the USFDA (2002), combinations of HIPEF treatment with 0.1 % of cinnamon bark oil in apple and pear juices, and with 0.05 % of that oil in strawberry juices were needed (Figures 2, 3 and 5); since in orange juice HIPEF treatment alone was able to reduce these pathogenic microorganisms at pasteurization levels (Table 6).

The greater effectiveness of the cinnamon bark oil on *S.* Enteritidis and *E. coli* O157:H7 in fruit juices was associated to the media pH, being more effective in media with lower pH. Burt (2004) reported that the bacterial susceptibility to the antimicrobial effect of essential oils appears to increase with a decrease in the pH of the food, because at low pH the hydrophobicity of an essential oil increases, enabling it to more easily dissolve in the lipids of the cell membrane of target bacteria.

Although, the mechanism of action of cinnamon oil on the microbial cells is still unclear, Wendakoon & Sakaguchi (1995) and Burt (2004) have indicated that the interaction of carbonyl group of the cinnamaldehyde, main compound of cinnamon oil from bark, on the cell proteins embedded in the cytoplasmatic membrane appear to inhibit the action of the enzymes amino acid decarboxylases, which are necessary for the amino acids biosynthesis and biodegradation. On the other hand, Oussalah et al. (2006) reported a release of the cell constituents, a decrease of intracellular ATP concentration and a decrease in intracellular pH due to an increase in the permeability of cell membrane when cinnamon oil was applied up to 0.1 %. Nonetheless, these authors did not observe an apparent change on the cell surface (by electron micrographs) when cinnamon oil was added. In the same way, Gill & Holley (2004, 2006) reported a rapid decrease of cellular ATP but no increase of extracellular ATP when 0.15 or 0.6 % of cinnamaldehyde was used. Thereby, the mechanism of action of the cinnamon oil and its main compound to inactivate microorganisms according to Gill & Holley (2004, 2006) and Oussalah et al. (2006) appear to be related to the cell membrane from which a slight disruption seem to occur, causing dispersion of the proton motive force by leakage of small ions without leakage of larger cell components, such as ATP, which are subsequently degraded by the ATPase enzyme.

Hence, the antimicrobial effect of HIPEF combined with cinnamon bark oil is enhanced due to the formation of pores on the cell membrane when HIPEF is applied, which may favor more easily the diffusion of the oil to cell inside and cause damage in the cell functions.

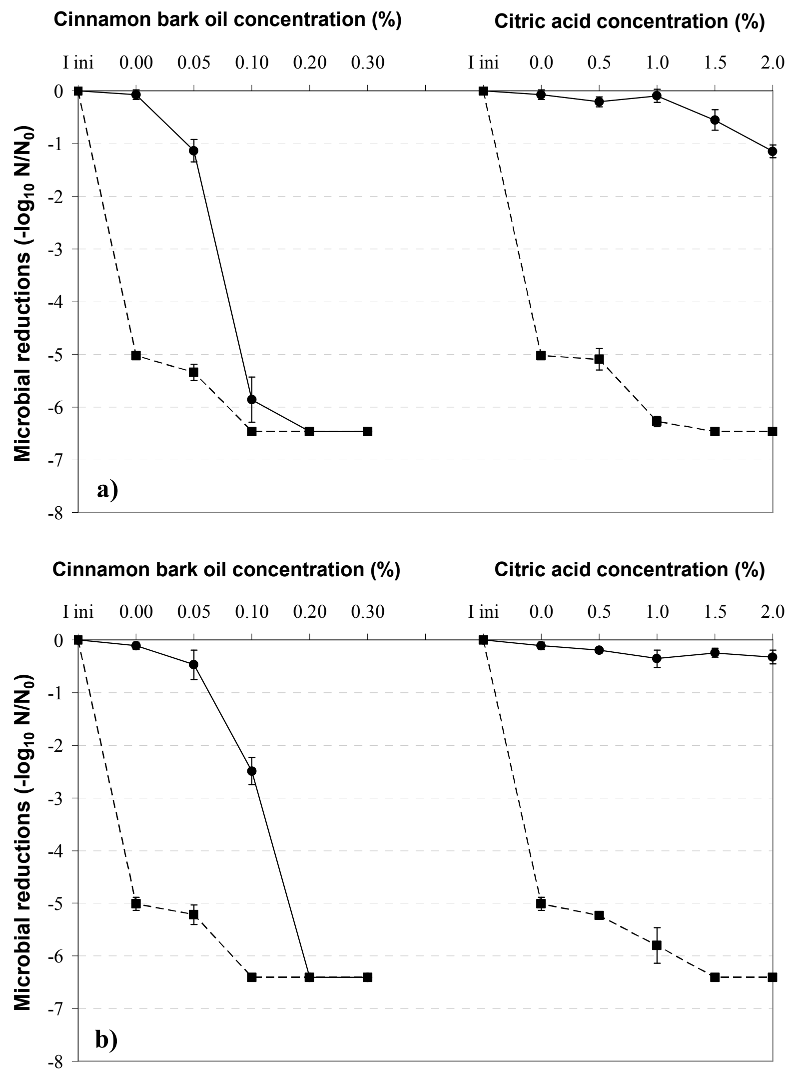

Figure 4.-*Microbial reductions of S. Enteritidis (a) and E. coli O157:H7 (b) in orange juice treated by antimicrobials (—●—) or by HIPEF and antimicrobials (- ■ -). Treatment conditions: 35 kV/cm and 4 µs pulse length in bipolar mode without exceeding 40º C. Symbols are means ± SD of two trials in duplicate (n = 4). I ini = Initial inoculums' level*

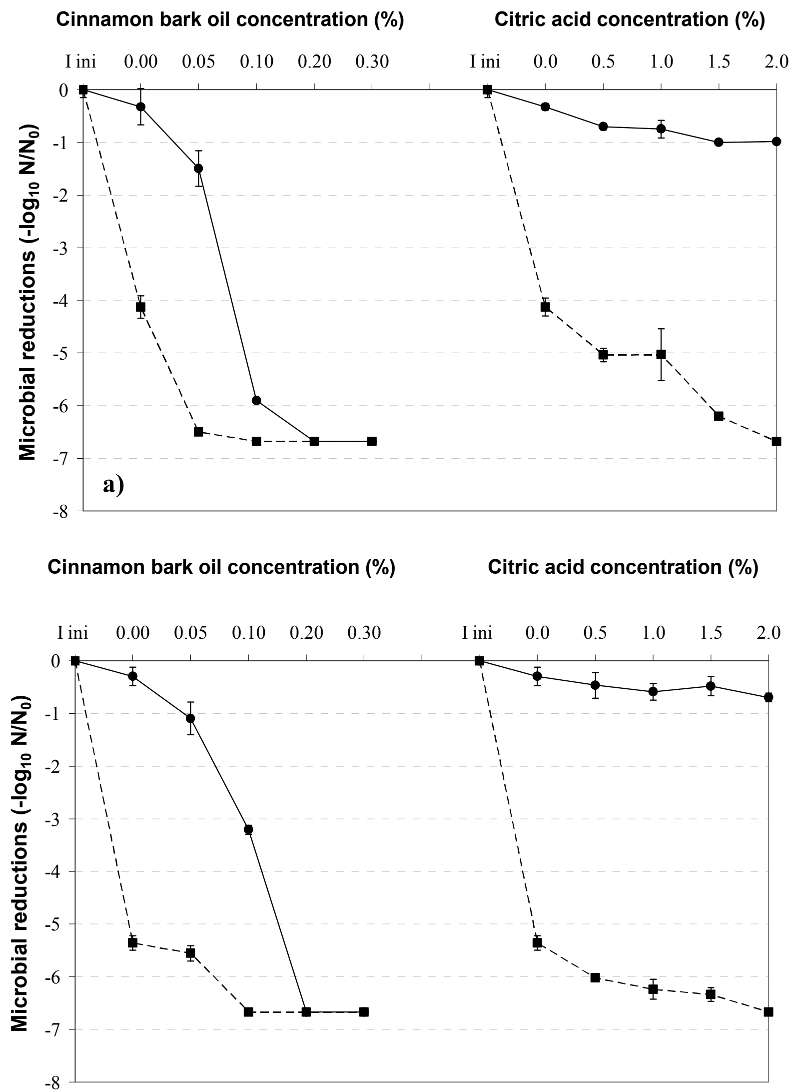

Figure 5.-Microbial reductions of S. Enteritidis (a) and E. coli O157:H7 (b) in strawberry juice treated by antimicrobials (●) or by HIPEF and antimicrobials (■). Treatment conditions: 35 kV/cm and 4 µs pulse length in bipolar mode without exceeding 37º C. Symbols are means ± SD of two trials in duplicate (n = 4). I ini = Initial inoculums' level

Application of HIPEF treatment in combination with essential oils to inactivate pathogenic microorganisms in fruit juices have not been found in the literature. Therefore, results given in this study contribute to the scientific knowledge about the effect of combined treatments of HIPEF with essential oils to ensure the microbiological safety and reduce the risk of food-borne illness caused by juices consumption.

4.-CONCLUSION

The results of this study clearly showed that *S.* Enteritidis and *E. coli* O157:H7 reductions in each fruit juice were more influenced by treatment time than by pulse frequency when HIPEF treatment was applied. Total energy density input was related to the microbial inactivation, but a lineal relation was not observed due to the occurrence of tailing in survival curves. Optimization of processing factors of HIPEF through a multiple response analysis resulted to be a good tool to search the minimal values of treatment time and pulse frequency needed to reduce the maximal levels of both pathogenic microorganisms in each fruit juice by HIPEF applying.

Populations of *S.* Enteritidis were more HIPEF-resistant than populations *E. coli* O157:H7 in all fruit juices studied under the same experimental conditions. In general, the sensibility of each pathogenic microorganism to HIPEF treatment was associated to the pH medium, being more sensible a lowest pH values.

On the other hand, *S.* Enteritidis was more susceptible to the citric acid and cinnamon bark oil than *E. coli* O157:H7. However, when HIPEF was combined with citric acid a higher inactivation of *E. coli* O157:H7 in all fruit juices used than *S.* Enteritidis was observed. In contrast, a lower inactivation of *E. coli* O157:H7 than *S.* Enteritidis was observed when HIPEF was combined with cinnamon bark oil.

5.-ACKNOWLEDGEMENTS

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Capítulo IV

Combination of high-intensity pulsed electric fields with natural antimicrobials to inactivate pathogenic microorganisms and extend the shelf-life of melon and watermelon juices

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ABSTRACT

The effect of high-intensity pulsed electric field (HIPEF) combined with citric acid (0.5- 2.0%) or cinnamon bark oil (0.05-0.30%) against populations of *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* in melon and watermelon juices were evaluated. Microbiological shelf-life and sensory attributes were also determined. Populations of *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* were reduced by more than 5.0 log_{10} CFU/ml in HIPEF-processed melon (35kV/cm for 1709µs at 193Hz and 4µs pulse duration) and watermelon (35kV/cm for 1682µs at 193Hz and 4µs pulse duration) juices containing 2.0 and 1.5% of citric acid, respectively, or 0.2% of cinnamon bark oil. In addition, these treatments were also able to inactivate mesophilic, psychrophilic and, molds and yeasts populations, leading to a shelf-life of more than 91 days in both juices stored at 5ºC. Hence, the microbiological quality and safety of these fruit juices by combining HIPEF and citric acid or cinnamon bark oil were ensured. However, the taste and odor in those HIPEF-treated melon and watermelon juices containing antimicrobials were significantly affected. Therefore, further studies are needed to decrease the impact on the sensory attributes by using antimicrobials.

***Keywords*:** HIPEF; *E. coli* O157:H7; *S.* Enteritidis; *L. monocytogenes*; melon; watermelon; citric acid; cinnamon bark oil; shelf-life

1.-INTRODUCTION

Consumption of just made melon and watermelon juices can provide potential health benefits due to the antioxidant and regulatory capacities of their naturally occurring pigments, vitamins and minerals (Edwards et al., 2003). However, these products without a minimal processing may be potential source of microbiological diseases, since the low acidity (pH 5.2 to 6.7) and high water activity (0.97 to 0.99) of these fruits can favor the growth of pathogenic microorganisms (USFDA, 2001). In addition, because melon and watermelon fruits are grown on the ground, it is difficult to prevent microbial contamination on the rind of the fruit. Hence, if melon and watermelon fruits with a contaminated rind are cut, pathogenic microorganisms such as *Salmonella* spp., *E. coli* O157:H7 and *L. monocytogenes* can be transferred to the edible part and juice causing diseases (Ukuku and Sapers, 2001; Sharma et al., 2005). Incidence, survival and growth of *Salmonella* spp., *E. coli* O157:H7 and *L. monocytogenes* on watermelon and melon slices and juices have been demonstrated by several researchers (Fernandez-Escartín et al., 1989, Golden et al., 1993, Del Rosario and Beuchat, 1995, Penteado and Leitao, 2004). On the other hand, outbreaks of *Salmonella* spp. and *E. coli* O157:H7 by consumption of fresh-cut melon and watermelon as well as their juices have also been reported (Mohle-Boetani et al., 1999, Powell and Luedtke, 2000, USFDA, 2001; CDC, 2007).

Although, thermal treatment effectively destroys pathogenic microorganisms in fruit juices, undesirable changes on the organoleptic and nutritional properties of juice are observed (Espach-Barroso et al., 2003; Min et al., 2003a; Elez-Martínez et al., 2006). For that reason, significant efforts are leading to the development of novel non-thermal processes such as high-intensity pulsed electric fields (HIPEF), an alternative preservation process that is proving to be able to inactivate spoilage and pathogenic microorganisms without significantly affect the organoleptic and nutritional properties of several foods (Hodgins et al., 2002; Min et al., 2003a; 2003b; Cserhalmi et al., 2006; Elez-Martínez et al., 2006). This technology involves the application of short pulses (1 to 10 μs) of high-intensity electric field (typically 20 to 80 kV/cm) to fluid foods placed between two electrodes in batch or continuous flow systems using low processing temperatures (<50º C) and low energy consumption with regard to the thermal treatment. The effectiveness of HIPEF treatment to reduce *Salmonella* spp., *E. coli* and *L. monocytogenes* populations in some fruit juices has been reported (Evrendilek et al., 2000; Iu et al., 2001, Liang et

al., 2002; Mosqueda-Melgar et al., 2007). The microbiological shelf-life extension of some HIPEF-treated acid juices such as orange, apple, cranberry and tomato in comparison with unprocessed juices (control) has also been demonstrated (Jin and Zhang, 1999; Evrendilek et al., 2000; Hodgins et al., 2002; Min et al., 2003a, 2003b; Elez-Martínez et al., 2006). However, the effect of HIPEF treatment on the naturally occurring microorganisms in non-acid juices such as those from melon and watermelon has not been found in the literature.

On the other hand, the use of HIPEF in combination with natural antimicrobials such as bacteriocins, enzymes, essential oils, spices and organic acids appears to enhance the killing effect on microorganisms in fruit juices (Iu et al., 2001; Liang et al., 2002; Liang et al., 2006; Raybaudi-Massilia et al., 2006a; Nguyen and Mittal, 2007). However, the combined effect of HIPEF treatment with citric acid or cinnamon bark oil against pathogenic and spoilage microorganisms in fruit juices have not been still studied. Citric acid and cinnamon bark oil are GRAS (Generally Recognized As Safe) substances permitted by the Food Additive Status List (USFDA 2006) and European Parliament and Council Directive Nr. 95/2/EC (1995). Citric acid is an organic acid naturally present in several fruits, including melon and watermelon fruits, although in very low concentrations (Lamikanra et al., 2000; Gil et al., 2006). This organic acid is used as flavoring (pleasant sour taste) and preservative in foods and beverages (Doores, 1993); moreover, it possesses antimicrobial properties attributed to chelating metal ions, which are essentials for microbial growth, and/or reduction of pH in the medium (Sharma, 2000; Stratford and Elkund, 2003; Nazer et al., 2005). Likewise, the essential oil from cinnamon bark is used as flavoring agent (pleasant sweet taste) in foods and beverages (Wright, 1999), and due to its content of antimicrobial compounds, among them cynnamaldehyde and eugenol, it is a potential natural agent for food preservation (Friedman et al., 2004; Burt, 2004; Gill and Holley, 2004; Raybaudi-Massilia, et al., 2006b; Gill and Holley, 2006; Oussalah, 2006; 2007).

The objectives of this study were to evaluate the effect of HIPEF treatment combined with different concentrations of citric acid or cinnamon bark oil against populations of *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* inoculated in melon and watermelon juices, as well as to evaluate the microbiological shelf-life of these juices under the most adequate processing conditions, having the conventional thermal treatment and the untreated juices as references. In addition, sensory evaluations of treated and untreated melon and watermelon juices were also conducted.

2.-MATERIALS AND METHODS

2.1.-Inactivation of pathogenic microorganisms

2.1.1.-Fruit juices preparation

Melon (*Cucumis melo* var. "Piel de sapo") and watermelon (*Citrullus lanatus* var. "Seedless") fruits at commercial ripeness were chosen from a supermarket of Lleida, Spain. The fruits were washed, dried, cut into slices and made juice through an Ufesa blender (Model BP 4512, Vitoria, Spain). Melon and watermelon juices were centrifuged at 12,500 rpm for 15 min at 4ºC in an Avanti™ J-25 Centrifuge (Beckman Instrument, Inc., USA). The supernatant juice was filtered, bottled and autoclaved in a Presoclave 75 (J.P. Selecta, S.A., Barcelona, Spain) at 121ºC for 15 min for obtaining free-microorganisms juice; afterwards, the samples of juice were cooled to room temperature (22ºC) for inoculating and subsequent processing by HIPEF. Analytical parameters such as electric conductivity (Testo 240 conductivimeter; Testo GmBh & Co; Lenzkirch, Germany), pH (Crison 2001 pH-meter; Crison Instruments S.A; Barcelona, Spain) and soluble solid content (Atago RX-1000 refractometer; Atago Company Ltd., Japan) were measured (Table 1) according to the Spanish Regulation (BOE., 1988).

Table 1.-Analytical characteristics of melon and watermelon juices used for inactivation studies containing different concentrations of citric acid and cinnamon bark oil

Fruit juice	Concentration (%)		Parameters [1]					
			pH		Electrical conductivity [c]		Soluble solid (%)	
	CA [a]	CBO [b]	CA	CBO	CA	CBO	CA	CBO
Melon	0.0		6.11 ± 0.03		6.46 ± 0.03		11.5 ± 0.1	
	0.5	0.05	.16 ± 0.02	6.00 ± 0.02	6.47 ± 0.01	6.46 ± 0.01	11.5 ± 0.2	11.5 ± 0.2
	1.0	0.10	.63 ± 0.01	5.98 ± 0.01	6.49 ± 0.01	6.46 ± 0.03	11.6 ± 0.1	11.5 ± 0.1
	1.5	0.20	.32 ± 0.02	5.92 ± 0.01	6.53 ± 0.01	6.46 ± 0.02	11.8 ± 0.1	11.5 ± 0.1
	2.0	0.30	.12 ± 0.02	5.83 ± 0.02	6.61 ± 0.02	6.46 ± 0.01	12.1 ± 0.1	11.5 ± 0.1
Watermelon	0.0		5.73 ± 0.03		4.11 ± 0.01		13.8 ± 0.1	
	0.5	0.05	.79 ± 0.02	5.72 ± 0.02	4.15 ± 0.01	4.04 ± 0.01	14.3 ± 0.1	13.8 ± 0.1
	1.0	0.10	.37 ± 0.01	5.73 ± 0.01	4.33 ± 0.01	4.08 ± 0.02	14.5 ± 0.1	13.9 ± 0.2
	1.5	0.20	.09 ± 0.03	5.71 ± 0.01	4.46 ± 0.01	4.10 ± 0.02	14.9 ± 0.1	14.0 ± 0.1
	2.0	0.30	.99 ± 0.01	5.72 ± 0.02	4.56 ± 0.02	4.17 ± 0.02	15.2 ± 0.1	14.0 ± 0.1

[1] Results are the mean of three measurements ± SD; [a] Citric acid (CA); [b] Cinnamon bark oil (CBO); [c] Electrical conductivity measured at 22º C (mS/cm).

2.1.2-Microbial culture preparation and inoculation onto fruit juices

Pure cultures of *Escherichia coli* O157:H7 (Colección Española de Cultivos Tipo (CECT) 4267; University of Valencia, Valencia, Spain), *Salmonella enterica* ser. Enteritidis 1.82 (National Collection of Type Culture (NCTC) 9001, PHLS Central Public Health Laboratory; London, UK) and *Listeria monocytogenes* 1.131 (CECT 932) were used for this study. Strains of *E. coli* O157:H7 and *L. monocytogenes* were grown in tryptone soy broth (TSB) (Biokar Diagnostics; Beauvais, France) plus 0.6% of yeast extract (Biokar Diagnostics); whereas, strain of S. Enteritidis was cultured in TSB. *E. coli* O157:H7 and *S.* Enteritidis were incubated at 37ºC with continuous agitation at 120rpm for 15 hours, while *L. monocytogenes* was incubated at 35ºC with continuous shaking at 200rpm for 15 hours to obtain cells in stationary growth phase. The final concentration achieved of microorganisms in the growth media was approximately 10^9-10^{10} colony forming units/milliliter (CFU/ml).

Each sample of melon and watermelon juices (100 ml) to be treated with or without HIPEF and antimicrobials was inoculated with 1 ml of each pathogenic microorganism to obtain thus, a final concentration of 10^7-10^8 CFU/ml approx. in each sample juice.

2.1.3.-HIPEF equipment and processing parameters

A continuous flow HIPEF system, model bench-scale OSU-4F (Ohio State University, Ohio, USA) which discharges square-wave pulses was used to process samples of melon and watermelon juices. The HIPEF system is composed of eight collinear field chambers connected in series and each one with two stainless steel electrodes separated by a distance of 0.292cm. Each chamber has a diameter of 0.23cm and a volume of 0.0121cm^3 with a cross section of 0.0415cm^2. The pulse waveform was monitored using a Tekscope THS 720 oscilloscope (Tektronix Inc., Oregon, USA) connected to the HIPEF apparatus. Pulse frequency and duration were controlled through of a Pulse Generator model 9410 (Quantum Composers, Inc., Bozeman MT, USA). The flow rate of the process was adjusted by a variable gear pump model 75210-25 (Cole Palmer Instrument Company; Illinois, USA). The circulating fruit juice during the HIPEF treatment was cooled through the heat exchangers connected to the chambers, which were immersed in an iced water bath (5ºC) (J.P. Selecta, S.A; Barcelona, Spain).

Samples of fruit juices were pumped across the HIPEF system at flow speed adjusted to 90-100 ml/min. Electric field intensity of 35kV/cm and pulse length of 4μs in bipolar mode during 1709μs treatment time and 193Hz pulse frequency (for melon juice), and 1682μs and 200Hz (for watermelon juice) were selected according to previous studies (Mosqueda-Melgar et al., 2007), as optimum processing parameters of HIPEF. The temperature of fruit juices during the HIPEF process did not exceed 40ºC.

2.1.4.-HIPEF treatment and antimicrobials

Prior to HIPEF processing, the effect of each antimicrobial such as citric acid (Scharlau Chemie, S.A., Barcelona, Spain) or cinnamon bark oil (Aceites Esenciales Dicana, Barcelona, Spain) added at different concentrations in fruit juices on *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* populations was evaluated. Citric acid at 0.0, 0.5, 1.0, 1.5 and 2.0% (wt/vol) and cinnamon bark oil at 0.00, 0.05, 0.10, 0.20, and 0.30% (vol/vol) were added into each sample of melon or watermelon juice. Samples of fruit juices with or without antimicrobial were inoculated with a cocktail of those pathogenic microorganisms and exposed during one hour at room temperature (22ºC) with continuous shaking through a magnetic stirrer to evaluate their effects on the microorganisms (Raybaudi-Massilia et al., 2006a). Once passed that time, HIPEF was applied, and the combined effect of each antimicrobial with HIPEF was evaluated.

2.1.5.-Enumeration of viable cells of pathogenic microorganisms

The injured and non-injured cells of *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* from melon and watermelon juices treated by HIPEF with or without natural antimicrobials were recovered in buffered peptone water (Biokar Diagnostic) during 20 min at 35ºC, and then serially diluted in saline peptone water (0.1% casein-meat peptone plus 0.85% Sodium chloride supplied by Biokar Diagnostic and Scharlau Chemie, S.A., respectively). Afterwards, the cells were spread plated by duplicate in selective media of MacConkey-Sorbitol agar (Biokar Diagnostic), Hektoen agar (Biokar Diagnostic) and Palcam agar (Biokar Diagnostic) for *E. coli* O157:H7, S. Enteritidis and *L. monocytogenes* counts, respectively. Plates were then incubated at 35ºC for 24-48 hours and counts were expressed as log_{10} CFU/ml. The recovery time (20 min) was selected according to generation time of each microorganism from growth curves previously made in the laboratory (data not shown).

2.2.-Microbiological shelf-life

2.2.1.-Fruit juices preparation

Melon and watermelon fruits were immersed in an aqueous solution of chlorine (200 µl/l, pH 6.8) for 5 min, then washed, dried, cut into pieces, and mixed through an Ufesa blender (Model BP 4512, Vitoria, Spain) to obtain the juice. The fruit juices were then filtered across a metal strainer and subsequently with a cloth filter to separate the larger particles of the juice, which may affect the HIPEF process. Afterwards, these fresh juices were degassed through a diaphragm vacuum pump (Vaccubrand, Wertheim, Germany) during 10 min to remove air burbles that may also affect the HIPEF process. From the juices, different portions were submitted to a) HIPEF, b) HIPEF and citric acid, c) HIPEF and cinnamon bark oil and d) thermal treatment. An unprocessed portion was also kept for being used as a reference. In addition, electrical conductivity, pH and solid soluble content of all the melon and watermelon juices were measured (Table 2).

Table 2.-Analytical characteristics of melon and watermelon juices used for microbiological shelf-life studies under different processing conditions

Juice	Process (conditions)	Values [a]		
		pH	Electrical conductivity [b] (mS/cm)	Soluble solids content (%)
Melon	Control	6.12 ± 0.02	6.13 ± 0.06	11.7 ± 0.1
	HIPEF (1709µs-193Hz)	6.09 ± 0.03	6.02 ± 0.03	11.2 ± 0.1
	HIPEF + CA (2.0%)	3.11 ± 0.02	6.38 ± 0.05	12.6 ± 0.1
	HIPEF + CBO (0.2%)	6.12 ± 0.01	6.12 ± 0.04	11.3 ± 0.1
	Thermal (90ºC x 1min)	6.07 ± 0.02	6.09 ± 0.01	11.2 ± 0.0
Watermelon	Control	5.76 ± 0.01	3.53 ± 0.03	10.6 ± 0.1
	HIPEF (1682µs-200Hz)	5.77 ± 0.01	3.54 ± 0.02	10.7 ± 0.0
	HIPEF + CA (1.5%)	3.05 ± 0.02	3.71 ± 0.03	11.8 ± 0.1
	HIPEF + CBO (0.2%)	5.78 ± 0.03	3.60 ± 0.02	10.7 ± 0.0
	Thermal (90ºC x 1min)	5.76 ± 0.01	3.61 ± 0.05	10.8 ± 0.1

[a] Values are the mean of three measurements ± SD; [b] Electrical conductivity measured at 22º C (mS/cm). HIPEF treatment conditions: 35 kV/cm and 4 µs pulse duration in bipolar mode without exceeding 40º C.

2.2.2.-HIPEF and thermal processing

OSU-4F HIPEF system was also used to treat samples of non-inoculated juice. The combination of HIPEF with the minimum concentration of citric acid and cinnamon bark oil able to inactivate populations of *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* up to 5.0 $\log_{10}$ cycles in each fruit juice was used to study the microbiological shelf-life. Melon and watermelon juices were exposed during one hour to the antimicrobials with continuous agitation at room temperature (22ºC) prior to HIPEF processing. Once passed that time, the samples of fruit juices were pumped and processed across the HIPEF system at a flow speed adjusted to 90-100 ml/min.

On the other hand, melon and watermelon juices were thermally processed at 90ºC during 1 min through a tubular stainless steel heat exchanger system immersed in a hot water bath with continuous shaking (Universitat de Lleida, Lleida, Spain). Those juices were pumped through the system with a peristaltic pump D-21V model (Dinko, Barcelona, Spain) and adjusted to a flow rate of 40 ml/min to ensure the complete thermal treatment. After thermal processing, the juice was immediately cooled in an ice water bath (Universitat de Lleida, Lleida, Spain). The heating temperature and exposure time employed in this study were found to be adequate to inactivate spoilage microorganisms (Shearer et al., 2002) and are in excess of those needed to inactivate pathogenic microorganisms such as *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* (Mazzotti, 2001; Sharma et al., 2005).

2.2.3.-Samples packaging and storage

Unprocessed and processed melon and watermelon juices were filled (leaving the minimum amount of headspace volume) and packaged into 30 ml sterile containers with polypropylene screw-cap (Deltalab, Barcelona, Spain) under a horizontal laminar air flow cabin (Telstar, S.A, Barcelona, Spain) in aseptic conditions. Finally, packaged fruit juices were stored at refrigeration temperature (5ºC) in darkness up to analysis.

2.2.4.-Microbiological Analysis

Enumeration of mesophilic microorganisms on plate count agar (PCA) according to ISO 4833:1991 (1991), molds and yeasts on chloramphenicol glucose agar (CGA) in accordance with ISO 7954:1987 (1987) and

psychrophilic microorganisms on PCA were carried out in the unprocessed and processed melon and watermelon juices. All culture media were purchased from Biokar Diagnostic. The microbiological analyses were performed each 7 days in duplicate during 91 days. Plate counts were expressed as $\log_{10}$ (CFU/ml).

2.2.5.-Sensory evaluation

Untreated melon and watermelon juices as well as juices processed by HIPEF, HIPEF and citric acid, HIPEF and cinnamon bark oil and thermally treated were given to panelists immediately after processing for sensory evaluation. The procedure carried out for this evaluation was similar to that described by Min et al. (2003a; 2003b). A total of 30 non-trainer panelists belonging to the Department of Food Technology at the University of Lleida (Spain) participated in the sensory tests. Fifteen milliliters of each sample were served into 20 ml ultra clear polypropylene containers with polyethylene screw-cap (Deltalab) coded with three digits randomly numbered; moreover, a glass containing potable water and a piece of non-salted cracker were provided to panelists for eliminating the residual taste between samples. The panelists were asked to rate the preference of odor, color, taste, sourness and overall acceptability in a hedonic scale from 0 (dislike extremely) to 10 (like extremely).

2.3.-Experimental design and Statistical Analysis

A multilevel factorial design was carried out in fruit juice samples inoculated with a cocktail of *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* populations treated or not with HIPEF and/or different concentrations of antimicrobials (citric acid at 0, 0.5, 1.0, 1.5 and 2.5% or cinnamon bark oil at 0, 0.05, 0.10, 0.20 and 0.30%). Analysis of variance (ANOVA) and multiple range tests (MRT) using a Fisher's least significant difference (LSD) procedure were used to determine significant differences ($P \leq 0.05$) among pathogenic microorganisms counts inoculated in melon and watermelon juices containing different concentrations of antimicrobials treated or not with HIPEF. ANOVA and MRT were also used to determine meaningful differences ($P \leq 0.05$) among odor, color, taste, sourness and overall attributes from those fruit juices treated by different processing conditions. The experiments were performed twice, and microbial count was made in duplicate (n = 4). Sensory evaluation was carried out once and sensory attributes were tested thirty times (n = 30). Means and standard

deviations were calculated for each treatment. All the statistical analyses were conducted with Statgraphics plus Centurion XV software Version 15.1.02.

2.3.1.-Microbial growth and shelf-life modeling

The microbial growth of naturally occurring microorganisms in melon and watermelon juices unprocessed and processed by HIPEF with or without antimicrobials, and thermally treated, was estimated through the Gompertz's equation modified by Zwietering et al. (1990) as follow (*Equation 1*):

$$Log_{10}(CFU/ml) = k + A \cdot exp\left\{-exp\left\{\left[(\mu_{max} \cdot e) \cdot \left(\frac{\lambda - t}{A}\right)\right] + 1\right\}\right\} \quad (1)$$

where log_{10} *(CFU/ml)*, is the current number of microorganisms present in the fruit juice; *k*, is the initial level of microorganisms (log_{10} CFU/ml); *A*, is the difference in log_{10} (CFU/ml) of microorganisms found between $t = 0$ days (initial population) and the maximum population density achieved at the stationary phase; μ_{max}, is the maximum growth rate (Δlog_{10} (CFU/ml)/day); λ, is the lag phase time (days); *t*, is the time (days) and *e*, is a constant of 2.7182 value.

Once estimated those Gompertz's parameters, a rearrangement of the *Equation 1* was done to calculate the value of the microbiological shelf-life (*MSL*; days) of untreated and treated melon and watermelon juices, in such a way that, *t*, expressed as *MSL*, is obtained as follow (*Equation 2*):

$$MSL(days) = \lambda - \frac{A \cdot \left\{ln\left[-ln\left(\frac{Log_{10}(10^7\,CFU/ml) - k}{A}\right)\right] - 1\right\}}{\mu_{max} \cdot e} \quad (2)$$

where 10^7 *CFU/ml*, is the maximum limit of microorganisms (at expiry date) considered by the Spanish Regulation (BOE, 2001) in raw foods from vegetal origin. All data modeling were made through the Statgraphics plus Centurion XV software Version 15.1.02.

3.-RESULTS

3.1.-Effect of HIPEF and citric acid on pathogenic microorganisms

Populations of *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* inoculated in melon and watermelon juices were slightly affected by the concentration of citric acid added without HIPEF treatment (Table 3). *L. monocytogenes* was more sensible to citric acid than *E. coli* O157:H7 and *S.* Enteritidis in watermelon juice, whereas, significant effects of the acid on those pathogens in melon juice were not observed (Table 3). Nonetheless, when HIPEF treatment was applied to these juices inoculated with a cocktail of *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* containing citric acid, additive effects up to 0.5% and synergistic effects from 1.0% of citric acid combined with HIPEF treatment in both fruit juices were found in the inactivation of the three studied pathogenic microorganisms (*Figure 1*). However, combinations of HIPEF with 1.5% or 2.0% of citric acid in watermelon and melon juices, respectively, were enough for reducing *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* populations by more than 5.0 log_{10} units (*Figure 1*), which are required for Regulatory Organizations as the USFDA (U.S. Food and Drug Administration) for pasteurization purpose of juice products when novel treatments are applied (USFDA 2002). In general, *L. monocytogenes* was more sensible to the combination of HIPEF treatment with citric acid followed by *S.* Enteritidis and *E. coli* O157:H7 populations in both juices.

3.2.-Effect of HIPEF and cinnamon bark oil on pathogenic microorganisms

Significant effects ($P \leq 0.05$) on the microbial reductions of *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* inoculated in melon and watermelon juices were found when different concentrations of cinnamon bark oil were tested without HIPEF treatment (Table 3). Population of *L. monocytogenes* showed a higher susceptibility to the essential oil than *E. coli* O157:H7 and *S.* Enteritidis populations in both studied fruit juices. *E. coli* O157:H7 population showed a higher essential oil-resistance than *S.* Enteritidis in melon and watermelon juice, being these microorganisms more susceptible to the oil in the latter juice (Table 3). But, when HIPEF treatment was applied to those fruit juices containing cinnamon bark oil, a greater antimicrobial effect against *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* populations was observed. Synergistic effects of HIPEF

treatment combined with cinnamon bark oil were detected from 0.05% on *S.* Enteritidis and *L. monocytogenes* populations, and from 0.10% on *E. coli* O157:H7 population both in melon and watermelon juices. Additive effect was only observed in population of *E. coli* O157:H7 inoculated in melon and watermelon juices when 0.05% of cinnamon bark oil combined with HIPEF were applied. Nevertheless, to inactivate *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* populations by more than 5.0 log_{10} units in both juices, as proposed by the USFDA (2002), combinations of HIPEF treatment with 0.20% of cinnamon bark oil were needed (*Figure 2*).

Table 3.-Effect of citric acid and cinnamon bark oil without HIPEF treatment on *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* populations inoculated in melon and melon and watermelon juices after one hour of exposure at 22º C

Anti-microbial	Concen-tration (%)	Microbial reduction ($-log_{10}$ N/N$_0$) [1]					
		Melon juice			Watermelon juice		
		E. coli O157:H7	*S.* Enteritidis	*L. monocytogenes*	*E. coli* O157:H7	*S.* Enteritidis	*L. monocytogenes*
Citric acid	0.0	0.079 ± 0.010 *a*	0.08 ± 0.06 *a*	0.19 ± 0.07 *a*	0.227 ± 0.015 *a*	0.03 ± 0.03 *a*	0.009 ± 0.003 *a*
	0.5	-0.02 ± 0.03 *b*	-0.06 ± 0.09 *b*	-0.051 ± 0.013 *a*	-0.33 ± 0.13 *b*	-0.11 ± 0.09 *a*	-0.36 ± 0.12 *b*
	1.0	-0.10 ± 0.11 *bc*	-0.232 ± 0.029 *c*	-0.16 ± 0.05 *a*	-0.74 ± 0.06 *c*	-0.21 ± 0.11 *a*	-0.937 ± 0.027 *c*
	1.5	-0.20 ± 0.03 *c*	-0.19 ± 0.08 *c*	-0.22 ± 0.08 *a*	-0.81 ± 0.27 *c*	-0.67 ± 0.12 *b*	-0.945 ± 0.007 *c*
	2.0	-0.40 ± 0.21 *c*	-0.46 ± 0.07 *d*	-0.24 ± 0.04 *a*	-0.85 ± 0.23 *c*	-1.03 ± 0.24 *c*	-1.5 ± 0.3 *d*
Cinnamon bark oil	0.00	0.079 ± 0.010 *a*	0.08 ± 0.06 *a*	0.19 ± 0.07 *a*	0.227 ± 0.015 *a*	0.03 ± 0.03 *a*	0.009 ± 0.003 *a*
	0.05	-0.05 ± 0.09 *b*	-0.10 ± 0.08 *b*	-0.22 ± 0.05 *b*	-0.28 ± 0.06 *a*	-0.16 ± 0.03 *a*	-0.180 ± 0.05 *a*
	0.10	-0.24 ± 0.07 *c*	-0.50 ± 0.07 *c*	-0.56 ± 0.21 *b*	-0.31 ± 0.26 *a*	-0.44 ± 0.03 *a*	-1.082 ± 0.026 *b*
	0.20	-0.29 ± 0.08 *c*	-1.06 ± 0.14 *d*	-2.03 ± 0.12 *c*	-0.624 ± 0.029 *b*	-1.7 ± 0.4 *b*	-3.34 ± 0.26 *c*
	0.30	-1.45 ± 0.06 *d*	-3.07 ± 0.22 *e*	-3.43 ± 0.34 *d*	-1.91 ± 0.29 *c*	-3.9 ± 0.3 *c*	-4.4 ± 0.5 *d*

[1] Values (log_{10} initial population / log_{10} final population) are the mean of four measurements ± SD. Different lower-case letters (*a*, *b*, *c*, *d*, *e*) on the same column indicate significant differences ($P \leq 0.05$) among concentrations by each antimicrobial and microorganism.

In general, *E. coli* O157:H7 population was more resistant to the combination of HIPEF treatment with cinnamon bark oil than *S.* Enteritidis and *L. monocytogenes* populations in both fruit juices; whereas, *L. monocytogenes* was always more sensible to those treatments both in melon and watermelon juices.

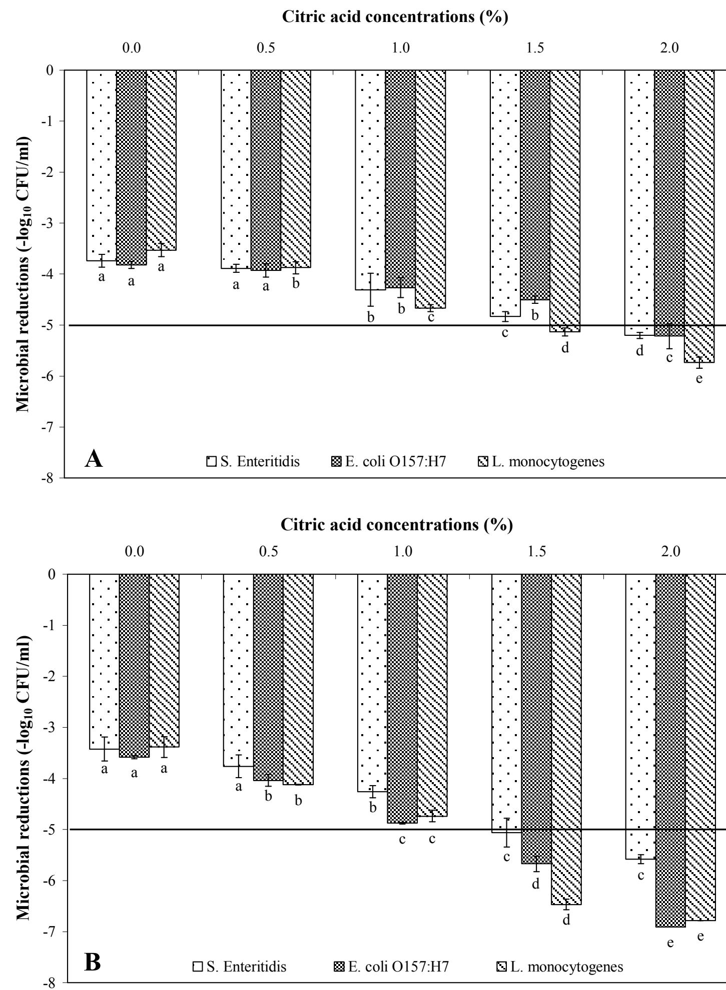

Figure 1.*-Microbial reductions of S. Enteritidis, E. coli O157:H7 and L. monocytogenes populations in melon and melon (A) and watermelon (B) juices exposed to HIPEF with different citric acids concentrations. Horizontal line (—) represent the minimum microbial reduction required to achieve the pasteurization level. Bars are the mean of four measurements ± SD. Different lower-case letters (a, b, c, d, e) indicate significant difference ($P \leq 0.05$) among concentrations by each microorganism.*

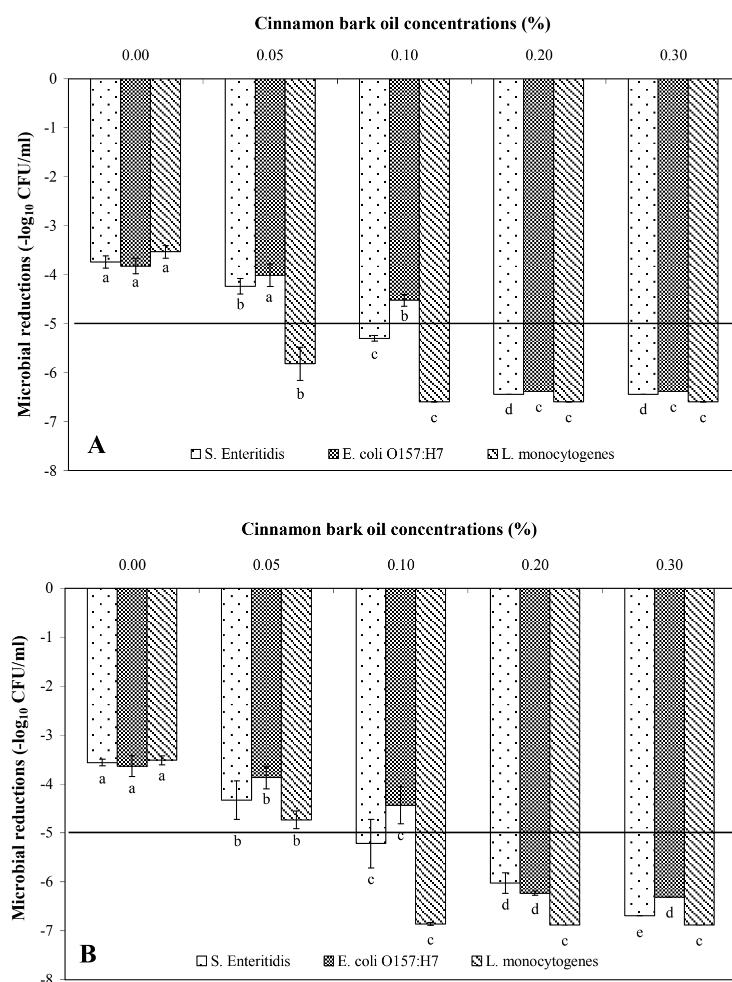

Figure 2.-*Microbial reductions of S. Enteritidis, E. coli O157:H7 and L. monocytogenes populations in melon and melon (A) and watermelon (B) juices exposed to HIPEF with different cinnamon bark oil concentrations. Horizontal line (—) represent the minimum microbial reduction required to achieve the pasteurization level. Bars are the mean of four measurements ± SD. Different lower-case letters (a, b, c, d, e) indicate significant difference (P ≤ 0.05) among concentrations by each microorganism.*

3.3.-Effect on the microbiological shelf-life

Based on the minimum antimicrobial concentration to inactivate more than 5.0 log_{10} units of pathogenic microorganisms in each fruit juice (*Figures 1* and *2*), the application of HIPEF to watermelon and melon juices containing 1.5 and 2.0% of citric acid, respectively, or 0.2% of cinnamon bark oil was carried out to study the microbiological shelf-life. Untreated juices as well as HIPEF-processed and thermally-processed juices were also evaluated.

The modified Gompertz's model (Equation 1) used to describe the growth of naturally occurring microorganisms in melon and watermelon juices fitted properly to our data, since determination coefficients (R^2) above 96% were found. A higher initial microbial load (*k*) and maximum growth rate (μ_{max}) of mesophilic and psychrophilic microorganisms than molds and yeasts in untreated melon and watermelon juices were estimated through the Gompertz's model (Table 4).

Table 4.-Estimated values of the Gompertz's parameters on the mesophilic, mold and yeast and psychrophilic growth in control and HIPEF-treated melon and watermelon juices stored at 5º C

Treatment	Parameters	Estimated values [a]					
		Melon juice			Watermelon juice		
		Mesophilic	Molds & Yeasts	Psychrophilic	Mesophilic	Molds & Yeasts	Psychrophilic
Control	***K***	2.611 ± 0.222	1.522 ± 0.117	2.630 ± 0.155	1.516 ± 0.079	0.356 ± 2.222	1.800 ± 0.113
	A	6.213 ± 0.297	5.595 ± 0.163	6.074 ± 0.191	7.289 ± 0.099	8.065 ± 2.342	6.743 ± 0.155
	μ_{max}	0.924 ± 0.561	0.539 ± 0.055	0.962 ± 1.272	1.085 ± 0.133	0.745 ± 0.103	0.751 ± 0.086
	λ	2.277 ± 2.985	2.877 ± 0.576	2.455 ± 2.418	3.041 ± 0.473	0.010 ± 4.173	2.469 ± 0.613
	R^2 **(%)**	99.14	99.69	99.48	99.81	99.29	99.81
HIPEF	***K***	0.028 ± 0.096	0.020 ± 0.046	0.049 ± 0.099	0.010 ± 0.087	0.003 ± 0.155	0.094 ± 0.154
	A	8.143 ± 0.233	7.390 ± 0.276	8.109 ± 0.308	9.227 ± 0.198	7.269 ± 0.293	7.620 ± 0.385
	μ_{max}	0.337 ± 0.028	0.248 ± 0.015	0.329 ± 0.031	0.314 ± 0.017	0.345 ± 0.051	0.118 ± 0.008
	λ	44.405 ± 1.063	58.342 ± 0.695	49.666 ± 1.188	37.028 ± 0.876	39.816 ± 1.602	37.247 ± 2.692
	R^2 **(%)**	99.12	99.51	98.82	98.53	96.69	96.73

[a] Estimated values ± asymptotic standard error given by the modified Gompertz's equation (Equation 1); ***k***, is the initial microbial load (log_{10} CFU/ml); ***A***, is the difference in log_{10} (CFU/ml) of microorganisms populations between t = 0 days (initial population) and the maximum population achieved at the stationary phase; μ_{max}, is the maximum growth rate (Δlog_{10} (CFU/ml)/day); λ, is the lag phase time (days); R^2; is the determination coefficient

In HIPEF-processed melon juice a higher μ_{max} and lower lag phase time (λ) of mesophilic and psychrophilic microorganisms than mold and yeast were observed; whereas, in HIPEF-processed watermelon juice a lower λ and higher microbial growth (A) of mesophilic microorganism than mold and yeasts was also detected (Table 4). Lower k and μ_{max} values and higher λ values in HIPEF-processed juices than control juices were observed. On the other hand, Gompertz's parameters on HIPEF-processed melon and watermelon juices containing citric acid or cinnamon bark oil, and those thermally processed were not estimated due to microbial growth was not detected throughout storage time (91 days) at 5ºC.

Unprocessed and HIPEF-processed melon and watermelon juices were mainly limited by mesophilic microorganisms at refrigeration temperature (5ºC), whereas, the naturally occurring microflora in both juices was inactivated by more than 91 days at 5ºC of storage when HIPEF treatment in combination with citric acid or cinnamon bark oil, and thermal treatment were applied (*Table 5, Figure 3*). Control melon and watermelon juices showed a shelf-life about 5 and 6 days, respectively, whereas, HIPEF-processed melon and watermelon juices exhibited a longer microbiological shelf-life extension regarding to those control fruit juices, since microbial load about 10^7 CFU/ml were achieved at 62 (for melon juice) and 50 (for watermelon juice) days of storage at 5ºC (Table 5).

Table 5.-Estimation of the microbiological shelf-life in melon and watermelon juices stored at 5ºC for 91 days submitted to different processing conditions

Fruit juice	Process (conditions)	MSL (days) [a]		
		Mesophilic	Mold & Yeast	Psychrophilic
Melon	Control	5.29	18.29	5.42
	HIPEF (1709µs-193Hz)	62.05	> 91	67.73
	HIPEF + CA (2.0%)	> 91	> 91	> 91
	HIPEF + CBO (0.2%)	> 91	> 91	> 91
	Thermal (90ºC x 1min)	> 91	> 91	> 91
Watermelon	Control	6.49	6.51	7.41
	HIPEF (1682µs-200Hz)	50.35	66.21	> 91
	HIPEF + CA (1.5%)	> 91	> 91	> 91
	HIPEF + CBO (0.2%)	> 91	> 91	> 91
	Thermal (90ºC x 1min)	> 91	> 91	> 91

[a] Estimated microbiological shelf-life according to Equation 2

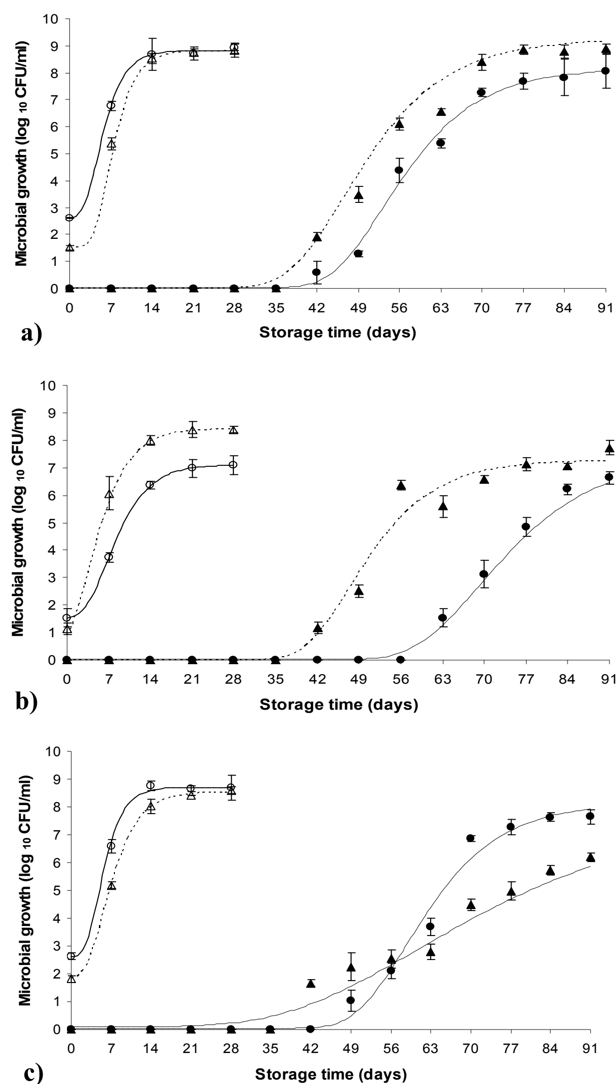

Figure 3.-*Microbial growth of a) mesophilic, b) mold and yeast and c) psychrophilic populations on untreated and HIPEF-treated melon (—) and watermelon (·····) juices during storage at 5º C for 91 days. Symbols and lines represent observed and modeled (modified Gompertz's equation) values, respectively. Symbols are means of four measurements ± SD. Untreated melon (○), untreated watermelon (Δ), HIPEF-treated melon (●), HIPEF-treated watermelon (▲) juices.*

3.4.-Effect on the sensory properties

Noticeable changes on the odor, color, taste, sourness and overall attributes of HIPEF-processed melon and watermelon juices with regard to the controls were not detected by panelists, at exception of the taste in melon juice; whereas, significant changes of those attributes were observed on both juices processed by HIPEF containing citric acid or cinnamon bark oil as well as those thermally treated (Table 6). Melon juice processed by HIPEF and citric acid or cinnamon bark oil exhibited the lower scores in odor, taste, sourness and overall; whereas, watermelon juice processed by HIPEF and citric acid or cinnamon bark oil displayed the lower rating in taste, sourness and overall by the panelists. Likewise, the color of thermal-processed watermelon juice received the lowest score. HIPEF-processed melon and watermelon juices without antimicrobials exhibited the better scores by the panelists in all attributes evaluated regarding to the others treatments (Table 6).

Table 6.-Effect on the organoleptic characteristics of melon and watermelon juices treated under different processing conditions

Fruit juice	Process (conditions)	Sensory attributes [1]				
		Odor	**Color**	**Taste**	**Sourness**	**Overall**
Melon	Control	7.7 ± 1.9 ^{a}	7.3 ± 1.5 ^{a}	7.9 ± 1.5 ^{a}	7.1 ± 1.6 ^{a}	7.7 ± 1.4 ^{a}
	HIPEF (1709μs-193Hz)	6.9 ± 1.9 ^{a}	7.3 ± 1.5 ^{a}	6.9 ± 1.9 ^{b}	7.1 ± 1.7 ^{a}	7.2 ± 1.7 ^{a}
	HIPEF + CA (2.0%)	2.2 ± 1.6 ^{c}	5.9 ± 2.1 ^{b}	2.2 ± 1.5 ^{d}	2.9 ± 2.4 ^{c}	3.3 ± 1.7 ^{c}
	HIPEF + CBO (0.2%)	2.8 ± 2.0 ^{c}	5.6 ± 1.9 ^{b}	1.6 ± 1.2 ^{d}	4.0 ± 2.1 ^{b}	2.2 ± 1.3 ^{d}
	Thermal (90ºC x 1min)	3.9 ± 2.0 ^{b}	5.9 ± 1.6 ^{b}	4.5 ± 1.8 ^{c}	6.1 ± 1.5 ^{a}	5.0 ± 1.3 ^{b}
Watermelon	Control	6.7 ± 1.2 ^{a}	8.3 ± 0.8 ^{a}	7.2 ± 1.8 ^{a}	7.1 ± 1.4 ^{a}	7.3 ± 1.3 ^{a}
	HIPEF (1682μs-200Hz)	6.7 ± 1.3 ^{a}	8.1 ± 1.0 ^{a}	7.6 ± 1.0 ^{a}	7.4 ± 1.4 ^{a}	7.2 ± 1.3 ^{a}
	HIPEF + CA (1.5%)	4.7 ± 1.8 ^{b}	7.2 ± 1.0 ^{b}	2.9 ± 1.8 ^{c}	3.1 ± 2.6 ^{c}	2.8 ± 1.6 ^{bc}
	HIPEF + CBO (0.2%)	3.2 ± 1.1 ^{c}	6.5 ± 2.0 ^{b}	2.1 ± 1.9 ^{c}	3.5 ± 2.3 ^{c}	2.0 ± 1.8 ^{c}
	Thermal (90ºC x 1min)	4.0 ± 2.0 ^{b}	2.7 ± 1.9 ^{c}	5.0 ± 1.7 ^{b}	5.8 ± 1.7 ^{b}	3.5 ± 1.6 ^{b}

[1] Values are the mean of thirty evaluations ± SD.
Different lower-case letters (*a*, *b*, *c*, *d*) on the same row indicate significant differences ($P \leq 0.05$) among processes by each sensory attribute and fruit juice.

4.-DISCUSSION

The minimal concentrations of citric acid to inactivate *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* populations by more than 5.0 $\log_{10}$ units in melon and watermelon juices treated by HIPEF could be related to the undissociated form of the acid molecule, which is assumed to possess the antimicrobial activity (Stratford and Eklund 2003). The citric acid molecules remain in undissociated form when the pH of the medium is below the pKa value (3.14) of the acid. In this study, those pH values were achieved from 2.0 and 1.5% of citric acid in melon (3.12) and watermelon (3.09) juices, respectively (Table 1). Similar results were obtained by Raybaudi-Massilia et al. (2006b), who reduced *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* populations by more than 5.0 $\log_{10}$ units in apple juice when HIPEF treatment (35kV/cm for 1500µs at 321Hz and 4µs pulse length in bipolar mode) was applied immediately or after one hour of exposure to 1.5 or 1.0% of malic acid, respectively. Likewise, Eswaranadan et al. (2004) reported that citric acid was lesser effective than malic acid at the same concentration for inactivating *E. coli* O157:H7, *S.* Gaminara and *L. monocytogenes* populations, as a consequence of the smaller molecular weight of the malic acid (134.09 g/mol) with regard to citric acid (192.14 g/mol). In addition, citric acid possesses a lower pKa value (3.14) than malic acid (3.40), thus, a lower concentration of organic acid is needed to remain in undissociated form and to exert the antimicrobial activity.

Citric acid is a hydrophilic molecule (-0.172 partition coefficient of octanol/water) and its access to microbial cell inside only occur across proteins (porins) inserted in outer membrane of Gram-negative bacteria, which permit the pass of hydrophilic molecules, or across of the thick peptidoglycan layer of Gram-positive bacteria. However, when HIPEF treatment is applied, pore formation on the cell membrane of the microorganism take place due to the great difference in the transmembrane potential when electric fields of high intensities are induced (Coster and Zimmerman, 1975). Therefore, the entrance of undissociated citric acid molecules towards the cell inside may occur more rapidly and, produces a decrease of the intracellular pH by dissociation of the acid molecule into charged anions which could causes microbial inactivation by damage in cell signaling, active transport and genetic material (Stratford and Eklund 2003).

On the other hand, the addition of cinnamon bark oil in melon and watermelon juices showed a higher effectiveness at a lower concentration

than citric acid against *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* populations when was combined or not with HIPEF. This fact could be attributed to the hydrophobic nature of the oil, which may directly diffuse across the lipid bilayer of the cell membrane, and/or gain access to the periplasm and deeper parts of the cell through the porins, which have also showed to allow the penetration of lipophilic substances of low molecular weight at significant rates, despite of its hydrophilic nature (Nikaido, 1996; Helander et al., 1998). Although, the mechanism of action of cinnamon oil on the microbial cells is still unclear, Wendakoon and Sakaguchi (1995) and Burt (2004) have reported that the interaction of carbonyl group of the cinnamaldehyde (main active compound of cinnamon oil from bark) on the cell proteins embedded in the cytoplasmatic membrane appear to inhibit the action of the enzymes amino acid decarboxylases, which are necessary for the amino acids biosynthesis and biodegradation. On the other hand, Oussalah et al. (2006) demonstrated that cinnamon oil at 0.1% was able to produce leakage out of the cell constituents, a decrease of the intracellular ATP concentration and intracellular pH, as a consequence of the increase in permeability of the cell membrane of *E. coli* O157:H7 and *L. monocytogenes*. However, these authors did not observe apparent changes on the cell surface (by electron micrographs) of those pathogens when cinnamon oil was added. Likewise, Gill and Holley (2004; 2006) reported a rapid decrease of cellular ATP without increase in extracellular ATP when 0.15 or 0.6% of cinnamaldehyde was used. Hence, the mechanism of action of the cinnamon oil and its main active compound on microorganisms according to Gill & Holley (2004, 2006) and Oussalah et al. (2006) appear to be related to the cell membrane from which a slight disruption seem to occur, causing dispersion of the proton motive force by leakage of small ions without leakage of larger cell components, such as ATP, which were subsequently degraded by the ATPase enzyme. On the other hand, the antimicrobial effect of cinnamon bark oil against *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* populations inoculated in melon and watermelon juices was enhancer when HIPEF was applied, due to the formation of pores on the cell membrane which could favor the diffusion of the oil to cell inside and cause damage in the cell functions. Other studies on the application of HIPEF treatment in combination with essential oils for inactivating pathogenic microorganisms in fruit juices were not found in the literature; therefore, this study to open a new way for ensuring juices safety.

In this study, population of *L. monocytogenes* was always more sensible to citric acid or cinnamon bark oil and its combination with HIPEF

than *E. coli* O157:H7 and *S.* Enteritidis populations inoculated in melon and watermelon juices. In contrast, *L. monocytogenes* showed to be slightly more resistant to HIPEF treatment than populations of *E. coli* O157:H7 and *S.* Enteritidis (*Figures 1* and *2*) as also reported by others researchers (Qin et al., 1998; Mosqueda-Melgar et al., 2007). The sensibility of these pathogenic microorganisms to antimicrobial substances could be attributed to the membrane composition of the bacteria, because, *L. monocytogenes* is a Gram-positive bacterium and *E. coli* O157:H7 and *S.* Enteritidis are Gram-negative bacteria. Brul and Coote (1999) and Burt (2004) reported that the latter bacteria possess an outer membrane (absent in Gram-positives) and a lipopolysacharides layer which play important roles in modulating the accessibility of preservatives and other small molecules toward the cell inside, thus serving, as a protector barrier. Nikaido (1996) and Helander et al. (1998) indicated that the diffusion of hydrophilic molecules and some lipophilic compounds of low molecular weight, which are essentials to maintain the cell functions, occur across water-filled channels formed by transmembrane proteins embedded into the lipid bilayer (porins). In contrast, Gram-positive bacteria possess a thick layer of peptidoglycan which is more permeable and less selective than outer membrane in Gram-negative bacteria. Therefore, the cell wall of Gram-positive bacteria is generally more sensible to substances than Gram-negative bacteria.

In addition, both antimicrobials were slightly more bactericidal in watermelon juice than in melon juice as a consequence of lower pH value in the former juice. Burt (2004) reported that the bacterial susceptibility to essential oils increase with a reduction in pH of the food, because at low pH the hydrophobicity of the oil increases, enabling it to more easily dissolve in the lipids of the cell membrane of the target bacteria. Likewise, organic acids are also more effective at lower pH value, because there are undissociate forms of acid molecules in greater levels, which are responsible of antimicrobial activity (Davidson, 2001).

On the other hand, mesophilic populations comprised the predominant and limiting flora more than molds and yeasts populations in unprocessed (control) melon and watermelon juices (*Table 5*, *Figures 1-3*). This fact could be a direct consequence of the high pH value (among 5.7 and 6.1) that possesses these fruits. Similar results were reported by Oms-Oliu et al. (2007a, 2007b) and Lamikanra et al. (2000) in "Piel de Sapo" and "Cantaloupe" fresh-cut melons, respectively. Ukuku and Fett (2002) reported that aerobic mesophilic bacteria followed by lactic acid bacteria and *Pseudomonas* spp. were the predominant classes of spoilage

microorganisms on “Cantaloupe” and “Honey Dew” fresh-cut melons. In the same way, Abbey et al. (1988) found that bacteria such as *Pseudomonas* spp., *Escherichia coli, Enterobacter* spp., and micrococci comprised the predominant microflora in sliced watermelon. However, when HIPEF treatment was applied to melon and watermelon juices, their naturally occurring microorganisms were reduced and inhibited; since, those microbial populations were not detected during the first 35 days of storage at 5º C. From that day, the microbial growth in both fruit juices was observed. Mesophilic more than molds and yeasts populations were the predominant microorganisms in melon and watermelon juices after HIPEF, as in control juices (Table 5). The observed microbial growth after applying HIPEF may be explained by two ways: a) recovery of injured microorganisms or b) germination of spore-forming microorganisms, since low influence of HIPEF treatment on spores inactivation of microorganisms has been reported (Raso et al., 1998; Pagán et al., 1998). Nevertheless, when melon and watermelon juices were treated by heat or HIPEF with citric acid (2.0 or 1.5%) or HIPEF with cinnamon bark oil (0.2%) those resident microorganisms and/or their spores were totally inactivated by more than 91 days of storage at 5º C. Liang et al. (2006) achieved the inactivation of mesophilic microorganisms previously grown in apple cider by 2.88 and 3.11 $\log_{10}$ CFU/ml when HIPEF (27kV/cm for 17.6μs at 200Hz and 1μs pulse duration) in combination with clove oil at 3 and 5% (vol/vol), respectively, were applied. Otherwise, Nguyen and Mittal (2007) reduced naturally occurring microorganisms in tomato juice heated at 50ºC by 3.89 and 4.77 $\log_{10}$ CFU/ml when clove oil (0.1% vol/vol) and mint extract (0.1% vol/vol) were used without HIPEF treatment. However, these authors did not study the behavior of those microbial populations throughout the time when antimicrobial substances combined or not with HIPEF were applied; therefore suitable comparisons with prior reports could not be made.

In this study, although HIPEF combined with citric acid or cinnamon bark oil could ensure the microbiological quality and safety of fruit juices in the same way than thermal treatment, changes on some organoleptic properties (odor and taste) of melon and watermelon juices were significantly affected. Hence, further studies about combinations of HIPEF with mix of natural antimicrobials or others substances are needed to reduce their impacts on the organoleptic characteristics of the juices.

5.-CONCLUSION

Results obtained in this study, demonstrated that the application of HIPEF treatment in combination with citric acid or cinnamon bark oil may be a good alternative to the thermal pasteurization for preserving the microbiological quality and safety in fruit juices and reducing the risk of food-borne illness caused by unpasteurized juices consumption. However, further studies are needed to reduce the negatives effects on taste, odor and sourness attributes when citric acid or cinnamon bark oil are added into melon or watermelon juice at concentrations that ensuring its safety, since HIPEF alone did not significantly affect the organoleptic properties of the juices. In addition, *E. coli* O157:H7 population showed to be more resistant to the combination of treatments than *L. monocytogenes* and *S.* Enteritidis, thus, the former bacterium may be considered as target pathogenic microorganism in these kinds of products.

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Capítulo V

Microbiological shelf-life extension in fruit juices by combining high-intensity pulsed electric fields and natural antimicrobials

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ABSTRACT

The effect of combining high-intensity pulsed electric fields (HIPEF) with citric acid or cinnamon bark oil on the microbiological shelf-life of strawberry, orange, apple, pear and tomato juices, as well as on the sensory properties of these products was evaluated. An extension of the microbiological shelf-life in those fruit juices treated by HIPEF with or without antimicrobials in comparison with those fruit juices without processing was observed. Naturally occurring microorganisms in strawberry and oranges juices were inactivated by more than 91 days of storage at 5ºC when only HIPEF treatment was applied. Resident microbial populations in apple, pear and tomato juices were inactivated by more than 91 days of refrigerated storage after applied HIPEF and antimicrobials, in the same way that thermal treatment. Negligible changes on the sensory attributes in all studied fruit juices processed by HIPEF were found; but, when HIPEF and natural antimicrobials (citric acid or cinnamon bark oil) were applied on those fruit juices, noticeable changes on some sensory attributes such as odor, taste and sourness were perceived.

Practical Aplications. The application of HIPEF treatment on fruit juices resulted to be effective to reduce spoilage microorganisms, however, when this technology is combined with antimicrobial substances such as citric acid (used commonly) and cinnamon bark oil products more microbiologically stable and durable are obtained. Therefore, combinations of those treatments may be an alternative process to the heat pasteurization to ensure the microbiological quality and safety in juice products, and to reduce the risk of food-borne illness caused by the consumption of these kinds of foods.

Keywords: high-intensity pulsed electric fields; citric acid; cinnamon bark oil; fruit juices; microbiological shelf-life

INTRODUCTION

Consumer's demand by fresh, ready-to-eat and nutritious foods such as fresh-cut fruit and unpasteurized fruit juices have increased in the last decades, as a consequence of the active health-promoting and balanced diet that can provide these products. Consumption of fresh fruit juices is integral part of a healthy diet, because they can supply much-needed antioxidants, vitamins and minerals, which play important roles in the prevention of heart diseases, cancers and diabetes (Matthews 2006). However, fresh fruit juices are highly susceptible to spoil, since fluid components (enzymes, organic acids, carbohydrates, etc.) are thoroughly mixed with air and microorganisms from the environment and handling during the fruit juice processing. Thus, if juices are not rapidly heated a fast microbial, enzymatic, chemical and physical deterioration and shorter shelf-life is observed (Bates *et al.* 2001). Nonetheless, thermal treatment may extensively damage the organoleptic, nutritional and physicochemical properties of fluid foods (Yeom *et al.* 2000; Espach-Barroso *et al.* 2003; Elez-Martínez *et al.* 2006). Those negative effects on foods have motivated a great interest in the development of novel technologies that can offer the advantages of using low processing temperatures, low energy consumption, and retention of nutritional and sensory properties of the food, while improves its microbiological quality and safety (Mertens and Knorr 1992; Mittal and Griffiths 2005).

High-intensity pulsed electric fields (HIPEF) treatment is one of those novel non-thermal technologies, which is able to inactivate microorganisms, to decrease the activity of enzymes and to extent the shelf-life of foods without significant loss of flavor, color and nutrients (Yeom *et al.* 2000; Hodgins *et al.* 2002; Cserhalmi *et al.* 2006; Elez-Matínez *et al.* 2006). This technology involve the application of short pulses (1 to 10 μs) of high-intensity electric field (typically 20 to 80 kV/cm) to fluid foods placed between two electrodes in batch or continuous flow system.

The effect of HIPEF treatment on the naturally occurring microbial populations in orange, orange-carrot, apple, tomato and cranberry juices has been widely studied (Jia *et al.* 1999; Jin and Zhang 1999; Evrendilek *et al.* 2000; Yeom *et al.* 2000; Hodgins *et al.* 2002; Min *et al.* 2003a; 2003b; Rivas *et al.* 2006; Elez-Martínez *et al.* 2006). Nevertheless, the effect of HIPEF in combination with antimicrobial substances against spoilage microorganisms in fruit juices has been few studied (Hodgins *et al.* 2002; Wu *et al.* 2005; Liang *et al.* 2006; Nguyen and Mittal 2007), and the

behavior on those microorganisms in the time (microbiological shelf-life) under those treatment combinations were not found in the literature.

Natural antimicrobials such as citric acid and cinnamon bark oil are GRAS (Generally Recognized As Safe) substances permitted by the Food Additive Status List (USFDA 2006) and European Parliament and Council Directive Nr. 95/2/EC (1995). Citric acid is an organic acid present naturally in several fruits, including strawberry, orange, apple, pear and tomato fruits, and it is used in the food industry as acidulant, flavoring and preservative (Doores 1993). Likewise, the essential oil from cinnamon bark is used as flavoring agent in foods and beverages (Wright 1999). In addition, these natural substances have demonstrated by themselves to possess antimicrobial activity against a great number of microorganisms (Sharma 2000; Stratford and Elkund 2003; Burt, 2004; Friedman *et al.* 2004; Gill and Holley 2004; Nazer *et al.* 2005; Raybaudi-Massilia, *et al.* 2006; Gill and Holley 2006; Oussalah, 2006; 2007; Mosqueda-Melgar *et al.* 2007). However, the effect of combining citric acid or cinnamon bark oil with HIPEF treatment against naturally occurring microorganisms in fruit juices have not been found in the literature.

The purpose of this study was to evaluate the effect of HIPEF combined with citric acid or cinnamon bark oil on the microbiological shelf-life (during 91 days) as well as to evaluate their effects on sensory characteristics (odor, color, taste, sourness, overall acceptability) of strawberry, orange, apple, pear and tomato juices stored at 5º C.

MATERIALS AND METHODS

Fruits and fruit juices preparation

Apple (*Malus domestica* Borkh var. "Fuji"), pear (*Pyrus communis* L. var. "Flor de invierno"), orange (*Citrus sinensis* L. var. "Valencia"), strawberry (*Fragaria* spp. var. "Camarosa") and tomato (*Solanum lycopersicum* var. "Roma") fruits at commercial ripeness were selected in a supermarket of Lleida, Spain. Fruits were immersed in chlorine (200 µl/l, pH 6.8) during 5 min for superficial disinfection, then washed with distilled water to eliminate remaining chlorine traces and subsequently dried with absorbent paper. Apple, pear, strawberry and tomato fruits were peeled, cut into pieces, and mixed through an Ufesa blender (Model BP 4512; Vitoria, Spain); whereas oranges were cut in two pieces and then squeezed with a Press-Citron model (ALJUAN S.L., Alicante, Spain) squeezer. Apple, pear

and strawberry puree were centrifuged at 12,500 rpm for 15 min at 4ºC in an Avanti™ J-25 Centrifuge (Beckman Instrument, Inc.; USA) to separate the pulp (fibers) of the remaining liquid (juice). Afterwards, the supernatant juice was filtered through a cloth strainer to remove the particles of minor size. Likewise, tomato and orange juices were filtered across of a metal strainer to separate the seeds, fibers and major particles of the juice. Finally, all fruit juices were then degassed through a diaphragm vacuum pump (Vacuubrand, Wertheim, Germany) for 10 min to remove air burbles which may affect the fruit juice processing. From this juice, different portions were served into bottles to the 1) HIPEF alone, 2) HIPEF and citric acid, 3) HIPEF and cinnamon bark oil and 4) thermal processing, and 5) control.

Analytical characteristics of the fruit juices

Measurement of pH (Crison pH-meter 2001 model; Crison Instruments S.A; Barcelona, Spain) and soluble solid content (Atago RX-1000 refractometer; Atago Company Ltd., Japan) of fruit juices treated by different processing conditions were carried out according to the Spanish Regulation (BOE, 1988). Electrical conductivity (Testo 240 conductivimeter; Testo GmBh & Co; Lenzkirch, Germany) was also measured.

HIPEF equipment

A continuous flow HIPEF system, bench-scale OSU-4F model (Ohio State University, Ohio, USA) which discharges square-wave pulses was used to process fruit juice samples (Figure 1). The HIPEF system is composed of eight collinear field chambers connected in series and each one with two stainless steel electrodes separated by a distance of 0.292 cm. Each chamber has a diameter of 0.23 cm and a volume of 0.0121 cm^3 with a cross section of 0.0415 cm^2. The pulse waveform, peak voltage and intensity of current were monitored using a Tekscope THS 720 oscilloscope (Tektronix Inc., Oregon, USA) connected to the HIPEF apparatus. Pulse frequency and width were controlled through of a Pulse Generator 9410 model (Quantum Composers, Inc., Bozeman MT, USA). The flow rate of the process was adjusted by a variable gear pump 75210-25 model (Cole Palmer Instrument Company; Illinois, USA). The circulating fruit juice

during the HIPEF treatment was cooled through heat exchangers connected to the chambers, which were immersed in an iced water bath (5ºC) (J.P. Selecta, S.A; Barcelona, Spain).

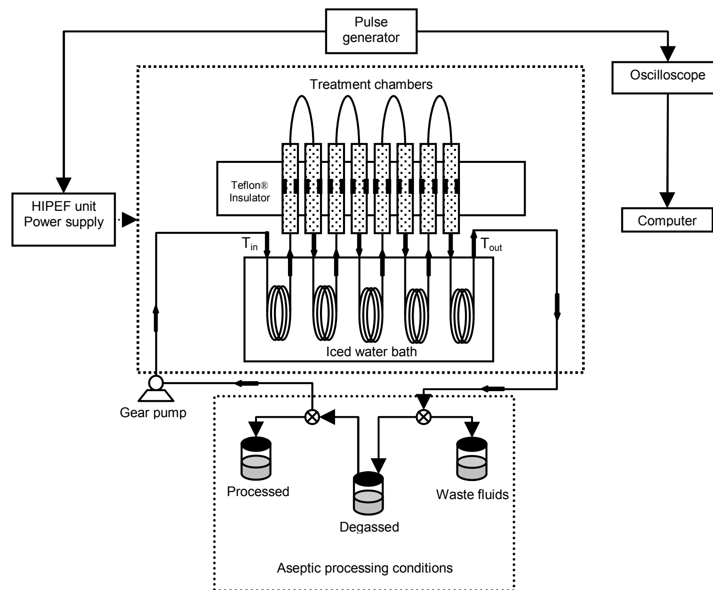

Figure 1.-*Schematic diagram of the fruit juice processing through a continuous flow bench-scale high-intensity pulsed electric fields (HIPEF) system (OSU – 4F).*

High-intensity pulsed electric fields and antimicrobials processing

Prior to each HIPEF processing the system was disinfected as suggested by Elez-Martínez et al. (2006). Afterwards, each fruit juice with or without antimicrobials was pumped through the HIPEF system at flow rate adjusted between 80-110 ml/min. Electric field intensity, treatment time, pulse frequency, pulse width and concentration of antimicrobials applied to each fruit juice were selected according to previous studies (Mosqueda-Melgar

et al., 2007) (Table 1). The combinations of those HIPEF processing parameters and antimicrobial concentrations were the needed minimal for ensuring the juices safety, since more than 5.0 log10 CFU/ml of *E. coli* O157:H7 and *Salmonella* were reached. Citric acid (Scharlau Chemie, S.A., Barcelona, Spain) and cinnamon bark oil (Aceites Esenciales Dicana, Barcelona, Spain) were the employed natural antimicrobials. These antimicrobials were mixed into the fruit juice at room temperature (22º C) for one hour using a magnetic stirrer prior to HIPEF treatment. The temperature during the HIPEF processing for all fruit juices was kept between 30 and 40º C to maximize the microbial lethality by HIPEF without undesirable heat effect.

Table 1.-High-intensity pulsed electric fields processing conditions and antimicrobial concentrations used to evaluate the microbiological shelf-life in fruit juices

Fruit juice	HIPEF processing parameters				Antimicrobials	
	E (kV/cm)	**t (µs)**	**f (Hz)**	**τ (µs)**	**CA (%)**	**CBO (%)**
Strawberry	35	1700	100	4	0.5	0.05
Orange	35	1700	235	4	0.5	0.05
Apple	35	1575	180	4	1.5	0.10
Pear	35	1600	215	4	1.5	0.10
Tomato	35	1000	100	4	2.0	0.10

E, electric field intensity; **t**, treatment time; **f**, pulse frequency; **τ**, pulse width; **CA**, citric acid; **CBO**, cinnamon bark oil.

Thermal Processing

Fruit juices were thermally processed at 90º C for 1 min through a tubular stainless steel heat exchanger coil system immersed in a hot water bath with continuous shaking (Universitat de Lleida, Lleida, Spain) as showed in Figure 2. Those fruit juices were pumped through the system with a peristaltic pump D-21V model (Dinko, Barcelona, Spain) and adjusted to a flow rate of 40 ml/min to ensure the completely thermal treatment. After thermal processing, the juice was immediately cooled in an iced water bath (Universitat de Lleida, Lleida, Spain). The applied thermal

temperature and exposure time were established to ensure the inactivation of spoilage microorganisms (Shearer et al., 2002).

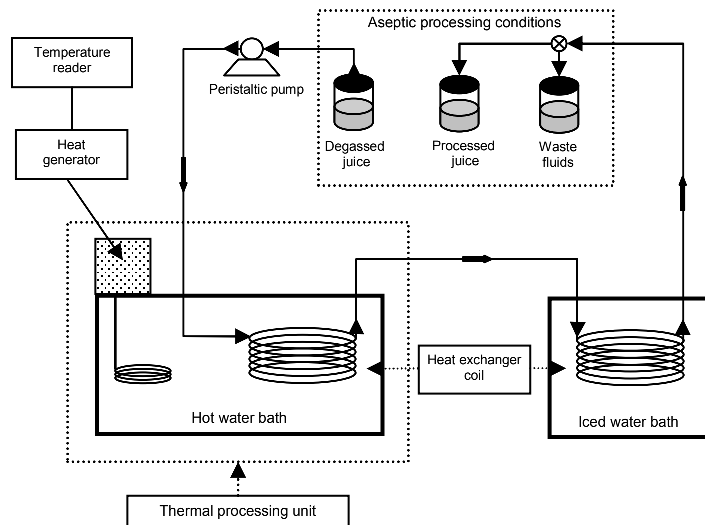

Figure 2.*-Schematic diagram of the fruit juice processing through the thermal system*

Packaging and storage

Unprocessed and processed fruit juices were filled (leaving the minimum amount of headspace volume) and packaged into 30 ml sterile containers with polypropylene screw-cap (Deltalab, Barcelona, Spain) under a horizontal laminar air flow cabin (Telstar, S.A, Barcelona, Spain) in aseptic conditions. Finally, packaged fruit juices were stored at refrigeration temperature (5º C) in darkness up to analysis.

Microbiological Analysis

Enumeration of mesophilic microorganisms on plate count agar (PCA) according to ISO 4833:1991 (1991), molds and yeasts on chloramphenicol

glucose agar (CGA) in accordance with ISO 7954:1987 (1987) and psychrophilic microorganisms on PCA were carried out in those unprocessed and processed fruit juices. Mesophilic microorganisms counts were noted after 24-48h of incubation at 35º C, molds and yeasts counts were made after 3-5 days at 22º C and, psychrophilic microorganisms counts were done after 12-15 days at 5º C. All culture media were purchased from Biokar Diagnostic. The microbiological analyses were performed each 7 days in duplicated during 91 days. Plate counts were expressed as $\log_{10}$ (CFU/ml).

Sensory evaluation

Untreated fruit juices as well as juices processed by HIPEF alone, HIPEF and citric acid, HIPEF and cinnamon bark oil, and thermally were given to panelists immediately after processing for sensory evaluation. The procedure performed for this evaluation was similar to described by Min et al. (2003a; 2003b). A total of 30 non-trainer panelists belonging to the Department of Food Technology at the University of Lleida (Spain) participated in the sensory tests. Fifteen milliliters of each sample were served into 20 ml ultra clear polypropylene containers with polyethylene screw-cap (Deltalab) coded with three digits randomly numbered; moreover, a glass containing potable water and a piece of non-salted cracker were provided to panelists for eliminating the residual taste between sample and sample. The panelists were asked to rate the preference of odor, color, taste, sourness and overall acceptability in a hedonic scale from 0 (dislike extremely) to 10 (like extremely).

Microbial growth and shelf-life modeling

Microbial growth of the naturally occurring microflora in those orange, apple, pear and tomato juices unprocessed and processed by HIPEF with or without antimicrobials, and thermal treatment, was estimated through the Gompertz's equation modified by Zwietering et al. (1990) as follow (Equation 1):

$$Log_{10}(CFU/ml) = k + A \cdot \mathbf{exp}\left\{-\mathbf{exp}\left\{\left[(\mu_{max} \cdot e) \cdot \left(\frac{\lambda - t}{A}\right)\right] + 1\right\}\right\} \quad \textbf{(1)}$$

where *log_{10} (CFU/ml)*, is the current number of microorganisms present in the fruit juice; *k*, is the initial level of microorganisms (log_{10} CFU/ml); *A*, is the difference in log_{10} (CFU/ml) of microorganisms found between $t = 0$ days (initial population) and the maximum population density achieved at the stationary phase; μ_{max}, is the maximum growth rate (Δlog_{10} (CFU/ml)/day); λ, is the lag phase time (days); *t*, is the time (days) and *e*, is a constant of 2.7182 value.

Once estimated those Gompertz's parameters, a rearrangement of the *Equation 1* was done to calculate the value of the microbiological shelf-life (*MSL*; days) of untreated and treated fruit juices, in such a way that, *t*, expressed as *MSL*, is obtained as follow (*Equation 2*):

$$MSL(days) = \lambda - \frac{A \cdot \left\{ \boldsymbol{ln}\left[-\boldsymbol{ln}\left(\frac{Log_{10}\left(10^7\ CFU/ml\right) - k}{A} \right) \right] - 1 \right\}}{\boldsymbol{\mu_{max}} \cdot e} \quad \textbf{(2)}$$

where *10^7 CFU/ml*, is the maximum limit of microorganisms (at expiry date) considered by the Spanish Regulation (BOE, 2001) in raw foods from vegetal origin. All data modeling were made through the Statgraphics plus Centurion XV software Version 15.1.02.

Statistical Analysis

Analysis of variance (ANOVA) and multiple range tests (MRT) using a Fisher's least significant difference (LSD) procedure were used to determine significant differences ($P \leq 0.05$) among the different processing conditions (unprocessed, HIPEF-processed, HIPEF and citric acid-processed, HIPEF and cinnamon bark oil-processed and thermally-processed) by each analytical characteristic and fruit juice. ANOVA and MRT were also led on the odor, color, taste, sourness and overall acceptability attributes of each fruit juice treated under different processing conditions. The experiments were performed twice, and microbial count was made in duplicated (n = 4). Sensory test was carried out once, and attributes were evaluated thirty times (n = 30). Means and standard deviations for analytical characteristics, microbiological analysis and sensory evaluation were calculated. All the statistical analyses were conducted with Statgraphics plus Centurion XV software Version 15.1.02.

RESULTS AND DISCUSSION

Analytical parameters of the fruit juices

Analytical characteristics such as pH, electrical conductivity and soluble solid content are showed in the Table 2 to offer information detailed about

Table 2.-Analytical characteristics of fruit juices under different processing conditions

Fruit juice	Process	Values [1] pH	Electrical conductivity [2] (mS/cm)	Soluble solids content (%)
Strawberry	Control	3.26 ± 0.01 ^{a}	3.78 ± 0.08 ^{a}	7.0 ± 0.1 ^{a}
	HIPEF	3.27 ± 0.02 ^{a}	3.80 ± 0.06 ^{a}	7.0 ± 0.1 ^{a}
	HIPEF + CA	2.93 ± 0.02 ^{b}	3.92 ± 0.01 ^{b}	7.3 ± 0.1 ^{b}
	HIPEF + CBO	3.24 ± 0.01 ^{a}	3.81 ± 0.04 ^{a}	7.1 ± 0.1 ^{a}
	Thermal	3.25 ± 0.02 ^{a}	3.91 ± 0.02 ^{b}	7.4 ± 0.2 ^{b}
Orange	Control	3.33 ± 0.01 ^{a}	4.01 ± 0.04 ^{a}	9.8 ± 0.1 ^{a}
	HIPEF	3.33 ± 0.02 ^{a}	4.03 ± 0.03 ^{ab}	9.9 ± 0.1 ^{a}
	HIPEF + CA	3.08 ± 0.01 ^{b}	4.06 ± 0.01 ^{b}	10.6 ± 0.1 ^{c}
	HIPEF + CBO	3.34 ± 0.01 ^{a}	4.01 ± 0.03 ^{a}	9.8 ± 0.1 ^{a}
	Thermal	3.33 ± 0.03 ^{a}	4.16 ± 0.05 ^{c}	10.2± 0.1 ^{b}
Apple	Control	4.21 ± 0.03 ^{a}	2.17 ± 0.13 ^{a}	14.1 ± 0.2 ^{a}
	HIPEF	4.21 ± 0.01 ^{a}	2.19 ± 0.04 ^{a}	14.1 ± 0.1 ^{a}
	HIPEF + CA	2.92 ± 0.02 ^{b}	2.52 ± 0.08 ^{b}	14.7 ± 0.1 ^{b}
	HIPEF + CBO	4.21 ± 0.01 ^{a}	2.18 ± 0.02 ^{a}	14.0 ± 0.1 ^{a}
	Thermal	4.21 ± 0.01 ^{a}	2.21 ± 0.03 ^{a}	14.2 ± 0.1 ^{a}
Pear	Control	4.85 ± 0.01 ^{a}	3.04 ± 0.03 ^{a}	15.4 ± 0.1 ^{a}
	HIPEF	4.87 ± 0.02 ^{a}	3.01 ± 0.04 ^{a}	15.3 ± 0.2 ^{a}
	HIPEF + CA	2.91 ± 0.01 ^{b}	3.15 ± 0.02 ^{b}	16.1 ± 0.2 ^{b}
	HIPEF + CBO	4.85 ± 0.01 ^{a}	2.99 ± 0.02 ^{a}	15.4 ± 0.1 ^{a}
	Thermal	4.84 ± 0.03 ^{a}	3.04 ± 0.02 ^{a}	15.6 ± 0.1 ^{a}
Tomato	Control	4.30 ± 0.02 ^{a}	5.51 ± 0.04 ^{a}	4.7 ± 0.2 ^{a}
	HIPEF	4.30 ± 0.01 ^{a}	5.55 ± 0.05 ^{ab}	4.6 ± 0.1 ^{a}
	HIPEF + CA	2.97 ± 0.02 ^{b}	5.93 ± 0.02 ^{c}	6.1 ± 0.1 ^{b}
	HIPEF + CBO	4.29 ± 0.01 ^{a}	5.55 ± 0.02 ^{ab}	4.7 ± 0.2 ^{a}
	Thermal	4.30 ± 0.01 ^{a}	5.61 ± 0.04 ^{b}	4.7 ± 0.1 ^{a}

[1] Values are the mean of three measurements ± SD; [2] Electrical conductivity measured at 22º C (mS/cm); **CA**, citric acid; **CBO**, cinnamon bark oil.
Different lower-case superscript letters (a, b, c) on the same column indicate significant differences (*P ≤ 0.05*) among processes by each analytical characteristic and fruit juice.

the used fruit juices in this study. The pH value of the different fruit juices processed did not significantly vary with regard to the unprocessed fruit juice, except in those fruit juices processed by HIPEF and citric acid, where a decrease of the pH value was observed, as a consequence of the organic acid added (Table 2). Electrical conductivity in those thermally-processed or HIPEF and citric acid-processed fruit juices lightly changed in comparison with unprocessed fruit juices. Although this fact could imply an increase in the temperature of the medium due to a higher number of particles in movement that transport the electric current when an electric field is applied, negligible effects on the effectiveness of HIPEF treatment and microbial inactivation may be considered. On the other hand, the soluble solids content into sample juices increased when citric acid was added; proving that the addition of organic acids contributes to the final soluble solids content of the product. The soluble solids values showed in this study about strawberry, orange, apple, pear and tomato juices are within the range from those reported in unpasteurized and commercial juices (FAO 2001; CODEX STAN 2005; Sánchez-Moreno *et al.* 2006; Sinha 2006; Sandhu and Minhas 2006) where citric acid and others preservatives are normally added.

Microbiological behavior and shelf-life in strawberry juice

Initial populations of molds and yeasts (3.38 $\log_{10}$ CFU/ml) were higher than mesophilic (2.40 $\log_{10}$ CFU/ml) and psychrophilic (2.58 $\log_{10}$ CFU/ml) populations in strawberry juice samples just before processing (time 0 day). The modeled microbial growth by the modified Gompertz's equation (1) also reflected a higher initial microbial load (k) in molds and yeasts than other populations (Table 3). Therefore, the microbiological shelf-life in that control juice was limited by molds and yeasts in a shorter time than mesophilic and psychrophilic microorganisms (Table 3). The predominance of molds and yeasts in this kind of juice is due to the high levels of sugars and others nutrients; moreover, its low pH makes it particularly susceptible to fungal spoilage, because a big part of the bacterial competition is eliminated under these conditions (Tournas and Katsoudas 2005). Deak and Beuchat (1996) and Tournas and Katsoudas (2005) reported that the most frequently isolated molds and yeasts in strawberries are *Botrytis* spp., *Rhizopus* spp., *Penicillum* spp., *Candida* spp., *Cryptococcus* spp., *Rhodotorula* spp., *Sporobolomyces* spp. and *Zygosaccharomyces* spp. However, when strawberry juice was treated

Table 3.-Estimated values of the Gompertz's parameters and microbiological shelf-life (MSL) of mesophilic, mold and yeast and psychrophilic populations from control fruit juices stored at 5º C for 35 days

Fruit juice	Microorganism	Estimated values [a]					
		K	***A***	μ_{max}	λ	R^2 (%)	MSL (days) [b]
Strawberry	**Mesophilic**	1.8 ± 0.5	6.0 ± 0.5	0.466 ± 0.021	0.009 ± 0.014	99.79	10.38
	Mold & Yeast	3.18 ± 0.28	5.2 ± 0.3	0.37 ± 0.04	0.9 ± 1.4	99.23	7.89
	Psychrophilic	2.52 ± 0.09	5.38 ± 0.13	0.297 ± 0.013	3.7 ± 0.5	99.83	16.25
Orange	**Mesophilic**	2.12 ± 0.21	5.4 ± 0.4	0.28 ± 0.04	7.1 ± 1.7	98.32	24.69
	Mold & Yeast	0.007 ± 0.029	7.5 ± 0.6	0.33 ± 0.03	2.4 ± 1.8	99.11	25.02
	Psychrophilic	1.19 ± 0.07	5.93 ± 0.13	0.353 ± 0.015	6.8 ± 0.5	99.85	31.58
Apple	**Mesophilic**	1.71 ± 0.13	6.28 ± 0.16	0.54 ± 0.03	4.5 ± 0.4	99.67	12.69
	Mold & Yeast	1.84 ± 0.16	6.0 ± 0.3	0.297 ± 0.026	6.4 ± 1.1	99.33	21.43
	Psychrophilic	1.58 ± 0.19	6.54 ± 0.22	0.83 ± 0.10	5.2 ± 0.4	99.33	12.87
Pear	**Mesophilic**	2.08 ± 0.21	6.12 ± 0.26	0.8 ± 0.4	0.6 ± 2.6	99.26	5.34
	Mold & Yeast	2.3 ± 0.4	6.4 ± 0.4	0.60 ± 0.05	0.3 ± 1.4	99.59	5.56
	Psychrophilic	2.3 ± 0.5	6.1 ± 0.5	0.66 ± 0.13	0.9 ± 1.9	99.36	5.92
Tomato	**Mesophilic**	2.05 ± 0.20	5.22 ± 0.23	0.77 ± 0.20	4.5 ± 0.7	98.79	12.42
	Mold & Yeast	1.00 ± 0.21	6.87 ± 0.24	1.2 ± 0.8	2.9 ± 2.8	99.11	7.54
	Psychrophilic	2.39 ± 0.20	5.23 ± 0.23	1.2 ± 1.8	4 ± 4	98.62	7.77

[a] Estimated values ± asymptotic standard error given by the modified Gompertz's equation (Equation 1); [b] Estimated microbiological shelf-life according to Equation 2; ***k***, is the initial level of microorganisms ($\log_{10}$ CFU/ml); ***A***, is the difference in $\log_{10}$ (CFU/ml) of microorganisms populations between $t = 0$ days (initial population) and the maximum population achieved at the stationary phase; μ_{max}, is the maximum growth rate ($\Delta\log_{10}$ (CFU/ml)/day); λ, is the lag phase time (days); R^2, is the determination coefficient

Table 4.-Estimated values of the Gompertz's parameters of mesophilic, mold and yeast and psychrophilic populations from HIPEF-treated fruit juices without antimicrobials stored at 5º C for 91 days

Fruit juice	Microorganism	Estimated values [a]				
		K	***A***	μ_{max}	λ	R^2 (%)
Apple	**Mesophilic**	0.57 ± 0.12	7.83 ± 0.18	0.273 ± 0.017	21.4 ± 1.1	99.33
	Mold & Yeast	0.77 ± 0.16	7.57 ± 0.24	0.278 ± 0.025	22.5 ± 1.5	98.59
	Psychrophilic	1.22 ± 0.14	7.23 ± 0.20	0.246 ± 0.019	21.5 ± 1.4	98.92
Pear	**Mesophilic**	0.01 ± 0.06	8.16 ± 0.08	0.571 ± 0.028	32.1 ± 0.4	99.78
	Mold & Yeast	0.02 ± 0.07	8.51 ± 0.11	0.443 ± 0.022	31.3 ± 0.5	99.71
	Psychrophilic	0.04 ± 0.13	7.86 ± 0.18	0.49 ± 0.05	29.9 ± 0.9	98.98
Tomato	**Mesophilic**	0.31 ± 0.11	7.61 ± 0.20	0.238 ± 0.015	28.7 ± 1.2	99.23
	Mold & Yeast	0.02 ± 0.12	8.31 ± 0.23	0.248 ± 0.016	28.0 ± 1.2	99.22
	Psychrophilic	0.02 ± 0.16	7.15 ± 0.24	0.31 ± 0.04	30.1 ± 1.5	98.07

[a] Estimated values ± asymptotic standard error given by the modified Gompertz's equation (Equation 1); ***k***, is the initial level of microorganisms ($\log_{10}$ CFU/ml); ***A***, is the difference in $\log_{10}$ (CFU/ml) of microorganisms populations between $t = 0$ days (initial population) and the maximum population achieved at the stationary phase; μ_{max}, is the maximum growth rate ($\Delta\log_{10}$ (CFU/ml)/day); λ, is the lag phase time (days); R^2, is the determination coefficient.

thermally or by HIPEF with or without antimicrobials, these microbial populations and their spores were totally inactivated; and microbial growth was not observed during 91 days of storage at 5º C (Tables 4-5, Figures 3-5). Comparison with others studies was impossible, since, reports about the use of HIPEF on strawberry juice were not found in the literature.

The microbial inactivation caused by HIPEF could be due to the formation of pores in the cell membrane when electric fields are applied ("electroporation"), as a consequence of the difference of transmembrane potential between the cell membrane and electric field induced (Coster and Zimmermann 1975). When the transmembrane potential to exceed the critical or threshold value, a reversible or irreversible membrane breakdown takes place, depending on the intensity of electric field induced (Barbosa-Cánovas *et al.* 1999) and/or the membrane organizational changes (Tsong 1990). Mild electric field intensities would form reversible pores in the cell membrane, whereas drastic electric field intensities would lead to the irreversibility of this phenomenon, which results in cellular death (Ho and Mittal 1996). On the other hand, thermal treatment has showed to cause damages in the cell cytoplasm by coagulation of genetic material and others proteins, without disruption of the cell wall as induced by HIPEF treatment (Pothakamury *et al.* 1997).

Microbiological behavior and shelf-life in orange juice

Higher microbial load of mesophilic microorganisms (2.07 $\log_{10}$ CFU/ml) than molds and yeasts (0.15 $\log_{10}$ CFU/ml) were observed in unprocessed orange juice at time 0 day. However, a longer lag phase time (λ) and a lower maximum growth rate (μ_{max}) was noted in those mesophilic populations in comparison with those mold and yeast populations when microbial growth was modeled (Table 3). In fact, molds and yeasts started to grow much faster than mesophilic and psychrophilic populations (Figures 3-5). But, both mesophilic and mold and yeast populations practically achieved levels of 10^7 CFU/ml (limit of shelf-life) after 25 days of storage at 5ºC (Table 3). Populations of molds and yeasts are more likely to be predominant in orange juice because of its low pH and high content in sugars and organic acids (Deak and Beuchat 1996; Keller and Miller 2006). Yeasts such as *Zygosaccharomyces* spp., *Saccharomyces* spp. and *Candida* spp. were the most frequently isolated species in orange juice (Deak and Beuchat 1996). The growth of lactic acid bacteria such as *Lactobacillus* spp. and *Leuconostoc* spp. has also been reported in orange juice (Parish and Higgins 1988; Hendrix and Red 1995). Nevertheless, these present

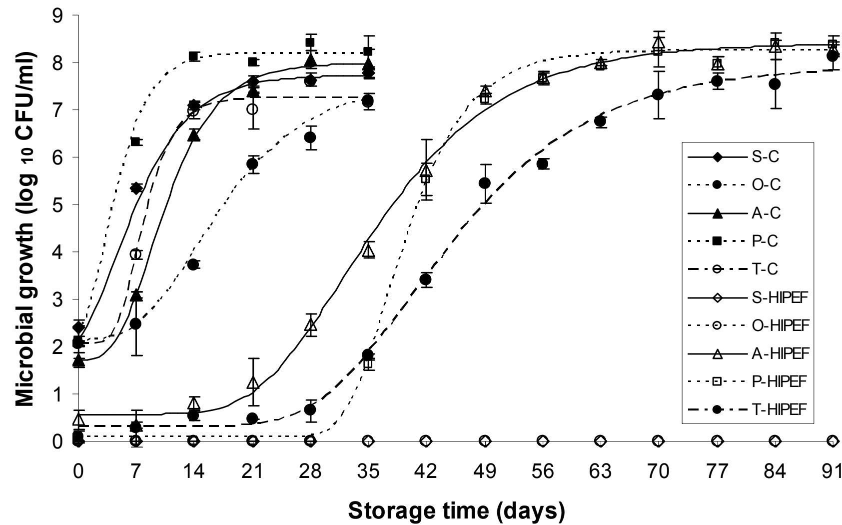

***Figure 3.**-Microbial growth of mesophilic microorganisms on control fruit juices (C) and HIPEF-treated fruit juices (HIPEF) during storage at 5º C for 91 days. S: strawberry; O: orange; A: apple; P: pear; T: tomato. Symbols and lines represent observed and modeled (modified Gompertz's equation) values, respectively. Symbols are means of four measurements ± SD.*

microbial populations in orange juice were totally inactivated (< 1 $\log_{10}$ CFU/ml) when HIPEF with or without antimicrobials and thermal treatment were applied; since microbial growth was not detected during 91 days of storage at 5º C (Table 5, Figures 3-5). Similar results were reported by Jia *et al.* (1999), Yeom *et al.* (2000), Min *et al.* (2003b) and Elez-Martínez *et al.* (2006) in orange juice treated by HIPEF or thermally. Nevertheless, Yeom *et al*. (2000) and Min et al. (2003b) achieved to extend the microbiological shelf-life by more than 112 and 196 days, respectively. This could be attributed to the increase of the temperature of the orange juice during HIPEF processing (between 58 and 60ºC); which could contribute in the inactivation of some microorganism, especially bacteria, because are less sensible to the heat than yeasts. In our study, treatment temperature did not exceed 40ºC, thus ensuring that all microorganisms were inactivated due to electroporation as also reported by Elez-Martínez *et al*. (2006).

Although, the microbiological shelf-life by combining HIPEF and antimicrobials have not been previously studied, the effect of both treatments on the naturally occurring microorganisms in orange juice has been reported by Hodgins *et al.* (2002). They reduced microbial populations of spoiled intentionally orange juice by 6.83 $\log_{10}$ (CFU/ml) when HIPEF (80 kV/cm for 60 µs at 44ºC) was combined with nisin (0.4%).

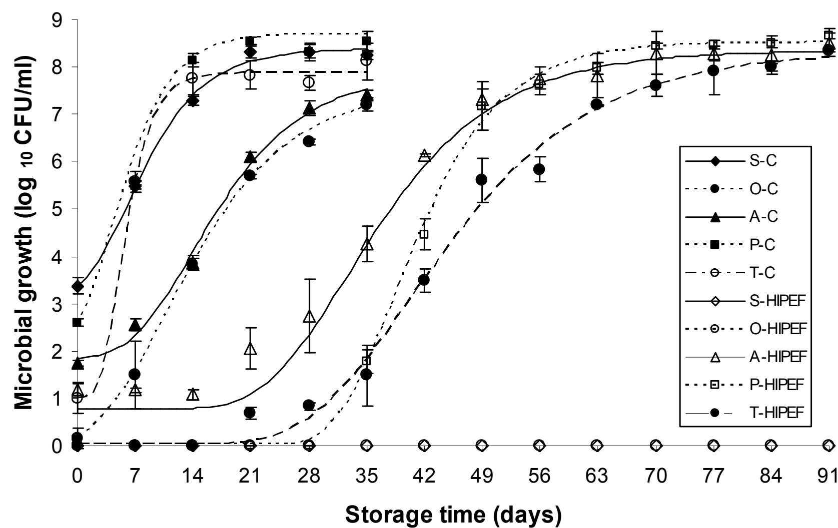

Figure 4.-*Microbial growth of molds and yeasts on control fruit juices (C) and HIPEF-treated fruit juices (HIPEF) during storage at 5º C for 91 days. S: strawberry; O: orange; A: apple; P: pear; T: tomato. Symbols and lines represent observed and modeled (modified Gompertz's equation) values, respectively. Symbols are means of four measurements ± SD.*

Microbiological behavior and shelf-life in apple juice

A similar initial microbial load of mesophilic, psychrophilic and, mold and yeast populations were observed in unprocessed apple juice (Table 3, Figures 3-5). But, shorter λ and higher μ_{max} values for mesophilic and

Table 5.-Microbiological shelf-life of fruit juices submitted to HIPEF treatment with or without antimicrobials and thermal treatment stored at 5ºC for 91 days.

Fruit juice	Process	MSL (days) [a]		
		Mesophilic	**Mold & Yeast**	**Psychrophilic**
Strawberry	HIPEF	> 91	> 91	> 91
	HIPEF + CA	> 91	> 91	> 91
	HIPEF + CBO	> 91	> 91	> 91
	Thermal	> 91	> 91	> 91
Orange	HIPEF	> 91	> 91	> 91
	HIPEF + CA	> 91	> 91	> 91
	HIPEF + CBO	> 91	> 91	> 91
	Thermal	> 91	> 91	> 91
Apple	HIPEF	39.86	40.21	40.97
	HIPEF + CA	> 91	> 91	> 91
	HIPEF + CBO	> 91	> 91	> 91
	Thermal	> 91	> 91	> 91
Pear	HIPEF	42.19	43.57	43.02
	HIPEF + CA	> 91	> 91	> 91
	HIPEF + CBO	> 91	> 91	> 91
	Thermal	> 91	> 91	> 91
Tomato	HIPEF	54.35	50.96	62.77
	HIPEF + CA	> 91	> 91	> 91
	HIPEF + CBO	> 91	> 91	> 91
	Thermal	> 91	> 91	> 91

[a] Estimated microbiological shelf-life according to Equation 2; **CA**, citric acid; **CBO**, cinnamon bark oil.

psychrophilic microorganisms than molds and yeasts to indicate their predominance in the control apple juice throughout the storage time. Hence, the former populations were the main limiting microorganismos of the shelf-life in those control samples. Owing to the composition of the apple juice, the main spoilage microflora is constituted by lactic acid bacteria, molds and yeasts. Species of yeasts such as *Saccharomyces*, *Zygosaccharomyces*, *Candida* and *Pichia* have frequently been isolated of apple juice (Deak and Beuchat 1996). But, when HIPEF was applied to apple juice these initial microbial populations were reduced, and the remaining microbial populations were inactivated or injured at least 21 days, as reflected in the λ values (Table 4). From that day, microbial growth was observed, and mesophilic populations more than mold and yeast

populations limited the shelf-life of the apple juice (Table 5). Evrendilek *et al.* (2000) reduced the initial mesophilic (3.21 log_{10} CFU/ml) and molds and yeasts (3.40 log_{10} CFU/ml) populations at 1.28 and 3.05 log_{10} (CFU/ml), respectively, after HIPEF treatment of 35 kV/cm for 94 µs at 27ºC was applied. In addition, these authors achieved to prolong the shelf-life of apple juice up to 37 days of storage at 4ºC; being molds and yeasts populations the limiting main flora of the microbiological shelf-life. This behavior is contrary to the observed in our study, because Evrendilek *et al.* (2000) obtained a higher level of molds and yeasts than mesophilics after HIPEF applied.

Nonetheless, when apple juice was treated thermally or by HIPEF and citric acid (1.5%) or cinnamon bark oil (0.1%), a total reduction and inactivation of the natural microflora for 91 days at 5º C was observed (Figures 3-5). These results demonstrated that the addition of antimicrobial substances to the apple juice were able to synergistically act with HIPEF treatment to completely inhibit and inactivate both vegetative cells and their spores by more than 91 days in refrigerated storage. The antimicrobial activity of the citric acid is attributed at its undissociated form, which is achieved when the medium pH (2.92) is below the pKa value (3.14) of the acid molecule. Once cell inside, the acid molecules are forced to dissociate into charged anions due to the near-neutral pH of the cell cytoplasm, and a gradual decrease in intracellular pH may occur and end in cell inactivation by damage in cell signaling, active transport and genetic material (Stratford and Eklund 2003). On the other hand, although the mechanism of antimicrobial action of cinnamon oil on the microbial cells is still unclear, several targets have been proposed. Wendakoon and Sakaguchi (1995) and Burt (2004) have indicated that the interaction of carbonyl group of the cinnamaldehyde, main compound of cinnamon oil from bark, on the cell proteins inserted in the cytoplasmatic membrane appear to inhibit the action of the enzymes amino acid decarboxylases, which are necessary for the amino acids biosynthesis and biodegradation. Oussalah *et al*. (2006) reported a release of the cell constituents, a decrease of intracellular ATP concentration and a decrease in intracellular pH due to an increase in the permeability of cell membrane when cinnamon oil was applied up to 0.1 %. Nonetheless, these authors did not observe an apparent change on the cell surface (by electron micrographs) when cinnamon oil was added. Likewise, Gill and Holley (2004; 2006) reported a rapid decrease of cellular ATP but no increase of extracellular ATP when 0.15 or 0.6 % of cinnamaldehyde was used. Thereby, the mechanism of action of the cinnamon oil and its main compound to inactivate microorganisms according to Gill and Holley

(2004; 2006) and Oussalah *et al*. (2006) appear to be related to the cell membrane from which a slight disruption seem to occur, causing dispersion of the proton motive force by leakage of small ions without leakage of larger cell components, such as ATP, which are subsequently degraded by the ATPase enzyme.

Nonetheless, when HIPEF is applied to fruit juices containing citric acid or cinnamon bark oil a higher antimicrobial effect take place, due to the pore formation on the cell membrane of the microorganism when electric fields are employed, which would permit a faster diffusion of both antimicrobial substances towards the cell inside and end in microbial death.

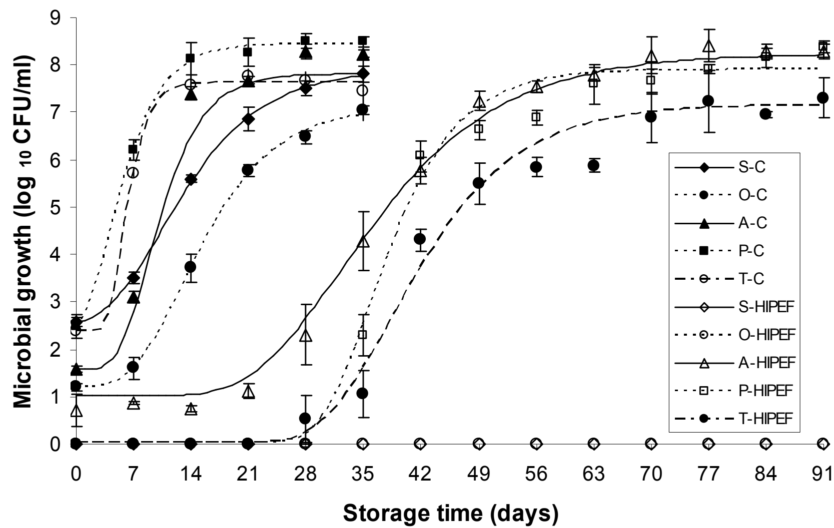

Figure 5.*-Microbial growth of psychrophilic microorganisms on control fruit juices (C) and HIPEF-treated fruit juices (HIPEF) during storage at 5º C for 91 days. S: strawberry; O: orange; A: apple; P: pear; T: tomato. Symbols and lines represent observed and modeled (modified Gompertz's equation) values, respectively. Symbols are means of four measurements ± SD.*

Microbiological behavior and shelf-life in pear juice

No significant predominance of any microbial populations in control pear juice was observed either at time 0 day or at end of its shelf-life (Table 3). Nonetheless, mesophilic microorganisms showed greater both μ_{max} and λ values than mold and yeast populations. The control pear juice was slightly limited by the growth of mesophilic populations, followed by mold and yeast and psychrophilic populations (Table 3). Corbo *et al.* (2004) indicated that mesophilic bacterial population in fresh-cut pear was constituted mainly by *Bacillus* spp., *Pseudomonas* spp. and *Enterobacter* spp., being the two latter the most dominants. They also indicated that yeasts populations such as *Cryptococcus* spp. and *Kloeckera* spp., and molds populations constituted an important part of the native microflora. Nevertheless, when pear juice was treated by HIPEF, those microbial populations were reduced to less than 1 $\log_{10}$ (CFU/ml) and, longer λ and lower μ_{max} values than those control juice samples were reached (Tables 3 and 4). The microbial growth in those HIPEF-processed pear juices began from the 35 days of storage at 5ºC and achieved levels above 10^7 CFU/ml from 42 days at 5ºC. This observed microbial growth is due probably to the germination of spores bacterial or ascospores of molds initially present in the juice, since HIPEF has demonstrated little effectiveness on these structures (Grahl and Märk 1996, Raso *et al.* 1998; Pagán *et al.* 1998). However, those microbial populations and /or their spores were inactivated by more than 91 days of storage at 5ºC when thermal treatment or HIPEF in combination with citric acid (1.5%) or cinnamon bark oil (0.1%) were applied (Figures 3-5).

Microbiological behavior and shelf-life in tomato juice

Mesophilic and psychrophilic microorganisms more than mold and yeast populations comprise the predominant flora in control tomato juice at time 0 day; since, microbial counts of 2.05 and 2.39 $\log_{10}$ (CFU/ml) of mesophilic and psychrophilic populations, respectively, and 1.00 $\log_{10}$ (CFU/ml) of molds and yeasts were found. However, the latter population exhibited shorter λ and higher μ_{max} values than those observed for mesophilic microorganisms in control tomato juice; therefore, they limited the shelf-life of the product more rapidly (Table 3). Min *et al.* (2003a) reported that the major microorganism in control tomato juice was yeast, when microscopic examination was carried out. Deak and Beuchat (1996)

indicated that molds and yeasts are the main spoilage microorganisms in juice products, due to their ability to survival and growth at low pH environments and rapidly degrade sugars and vitamins into secondary metabolites that may inhibit the growth of several bacteria.

Those microbial populations in tomato juice were reduced by less than 1 $\log_{10}$ (CFU/ml) at least 28 days at 5ºC when HIPEF was applied. From that moment, mesophilic and molds and yeast populations began to grow, but a higher μ_{max} in those molds and yeasts populations (Table 3) showed that their growth was much faster; and thus, limited the shelf-life of the tomato juice in a shorter time than mesophilic and psychrophilic populations (Table 5). That occurred microbial growth in HIPEF-processed tomato juice could be due to the germination of the surviving spores during storage time; since, several studies have demonstrated that HIPEF does not efficiently inactivate bacterial spores and ascospores of molds (Grahl and Märk 1996; Raso *et al.* 1998; Pagán *et al.* 1998). Ascospores of molds from *Byssochlamys nivea*, *Byssochlamys fulva* and *Neosartorya fischeri* isolated of spoiled tomato products was reported by Kotzekidou (1997). Likewise, Anderson (1984) found both vegetative cell and spores of *Bacillus coagulans* in tomato products. Whereas, Min *et al.* (2003a) reported the occurrence of spores and ascospores from *Bacillus* and molds and yeasts, respectively, in HIPEF-processed tomato juice by microscopic examination.

In this study, microbial counts in HIPEF-processed tomato juice achieved levels of 10^7 CFU/ml after 50 days of storage at 5ºC; being those molds and yeasts populations the first in reach such level as in the control sample (Table 5). Nguyen and Mittal (2007) reached to keep the microbial counts below 1 $\log_{10}$ (CFU/ml) at least 28 days of storage at 4ºC when processed tomato juice by HIPEF (87 kV/cm for 80 µs at 50ºC). Min et al. (2003a) reported a longer extension of the microbiological shelf-life in tomato juice (> 112 days at 4ºC) processed by HIPEF (40 kV/cm for 57 µs at 45ºC). This fact could be attributed to the kind of juice, because these authors used a tomato juice previously heated at 88ºC during 2 min and then cooled to 45ºC just before apply HIPEF treatment, thus, the majority of the native microflora and a part of the microbial spores could be destroyed by the previous thermal processing.

On the other hand, when tomato juice was thermally-processed or HIPEF-processed and citric acid (2.0%) or cinnamon bark oil (0.1%) a longer extension of the microbiological shelf-life (> 91 days at 5ºC) was found. These results demonstrated that the added antimicrobials substances had an inhibiter significant effect on the vegetative cells and their spores. A

similar reduction of the naturally occurring microflora of tomato juice processed by HIPEF (80 kV/cm for 40 µs at 50ºC) and nisin (0.4%) was reported by Nguyen and Mittal (2007); however, these authors did not report the effect of these combination of treatments on those microbial populations throughout the storage time.

Effect on the sensory characteristics

The influence of the HIPEF-processed fruit juices with or without antimicrobials, or thermally-processed on the sensory attributes (odor, color, taste, sourness and overall acceptability) in comparison with those unprocessed (control) fruit juice samples are showed in the Table 6.

Table 6.-Effect on the organoleptic characteristics of fruit juices treated under different processing conditions.

Fruit juice	Process	Sensory attributes [1]				
		Odor	Color	Taste	Sourness	Overall
Strawberry	Control	7.9 ± 1.0 ^{a}	8.2 ± 0.9 ^{a}	8.2 ± 0.9 ^{a}	7.9 ± 1.0 ^{a}	8.4 ± 0.4 ^{a}
	HIPEF	7.4 ± 1.2 ^{ab}	7.9 ± 1.2 ^{a}	6.8 ± 1.4 ^{b}	6.4 ± 1.2 ^{b}	6.7 ± 1.3 ^{b}
	HIPEF + CA	7.2 ± 0.9 ^{b}	7.6 ± 1.2 ^{a}	5.6 ± 1.4 ^{c}	5.2 ± 0.9 ^{c}	5.7 ± 1.2 ^{c}
	HIPEF + CBO	5.9 ± 0.9 ^{c}	7.9 ± 1.1 ^{a}	3.9 ± 1.7 ^{d}	4.5 ± 1.4 ^{d}	4.4 ± 1.0 ^{d}
	Thermal	7.9 ± 1.2 ^{a}	7.5 ± 1.4 ^{a}	5.4 ± 1.4 ^{c}	3.9 ± 1.2 ^{e}	5.4 ± 1.3 ^{c}
Orange	Control	5.6 ± 1.8 ^{a}	8.4 ± 0.8 ^{a}	6.0 ± 1.8 ^{a}	6.4 ± 1.5 ^{a}	6.2 ± 1.9 ^{a}
	HIPEF	6.1 ± 1.3 ^{a}	8.2 ± 1.0 ^{a}	5.8 ± 1.2 ^{a}	6.8 ± 1.2 ^{a}	6.1 ± 1.6 ^{a}
	HIPEF + CA	4.5 ± 2.2 ^{b}	7.8 ± 1.8 ^{a}	4.9 ± 1.7 ^{b}	4.5 ± 1.5 ^{b}	4.8 ± 1.9 ^{b}
	HIPEF + CBO	3.1 ± 2.1 ^{c}	7.3 ± 2.2 ^{a}	3.9 ± 1.7 ^{c}	5.3 ± 2.0 ^{b}	4.2 ± 1.7 ^{b}
	Thermal	5.8 ± 2.0 ^{a}	7.5 ± 1.8 ^{a}	6.6 ± 1.4 ^{a}	6.9 ± 1.2 ^{a}	6.5 ± 1.4 ^{a}
Apple	Control	7.1 ± 1.7 ^{a}	6.8 ± 1.3 ^{a}	7.6 ± 1.6 ^{a}	7.5 ± 1.6 ^{a}	7.9 ± 1.3 ^{a}
	HIPEF	6.4 ± 1.8 ^{ab}	6.4 ± 1.8 ^{a}	6.2 ± 2.1 ^{b}	6.9 ± 1.8 ^{a}	6.6 ± 1.8 ^{b}
	HIPEF + CA	6.0 ± 1.4 ^{b}	7.1 ± 1.3 ^{a}	2.9 ± 1.3 ^{c}	1.7 ± 1.7 ^{d}	2.9 ± 1.8 ^{c}
	HIPEF + CBO	2.7 ± 2.0 ^{c}	6.8 ± 1.5 ^{a}	3.5 ± 1.5 ^{c}	4.0 ± 1.8 ^{c}	2.9 ± 1.4 ^{c}
	Thermal	6.2 ± 1.7 ^{ab}	6.9 ± 1.5 ^{a}	5.8 ± 1.8 ^{b}	5.8 ± 1.5 ^{b}	5.9 ± 1.7 ^{b}
Pear	Control	6.9 ± 1.7 ^{a}	6.0 ± 1.6 ^{a}	7.2 ± 1.5 ^{a}	5.8 ± 2.3 ^{a}	7.3 ± 1.6 ^{a}
	HIPEF	6.2 ± 5.8 ^{ab}	6.0 ± 1.7 ^{a}	6.9 ± 1.5 ^{a}	6.1 ± 2.2 ^{a}	6.4 ± 1.9 ^{a}
	HIPEF + CA	6.1 ± 1.8 ^{ab}	6.8 ± 1.8 ^{a}	4.1 ± 2.1 ^{b}	2.9 ± 1.9 ^{b}	3.5 ± 2.1 ^{b}
	HIPEF + CBO	2.9 ± 2.4 ^{c}	5.6 ± 1.9 ^{a}	2.6 ± 1.7 ^{c}	3.6 ± 1.2 ^{b}	2.6 ± 1.2 ^{b}
	Thermal	5.8 ± 1.7 ^{b}	6.1 ± 1.6 ^{a}	6.8 ± 1.6 ^{a}	6.2 ± 2.2 ^{a}	6.6 ± 1.8 ^{a}
Tomato	Control	8.0 ± 6.1 ^{a}	6.1 ± 1.1 ^{a}	6.1 ± 2.0 ^{a}	5.7 ± 1.4 ^{a}	6.2 ± 1.5 ^{a}
	HIPEF	7.5 ± 1.9 ^{a}	6.1 ± 0.9 ^{a}	5.2 ± 1.7 ^{a}	5.4 ± 1.7 ^{a}	5.1 ± 1.9 ^{b}
	HIPEF + CA	5.8 ± 1.9 ^{b}	5.3 ± 1.2 ^{a}	2.2 ± 1.2 ^{b}	1.9 ±1.2 ^{d}	2.6 ± 1.2 ^{c}
	HIPEF + CBO	2.9 ± 1.8 ^{c}	5.9 ± 1.7 ^{a}	2.3 ± 1.8 ^{b}	3.7 ± 1.5 ^{c}	2.5 ± 1.5 ^{c}
	Thermal	7.4 ± 1.3 ^{a}	6.0 ± 2.0 ^{a}	6.0 ± 2.2 ^{a}	6.3 ± 1.3 ^{a}	5.9 ± 1.3 ^{ab}

[1] Values are the mean of thirty evaluations ± SD; **CA**, citric acid; **CBO**, cinnamon bark oil.
Different lower-case superscript letters (a, b, c, d, e) on the same column indicate significant differences (*P ≤ 0.05*) among processes by each sensory attribute and fruit juice.

Significant changes ($P \leq 0.05$) on the color of all processed fruit juices were not observed by the panelists. Likewise, significant differences ($P \leq 0.05$) on the odor of the HIPEF-processed fruit juices were not detected by the panelists in comparison with those control samples. Taste and sourness of HIPEF-processed orange, pear and tomato juices were not significantly different ($P \leq 0.05$) of those control samples. Odor in thermally-processed fruit juices were not meaningful affected ($P > 0.05$) with the exception of pear juice, where noticeable changes ($P \leq 0.05$) were perceived. Thermally-processed strawberry and apple juices significant affected ($P \leq 0.05$) their taste and sourness attributes. Combinations of HIPEF and citric acid or HIPEF and cinnamon bark oil had significant effects ($P \leq 0.05$) on odor, taste, sourness and overall acceptability of the assayed fruit juices, being the HIPEF-processed fruit juices containing cinnamon bark oil those of lowest score according to the panelists. In general, the HIPEF-processed fruit juices received the lowest impacts on the studied sensory attributes; whereas, fruit juices processed by HIPEF and antimicrobials significantly affected their organoleptic characteristics in comparison with those control juice samples.

CONCLUSION

The microbiological quality of the fruit juices processed by HIPEF with or without antimicrobials was assured at least 91 days of storage at 5ºC, in the same way that thermal treatment. Therefore, HIPEF treatment or their combinations with natural antimicrobials such as citric acid or cinnamon bark oil may be a good alternative to the heat pasteurization for obtaining fruit juices more stable and durable. However, in those fruit juices treated by HIPEF and antimicrobials, significant changes on some sensory attributes were detected; thus, further studies are required to evaluate combinations of treatments that improving the microbiological quality of the food while have the lowest impacts on those organoleptic properties.

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DISCUSIÓN GENERAL

La demanda de los consumidores por adquirir zumos de frutas frescos o sin tratamiento ha venido incrementándose en los últimos años. Este hecho es debido en parte al alto valor nutricional y organoléptico que ofrecen estos productos frescos, además de ser una parte importante en la dieta de los consumidores, ya que estos pueden aportar las vitaminas, minerales y antioxidantes que nuestro cuerpo necesita para mantener una vida más saludable y duradera. Sin embargo, si estos productos frescos no van acompañados de un procesamiento o cualquier paso efectivo de eliminación microbiana, entonces el riesgo de enfermedades asociadas a microorganismos irá en aumento (Balla y Farkas, 2006). De hecho, el número de brotes de enfermedades asociados a microorganismos patógenos por consumo de zumos de frutas sin pasteurizar ha aumentado también en los últimos años (Powell y Luedtke, 2000; CDC, 2007). Los patógenos emergentes más importantes y de mayor incidencia asociado a estos tipos de productos son *Escherichia coli* O157:H7, *Salmonella* spp. y *Listeria monocytogenes*. Aunque estos microorganismos patogénicos pueden ser eliminados fácilmente a través de la pasteurización térmica, las propiedades organolépticas, fisicoquímicas y nutricionales del alimento pueden ser afectadas extensivamente (Elez-Martínez y Martín-Belloso, 2005).

Por lo tanto, nuevas tecnologías de procesamiento no térmico de alimentos están surgiendo y siendo probadas para contrarrestar estos efectos negativos creados por el tratamiento térmico. Uno de ellos es el uso de pulsos eléctricos de alta intensidad de campo (PEAIC), el cual usa bajo consumo de energía y es capaz de inactivar microorganismos sin pérdidas significativas sobre los atributos nutricionales y sensoriales de los alimentos (Elez-Martínez y col. 2006; Cserhalmi y col. 2006). Aunque, actualmente, el uso de PEAIC está siendo aplicado a nivel industrial para unos pocos zumos de frutas, éste necesita garantizar aún un nivel de seguridad microbiológica similar o superior al que se obtiene con los métodos tradicionales. Por lo tanto, el objetivo de la pasteurización de los alimentos por PEAIC debe ser inactivar todos los microorganismos patógenos por al menos 5 unidades logarítmicas, y reducir o inactivar la carga de los microorganismos deteriorativos para extender la vida útil o tiempo de conservación del producto. Para lograr estas metas, es necesario cuantificar la eficacia de los PEAIC sobre los microorganismos, tanto patógenos como deteriorativos, de mayor preocupación en los alimentos. De este modo será posible establecer los parámetros de procesado que aseguren que el producto final cumpla con los requisitos establecidos por las autoridades sanitarias.

Efecto del tratamiento de PEAIC sobre las poblaciones de *E. coli* O157:H7, *S.* Enteritidis y/o *L. monocytogenes* inoculadas en los zumos de melón, sandía, manzana, pera, naranja, fresa y tomate.

La aplicación de los PEAIC a los zumos de frutas inoculados con microorganismos patogénicos fue efectiva para reducirlos o inactivarlos por más de 3 reducciones logarítmicas en los zumos de melón y sandía, y por más de 4 reducciones logarítmicas en los zumos de fresa, manzana, pera y tomate; mientras que el zumo de naranja pudo ser pasteurizado completamente por la aplicación de PEAIC, ya que se lograron reducir más de 5 unidades logarítmicas de las poblaciones de microorganismos patogénicos objetivo.

De los parámetros evaluados, el tiempo de tratamiento fue más influyente que la frecuencia de pulsos sobre las poblaciones de microorganismos patogénicos inoculadas en los zumos de frutas, ya que se encontraron valores más altos de *F-ratio* (Tabla 2 – Cap. I; Tabla 4 – Cap. III). En general, se obtuvieron mayores reducciones microbianas cuando se aplicaron tiempos de tratamientos más largos, independientemente del tipo de zumo de fruta y microorganismo empleado, sin embargo, estas reducciones microbianas no siguieron una cinética de primer orden o una relación lineal entre el logaritmo del número de supervivientes y el tiempo de tratamiento cuando el resto de los parámetros de procesamiento de PEAIC se mantuvieron constantes. En cambio, se observó un comportamiento logarítmico o cóncavo, es decir, una rápida reducción microbiana al comienzo del tratamiento y luego una reducción microbiana gradual cuando se aplicaron tiempos más largos de tratamiento. Este hecho, podría atribuirse a la existencia de una distribución de resistencias al tratamiento de PEAIC dentro de una misma población, causada posiblemente por los diferentes estados fisiológicos de cada célula microbiana dentro de la misma población bacteriana (van Boekel, 2002; Chen, 2007), por lo tanto, una parte de esa población pudo crear una mayor resistencia a PEAIC que el resto de la población y originar estas curvas de supervivencia con colas o “tailing”. Álvarez y col. (2003) reportaron que la presencia de colas en curvas de supervivencia pudiera ser debido a una acumulación de células muertas alrededor de esas células supervivientes que quedaron remanentes, sirviendo así de escudo contra el tratamiento. Mañas y col. (2001) indicaron que la heterogeneidad en la intensidad del tratamiento causada por la falta de distribución uniforme del campo eléctrico en la cámara de tratamiento o por la existencia de espacios

muertos donde los microorganismos no reciben el tratamiento, pueden ser las causas de colas en curvas de supervivencia.

En este estudio, el comportamiento aletargado de la inactivación microbiana por PEAIC comenzó en muchos casos a partir de 1250 µs de tiempo de tratamiento, a partir de ese tiempo, las reducciones microbianas conseguidas fueron poco significativas, por lo tanto, prolongar el tratamiento por mucho más tiempo bajo este fenómeno no sería deseable, ya que se observaría un aumento considerable de la densidad de energía. Este hecho es debido a que la densidad de energía es directamente proporcional al voltaje aplicado, la intensidad de corriente y el tiempo de tratamiento aplicado, e inversamente proporcional al volumen de las cámaras de tratamiento (Martín y col., 1994); de este modo, al aumentar el tiempo de tratamiento manteniendo constante el resto de los parámetros, entonces la densidad de energía recibida por el alimento será mayor (Tabla 3 - Cap. III).

Korolczuck y col. (2006) reportaron una relación directa entre la cantidad de energía recibida por el alimento y las reducciones microbianas. Este hecho pudiera ser atribuido al incremento de la temperatura del medio, el cual pudiera influenciar sobre las propiedades de fluidez de membrana, ya que a mayor temperatura (> 30ºC) los fosfolípidos de la bicapa lipídica están menos ordenados y la membrana celular tiene una estructura líquida cristalina (Arosson y Rönner, 2001), por lo tanto, una mayor susceptibilidad a la electroporación por tratamiento de PEAIC puede ser observada. Sin embargo, la temperatura durante el tratamiento de PEAIC no debiera exceder de 40ºC porque inactivación celular inducido por calor pudiera ser observado, ya que *E. coli* O157:H7, entre los microorganismos seleccionados, es sensible generalmente a temperaturas superiores a los 46ºC. Estas razones nos llevaron a elegir combinaciones de los parámetros de procesamiento de PEAIC que estén dentro del rango de mayor eficiencia del tratamiento de PEAIC para inactivar a los microorganismos sin efectos indeseables de calor.

Por el otro lado, aunque una mayor frecuencia de pulsos es pensado por causar una mayor inactivación microbiana, debido al mayor número de pulsos en modo bipolar por segundo aplicado sobre la célula, los resultados obtenidos en esta investigación revelaron que el efecto de la frecuencia de pulso dependió del tipo de microorganismo y zumo de fruta utilizado, y del número de veces o pases por el que el zumo de fruta fue sometido a PEAIC. *E. coli* y *Salmonella* en zumo de naranja y, *E. coli* y *L. monocytogenes* en zumos de melón y sandía, respectivamente, fueron reducidos a una frecuencia de pulso más alta, mientras que, *Salmonella* en zumo de sandía y

tomate fue reducido ligeramente a menores frecuencias de pulsos. Por lo tanto, más estudios son necesarios para entender el efecto de este parámetro de procesamiento de PEAIC sobre los microorganismos.

Por otra parte, la frecuencia de pulsos puede jugar un papel importante en la energía añadida al medio, porque un aumento de este parámetro produce un incremento en el poder eléctrico del sistema (Table 3 – Cap. III), y por ende, un aumento de la temperatura del medio. Por consiguiente, es muy importante controlar el rango de frecuencia de pulso a utilizar, porque un efecto indeseable de calor sobre los microorganismos podría ser inducido.

En esta investigación, combinaciones de tiempo de tratamiento y frecuencia de pulso tuvieron diferentes influencias sobre cada microorganismo en cada zumo de fruta a distintos niveles. Por lo tanto, un análisis de repuesta múltiple dado por el diseño experimental empleado fue necesario para encontrar los valores óptimos de estos parámetros. Está herramienta sirvió para encontrar los valores mínimos necesarios del tiempo de tratamiento y de frecuencia de pulso para obtener las reducciones más altas de *E. coli* O157:H7, *Salmonella* y/o *L. monocytogenes* en cada zumo de fruta estudiado.

Cuando los zumos de frutas inoculados con *E. coli* O157:H7, *Salmonella* y/o *L. monocytogenes* fueron procesados por PEAIC bajos condiciones óptimas de tratamiento. La población de *L. monocytogenes* mostró la mayor resistencia a los PEAIC seguido por *Salmonella* y luego *E. coli* O157:H7. Esta mayor resistencia de *L. monocytogenes* a los PEAIC es debido a la composición de su membrana y al tamaño celular. Varios autores han demostrado que, dentro de las bacterias, las Gram-negativas son más sensibles a los PEAIC que las Gram-positivas, debido a que poseen una capa gruesa de peptidoglicano, el cual le confiere una mayor rigidez de su membrana, y por ende, mayor resistencia (Castro y col., 1993; Qin y col., 1998; Aronsson y col., 2001). Además, el menor tamaño celular de *L. monocytogenes* le otorga una protección adicional contra los PEAIC, porque el voltaje inducido a través de la membrana celular es proporcional al tamaño geométrico de la célula (Qin y col., 1998).

La efectividad de los PEAIC sobre los microorganismos patógenos dependió además del pH del zumo de fruta evaluado, siendo más efectiva en los zumos de fruta de acidez más alta (naranja > fresa > manzana = pera = tomate > sandía = melón). Este hecho es debido al desequilibrio osmótico producido entre el citoplasma celular y el medio circundante, por el cual las células deben consumir más energía para mantener el pH del citoplasma cerca de la neutralidad. Este estrés producido por las altas concentraciones

de protones (H^+) circundante aunado a la aplicación de PEAIC, producirá a su vez, una mayor inactivación microbiana (Aronsson y Rönner, 2001), como ocurrió en el presente estudio, y el cual se explicará con detalle más adelante. Por lo tanto, estos microorganismos patógenos fueron más resistentes a la inactivación por PEAIC en los zumos de melón y sandiá que en los zumos de naranja, fresa, manzana, pera y tomate, debido a la menor acidez de los primeros zumos de fruta.

Efecto del tratamiento de PEAIC y ácido cítrico o aceite esencial de canela sobre las poblaciones de *Escherichia coli* O157:H7, *Salmonella* Enteritidis y/o *Listeria monocytogenes* inoculados en los zumos de melón, sandía, manzana, pera, naranja, fresa y tomate.

Antes de evaluar el efecto combinado de PEAIC y sustancias antimicrobianas, se evaluó primeramente el efecto de cada antimicrobiano (ácido cítrico o aceite esencial de canela) sobre los microorganismos patogénicos inoculados en el zumo de fruta; ya que el efecto del tratamiento de PEAIC fue evaluado previamente. Los antimicrobianos usados en esta investigación son sustancias generalmente reconocidas como seguras, de sus siglas en ingles "GRAS", por lo tanto, su uso en alimentos es permitido (USFDA, 2006; European Parliament and Council Directive Nr. 95/2/EC., 1995). Para evaluar el efecto bactericida de estas sustancias antimicrobianas, el ácido cítrico y el aceite esencial de canela fueron añadidos a las muestras de zumos de frutas inoculadas con los microorganismos patógenos y mantenidos por una hora con agitación continua a temperatura ambiente (22ºC). Los resultados indicaron que el ácido cítrico en concentraciones que variaron desde 0.5 hasta 2.0% no disminuyeron significativamente las poblaciones de *E. coli* O157:H7, *Salmonella* y/o *L. monocytogenes* (alrededor de 1.0 ciclo logarítmico) inoculadas en los diferentes zumos de frutas. Sin embargo, reducciones significativas fueron observadas cuando se usó el aceite esencial de canela (a partir de 0.1%). Estas diferencias observadas entre los antimicrobianos usados son debidas en parte, a la liposolubilidad de las sustancias, siendo el aceite esencial de canela más liposoluble que el ácido cítrico, el cual es de naturaleza hidrofílica. Esto le confiere al aceite esencial de canela una mayor ventaja como antimicrobiano sobre las poblaciones de patógenos, ya que éste puede difundir directamente a través de la bicapa lipídica de la membrana celular, y/o ganar acceso al periplasma y a las partes más profundas de la célula a través de proteínas de membrana denominadas

"porinas", el cual también ha mostrado permitir la entrada de sustancias lipofílicas de bajo peso molecular a tasas significativas a pesar de su naturaleza hidrofílica (Nikaido, 1996, Helander y col., 1998).

Cuando tratamiento de PEAIC fue usado en combinación con los antimicrobianos, se lograron reducir más de 5 unidades logarítmicas de los microorganismos patogénicos objetivo en todos los zumos de frutas estudiados, cumpliéndose así con lo propuesto por las Organizaciones Internacionales (USFDA, 2002). Además se obtuvieron efectos aditivos y sinergísticos por combinación de tratamientos.

Las concentraciones mínimas de ácido cítrico en combinación con tratamiento de PEAIC para inactivar a las poblaciones de microorganismos patogénicos por más de 5 unidades logarítmicas en los zumos de frutas, estuvo relacionado a la forma no disociada de la molécula de ácido, el cual es asumida por poseer la actividad antimicrobiana (Stratford y Elkund, 2003). La molécula de ácido cítrico permanece en forma no disociada cuando el pH del medio se encuentra por debajo del valor de la constante de disociación del ácido (pKa), en este caso, ese valor corresponde a 3.14. Por lo tanto, menores concentraciones de ese ácido fueron requeridas en los zumos de naranja y fresa que en los zumos de melón, tomate, sandía, manzana y pera. Como el ácido cítrico es una molécula hidrofílica, es decir, de baja solubilidad lipídica, su acceso al interior celular está limitado a proteínas de naturaleza hidrofílica (porinas) que se encuentran insertadas en la membrana externa de las bacterias Gram negativas o a través de la gruesa capa de peptidoglicano de las bacterias Gram positivas (Davidson, 2001). Sin embargo, cuando PEAIC es aplicado, la formación de poros sobre la membrana celular de los microorganismos tiene lugar, como una consecuencia de la diferencia de potencial transmembrana entre la membrana celular y el campo eléctrico inducido (Coster y Zimmerman, 1975). Por lo tanto, la entrada de moléculas de ácido cítrico sin disociar hacia el interior celular ocurre más fácilmente y, una disminución del pH intracelular es producida por la disociación de la molécula de ácido en aniones cargados, el cual a su vez puede causar la inactivación microbiana por daños en: la señalización celular, transporte activo y material genético.

Por otro lado, las concentraciones mínimas necesarias de aceite esencial de canela en combinación con PEAIC para inactivar a los microorganismos patogénicos por más de 5 ciclos logarítmicos, dependió principalmente de la acidez del zumo de fruta utilizado, requiriéndose una menor concentración del aceite en aquellos zumos de frutas de mayor acidez (fresa = naranja < manzana = pera = tomate < sandía = melón). Burt (2004) indicó que la susceptibilidad de las bacterias a los aceites esenciales

incrementa con una reducción en el pH del medio, porque a pH bajo la hidrofobicidad del aceite aumenta, y en consecuencia una mayor tasa de difusión a través de la membrana celular puede ocurrir. Aunque el mecanismo de acción del aceite esencial de canela sobre células microbianas es aún incierto, una serie de posibles explicaciones han sido sugeridas. Una de ellas, es la interacción del grupo carbonilo del cinamaldehido, principal componente activo del aceite esencial de canela de corteza, sobre las proteínas de membrana para inhibir la acción de enzima amino descarboxilasa, el cual es necesaria para la biosíntesis y degradación de aminoácidos (Wendakoon y Sakaguchi, 1995; Burt, 2004). Otro posible mecanismo de acción es atribuido a una disminución de ATP y pH intracelular, como una consecuencia del incremento en la permeabilidad de la membrana celular, sin embargo, no se observaron cambios aparentes sobre la superficie celular de la membrana (Gill y Holley, 2004; Gill y Holley 2006; Oussalah y col., 2006). No obstante, cuando se aplican PEAIC, la formación de poros en la membrana celular pudo favorecer la difusión del aceite esencial hacia el interior celular y causar daños en las funciones celulares.

En este estudio, población de *L. monocytogenes* fue siempre más sensible al ácido cítrico o aceite esencial de canela y a su combinación con PEAIC, que poblaciones de *E. coli* O157:H7 y *Salmonella*. Este hecho es atribuido a la composición de la membrana de cada microorganismo, siendo *L. monocytogenes* la más sensible a las sustancias antimicrobianas, por que carecen de una membrana externa, el cual es más selectiva y menos permeable que la capa de peptidoglicano de las bacterias Gram-positivas, el cual es más permeable y porosa.

Efecto del tratamiento de PEAIC en combinación con ácido cítrico o aceite esencial de canela para extender la vida útil microbiológica de los zumos de frutas, y evaluar sus impactos sobre las propiedades sensoriales de estos productos.

El estudio sobre la vida útil microbiológica en cada zumo de fruta tratado por PEAIC y antimicrobianos, fue llevado a cabo sobre esas combinaciones óptimas de tratamientos para garantizar la inocuidad microbiana (más de 5 reducciones logarítmicas en los microorganismos patogénicos) en cada zumo de fruta. Basándonos en estos resultados, la vida útil desde un punto de vista microbiológico fue analizada.

Para ello, se modelizó el crecimiento microbiano ocurrido en las muestras de zumo con o sin tratamiento a través de la ecuación de Gompertz modificada por Zwietering y col. (1990) como sigue (Ecuación 1)

$$Log_{10}(CFU/ml) = k + A \cdot \mathbf{exp}\left\{ -\mathbf{exp}\left\{ \left[(\mu_{max} \cdot e) \cdot \left(\frac{\lambda - t}{A} \right) \right] + 1 \right\} \right\} \qquad (1)$$

donde Log_{10} (CFU/ml), es el número de microorganismos presentes en la muestra de zumo de fruta; k, es el nivel inicial de microorganismos (log_{10} CFU/ml); A, es la diferencia en log_{10} (CFU/ml) de microorganismos encontrados entre t = 0 días (población inicial) y la máxima densidad de población microbiana alcanzada en la fase estacionaria de crecimiento; μ_{max}, es la máxima tasa de crecimiento (Δlog_{10} (CFU/ml)/day); λ, es la fase de latencia; t, es el tiempo (días) y; e, es una constante de valor 2,7182.

Una vez estimados los parámetros de Gompertz, se procedió a calcular la vida útil microbiológica como sigue (Ecuación 2):

$$MSL(days) = \lambda - \frac{A \cdot \left\{ \mathbf{ln}\left[-\mathbf{ln}\left(\frac{Log_{10}(10^7 CFU/ml) - k}{A} \right) \right] - 1 \right\}}{\mu_{max} \cdot e} \qquad (2)$$

donde 10^7 *CFU/ml*, es el límite máximo de microorganismos permitido en estos tipos de alimentos al día de caducidad, según la regulación Española (B.O.E. 2001).

Los zumos de frutas sin procesamiento presentaron una carga microbiana inicial de entre 1,8 (sandía) y 3,2 (fresa) unidades logarítmicas, y la flora microbiana inicial predominante dependió del tipo de zumo y manipulación durante su procesamiento para la obtención del zumo. La aplicación de PEAIC sin antimicrobianos a los zumos de fruta logró extender la vida útil microbiológica por mucho más tiempo que en esas muestras controles o sin tratamiento, y reducir completamente la microflora inicial presente en el zumo, excepto en los zumo de manzana y pera, donde una parte de esa flora no pudo ser inactivada, por lo tanto, la extensión de la vida útil en estos zumos de fruta fue algo más corta (entre 39 y 42 días) que el resto de los zumos evaluados (de 50 a > 91días) tratados por PEAIC. Por otra parte, se logró inactivar totalmente a la población microbiana presente en los zumos de fresa y naranja por la aplicación de PEAIC sin

antimicrobianos por más de 91 días a temperaturas de refrigeración (5ºC). Asimismo, la flora microbiana en los zumos de sandía, melón y tomate tratados por PEAIC no fue detectada sino hasta después de aproximadamente 28 días de almacenamiento. A partir de ese momento, se observó crecimiento microbiano. Este crecimiento microbiano observado puede ser una consecuencia de la recuperación de células que estuvieron lesionadas por la aplicación de PEAIC, o por la germinación de esporas de microorganismos esporulados presentes en el zumo de fruta, ya que efectos pocos significativos sobre esas estructuras por aplicación de tratamientos PEAIC ha sido demostrada (Grahl y Märkl, 1996; Raso y col., 1998; Pagán y col., 1998). Sin embargo, cuando estos zumos de frutas fueron tratados con una combinación de PEAIC y ácido cítrico o aceite esencial de canela, estos microorganismos deteriorativos y/o sus esporas fueron inactivados totalmente por más de 91 días a temperaturas de refrigeración (5ºC). Este mismo comportamiento fue observado también en esos zumos de frutas tratados térmicamente. Por lo tanto, esta combinación de tratamientos (PEAIC y antimicrobianos) tiene el potencial de pasteurizar zumos de frutas al igual que con el método tradicional (tratamiento térmico) pero a temperaturas muchos más bajas.

Aunque, la calidad e inocuidad microbiológica de los zumos de frutas estudiados en esta investigación fue asegurada por la aplicación de PEAIC y ácido cítrico o aceite esencial de canela, de la misma manera que el tratamiento térmico, cambios significativos sobre algunas propiedades organolépticas como el olor y el sabor fueron detectados por los panelistas. En contraste, cuando PEAIC sin antimicrobianos fue aplicado a los zumos de frutas utilizadas, no se detectaron diferencias significativas en los atributos sensoriales cuando se comparó con las muestras de zumos de frutas sin tratamiento.

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CONCLUSIONES

CONCLUSIONES

- ✓ La optimización del tiempo de tratamiento y frecuencia del pulso, como factores de procesamiento de PEAIC, usando un análisis de respuesta múltiple, fue una herramienta necesaria para estimar la máxima reducción de una mezcla microorganismos patogénicos usando los valores mínimos de procesamiento.

- ✓ La efectividad de los PEAIC dependió principalmente del tipo de zumo usado, siendo más efectivo o letal contra los microorganismos patogénicos en los zumos de mayor acidez.

- ✓ La población de *Listeria monocytogenes* inoculada en zumo de melón y sandía fue más resistente a la aplicación de PEAIC que *E. coli* O157:H7 y *Salmonella* Enteritidis. Mientras que *S.* Enteritidis fue más resistente a los PEAIC que *E. coli* O157:H7 en los zumos de naranja, fresa, manzana y pera.

- ✓ La inactivación microbiana de *E. coli* O157:H7, *Salmonella* Enteritidis y/o *Listeria monocytogenes* inoculada en los zumos de frutas estudiados aumentó cuando se aplicaron tiempos más largos de tratamiento. Sin embargo, el efecto de la frecuencia de pulso fue menos concluyente, por lo tanto, más estudios son necesarios para evaluar el efecto de este parámetro en un rango de intervalo más amplio.

- ✓ El zumo de naranja resultó pasteurizado por la aplicación de PEAIC solo, ya que, se lograron más de 5 reducciones logarítmicas de *E. coli* O157:H7 y *S.* Enteritidis.

- ✓ Los zumos de melón, sandía, fresa, naranja, manzana, pera y tomate resultaron pasteurizados al combinar PEAIC con ácido cítrico o aceite esencial de canela.

- ✓ La concentración efectiva de las sustancias antimicrobianas que en combinación con PEAIC redujeron más de 5 unidades logarítmicas las poblaciones de microorganismos patógenos, dependió del pH del zumo; siendo menor su concentración en aquellos zumos de frutas de mayor acidez.

- ✓ La población de *E. coli* O157:H7 fue más resistente al tratamiento de PEAIC combinado con antimicrobianos que *S.* Enteritidis y/o *L. monocytogenes* en todos los zumos de frutas estudiados.

- ✓ Se logró una extensión de la vida útil microbiológica mucho más larga en los zumos de fruta tratados con PEAIC que en los zumos sin tratar. Cuando PEAIC fue combinado con ácido cítrico o aceite esencial de canela la vida útil microbiológica observada en los zumos fue aún mayor (> 91 días), siendo similar a esos obtenidos por tratamiento térmico.

- ✓ Las propiedades sensoriales de todos los zumos de frutas tratados por PEAIC sin antimicrobianos no fueron alteradas significativamente por el tratamiento cuando se comparó con esos zumos de frutas sin tratar.

- ✓ Aunque se garantizó la calidad e inocuidad microbiológica de los zumos de frutas tratados con PEAIC y sustancias antimicrobianas, se detectaron cambios significativos sobre algunas propiedades sensoriales tales como el sabor, el olor y la acidez.

INVESTIGACIONES FUTURAS

- ✓ Aplicar combinaciones de PEAIC con alguna sustancia natural antimicrobiana que tenga un menor impacto sobre las propiedades sensoriales del alimento, o aplicar mezclas de dos o más antimicrobianos en muy baja concentración para evitar alguna alteración perceptible en el sabor del alimento.

- ✓ Utilizar combinaciones de PEAIC con otras tecnologías no térmicas, como: altas presiones, pulsos de luz, luz UV, irradiación, etc., para lograr la pasteurización requerida por las Organizaciones Reguladoras.

- ✓ Estudiar el efecto de la frecuencia de pulsos sobre estos microorganismos patógenos en alimentos fluidos en un rango mucho más amplio, para conocer su efecto real sobre estos.