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Efecto de la Aplicación de Pulsos Eléctricos de Alta Intensidad de Campo sobre Enzimas y Vitaminas en Leche

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Capítulo 6

Reduction of protease activity in simulated milk ultrafiltrate by continuous flow high intensity pulsed electric field treatment

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ABSTRACT

A protease from *Bacillus subtilis* suspended in SMUF was subjected to HIPEF treatments of up to 6786.8 kJ/l applying field strengths from 19.7 to 35.5 up to 895.8 μs to evaluate the feasibility of this treatment on inactivating the enzyme. In addition, the influence of the pulse repetition rate (66.66, 88.88 and 111.11 Hz) and pulse width (4 and 7 μs) on the effectiveness of HIPEF treatments was tested. Protease activity was considerably reduced, a maximum inactivation of 62.7% was achieved after a 895.8-μs treatment at 35.5 kV/cm and 111.11 Hz. Protease activity decreased exponentially with the increase of the input energy density, treatment time and field strength when exposed to HIPEF processing. Moreover it was observed that the higher the pulse repetition rate the higher the inactivation. As regards pulse width, no significant changes in protease inactivation were observed if total treatment time was considered.

Keywords: High intensity pulsed electric fields, continuous flow system, protease, simulated milk ultrafiltrate.

INTRODUCTION

The purpose of all preservation methods is, initially, to inactivate the pathogen or spoiling microorganisms present in food. In the development of HIPEF technology, there are promising results about the inactivation of microorganisms inoculated in milk or SMUF and at this stage of research, the knowledge of the effects of HIPEF on other food spoiling products such as enzymes is essential.

Successful results on destruction of pathogenic microorganisms in SMUF or milk have been achieved by HIPEF processing (Bendicho et al. 2002a). Significant inactivation levels have been attained in several microorganisms inoculated in SMUF. When the treatment was applied to samples inoculated with *Escherichia coli, Lactobacillus delbrueckii, Bacillus subtilis,* and *Staphylococcus aureus,* reductions from 4 to 9 log cycles were reached (Zhang et al., 1995; Pothakamury et al., 1995a and 1995b; Qin et al., 1998).

As regards enzyme inactivation, few studies exist made in SMUF or milk. However, it has been observed that, in general, enzymes require more severe HIPEF treatment than microorganisms to obtain a significant reduction (Ho et al., 1997).

Some indigenous and endogenous enzymes from milk have been studied and controversial results have been obtained, whereas great inactivation can be achieved for some enzymes at certain conditions, no inactivation or even enhancement of the activity has been attained at different HIPEF conditions (Table 1). Variation in enzymatic activity depends on the electric field intensity, the treatment length, the treatment temperature, the HIPEF equipment characteristics, the type of enzyme, the enzyme concentration and the media containing the enzyme (Castro et al., 2001; Vega-Mercado et al., 1995 and 2001; Grahl and Märkl, 1996; Bendicho et al., 2002b and 2002c).

Castro et al. (1994) studied the HIPEF inactivation of alkaline phosphatase in SMUF, nonfat milk, 2% fat milk, and whole milk. HIPEF treatment was able to reduce its activity up to 65% in SMUF and in skim milk, but when 2% milk and whole milk were treated, ALP activity just was reduced up to 59% (Table 1). However, Grahl and Märkl (1996), Ho et al. (1997) and Van Loey et al. (2002) did not observe any significant enzyme reduction in either milk or an aqueous solution (Table 1). Another milk enzyme such as peroxidase, showed no inactivation when suspended in milk (Grahl and Märkl, 1996; Van Loey et al., 2002), whereas a significant reduction was observed when suspended in a phosphate aqueous solution (Ho et al., 1997) (Table 1).

Another dairy enzyme whose response to HIPEF processing has been evaluated is lipase. A lipase from *Pseudomonas fluorescens* suspended in SMUF could be inactivated up to 62.1% after a batch mode treatment. However, only a 13% inactivation was reached with a continuous process (Bendicho et al., 2002c) (Table 1).

The effect of HIPEF on dairy proteases is also being studied. The effect of HIPEF on plasmin was evaluated in SMUF by applying continuous HIPEF treatments and its activity was reduced a 90% (Vega-Mercado et al., 1995) (Table 1). As regards microbial proteases, Vega-Mercado et al. (2001) achieved up to 80% inactivation of an extracellular protease from *Pseudomonas fluorescens* by HIPEF processing when the enzyme was suspended in tryptic soy broth enriched with a yeast extract. Inactivation levels changed significantly when media was skim milk or casein-Tris buffer, where lower levels of inactivation were reached (Table 1). Bendicho et al. (2002b) studied the effect of HIPEF on a protease from *Bacillus subtilis* suspended in SMUF and in milk. When the protease was suspended in SMUF its activity decreased a maximum of 10% and 15% after a batch or a in-continuous treatment, respectively. However, when the enzyme was suspended in skim milk no

Table 1 Effects of high intensity pulsed electric field processing on milk enzymes

Enzyme	Media[1]	Treatment conditions	Effects	Reference
Alkaline phosphatase	SMUF Skim milk (SM) 2% fat milk (SSM) Whole milk (WM)	[2]18 8 and 22 kV/cm, up to 70 pulses	SMUF and SM 65% inactivation WM and SSM 59% inactivation	Castro et al , 1994
	Milk 1 5% fat Milk 3 5% fat	[2]21 5 kV/cm, up to 20 pulses	No significant effects	Grahl and Markl, 1996
	Buffer pH 9 8	[2]12-24 kV/cm, 30 pulses	Near 5% inactivation	Ho et al , 1997
	Milk	[2]6 7-20 kV/cm, up to 200 pulses	No inactivation	Van Loey et al , 2002
Lipase	Milk	[3]21 5 kV/cm, up to 20 pulses	Inactivation	Grahl and Markl, 1996
Lipase from *P fluorescens*	SMUF	[2]16 4 –27 4 kV/cm, up to 100 pulses	62% inactivation	Bendicho et al , 2002c
		[3]26 1-37 3 kV/cm, up to 100 pulses	13% inactivation	
Peroxidase	Milk	[2]21 5 kV/cm, 30 and 100 pulses	No significant effects	Grahl and Markl, 1996
Plasmin	SMUF	[3]15- 45 kV/cm, 10-50 pulses	Up to 90% inactivation	Vega-Mercado et al , 1995
	Potasium phosphate 100 mM	[2]12-22 kV/cm, 30 and 100 pulses	Near 30% inactivation	Ho et al , 1997
	Milk	[2]13-19 kV/cm, up to 200 pulses	<3% inactivation	Van Loey et al , 2002
Protease from *P fluorescens*	Casein solution (CS) Skim milk (SM)	[3]14-15 kV/cm, up to 98 pulses	SM 60% inactivation CS no significant effects	Vega-Mercado et al , 2001
		[3]25 kV/cm, up to 98 pulses	SM enzyme activation CS no significant effects	
Protease from *B subtilis*	SMUF Skim milk (SM)	[2]16 4 –27 4 kV/cm, up to 80 pulses	SMUF and SM no significant effects	Bendicho et al , 2002b
		[3]26 1-37 3 kV/cm, up to 84 pulses	SMUF 13% inactivation SM effects depended on the frequency	

[1]SMUF Solution similar to milk ultrafiltrate, [2]Batch mode HIPEF treatment, [3]Continuous mode HIPEF treatment

reduction in enzymatic activity was reported after a batch mode treatment and, when treatment was in continuous flow, the variation in activity depended on the frequency (Bendicho et al., 2002b) (Table 1). HIPEF treatments of higher input energy should be tried to evaluate the feasibility of improving the levels of enzyme inactivation achieved in the studies currently available. SMUF has been believed to be an ideal media to perform studies on enzyme inactivation, since it has very simple composition and would allow the study of the effect of electrical parameters without the influence of food components. So, in this study, the effectiveness of continuous HIPEF treatments of high energy inputs has been evaluate to inactivate a protease from *B. subtilis* in SMUF. Also, the influence of several parameters that are believed to be important on enzyme inactivation has been tested.

MATERIAL AND METHODS

Sample preparation

This study was performed in SMUF, that is a salt solution proposed by Jeness and Koops (1962) and whose salt composition is similar to that encountered in milk ultrafiltrates (Table 2).

Table 2. Composition of simulated skim milk ultrafiltrate (SMUF)[†] (Jeness and Koops, 1962).

Lactose	50.00
Potassium phosphate	1.58
Tri-potassium citrate	0.98
Potassium sulphate	0.18
Tri-sodium citrate	1.79
Calcium chloride dehydrate	1.30
Magnesium citrate	0.38
Potassium carbonate	0.3
Potassium chloride	1.00

[†] The values are expressed in g/l.

The protease from *Bacillus subtilis* was purchased in its liquid commercial form (Aldrich, Steinheim, Germany). According to the supplier information it contained 728 U/ml protease activity. Enzyme activity was expressed in mU/ml after verifying that there was a linear correlation between enzyme activity in mU/ml (from commercial information) and enzyme concentration in mg/ml.

Prior to apply the treatments, SMUF solution was added with 50 mU/ml of protease.

Enzyme determination

Reagents

Azocasein was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dihidrogenphosphate, disodium hidrogenphosphate and tricloracetic acid were from Prolabo (Fontenay S/Bois, France).

Solutions

Prior to run the analysis to determine protease activity, a pH 7.2 buffer solution was prepared mixing 36 ml of sodium hidrogenphosphate 5 mM and 14 ml of sodium dihidrogenphosphate 5 mM and diluting with distilled water until 1000 ml. This buffer was used to prepare a 1% azocasein solution. A 5% of tricloracetic acid aqueous solution was also required.

Protease activity determination

The assay for proteolytic activity using azocasein as substrate was described by Christen and Marshall (1984) and it has been validated for being used in SMUF by Bendicho et al. (2002d). It consisted on combining 1 ml of the azocasein solution with 100 µl of the sample that contains the enzyme. The contents of the tubes were mixed and incubated at 35.5 °C for 15 min. The reaction was stopped by the addition of 2 ml of 5% TCA. The absorbance of the supernatant fraction was read at 345 nm in a spectrophotometer UV/Visible (CECIL, CE 1021, England).

Pulsed electric field treatments

Pulse treatments were carried out using a in-continuous bench scale system (OSU-4F, Ohio State University, Columbus, OH, USA) (Figure 1), that held positive monopolar squared wave pulses. The treatment chamber device consisted on 8 colinear chambers disposed in series and everyone contained two stainless steel electrodes separated by a gap of 0.29 cm whose treatment volume was 0,012 cm^3 (Figure 1).

The flow rate of the sample was 60 ml/min, controlled by a variable speed pump (model 75210-25, Cole Palmer, Vernon Hills, IL, USA). The product was refrigerated in the space provided between the chambers by means of iced water. The temperature of the sample was recorded by a thermocouple and it never exceeded 40°C.

Treatments of up to 6786.8 kJ/l that consisted on apply field strengths of 19.7, 23.7, 27.6, 31.6 and 35.5 up to 895.8 µs were tested. Each pulse had a duration of 7 µs and the pulse repetition rate was set up at 66.66 Hz. On the other hand, samples were subjected to treatments of different pulse widths (4 and 7 µs) and different pulse repetition rates (66.66, 88.88 and 111.11 Hz) at the highest field strength (35.5 kV/cm) to check the influence of these parameters on the effectiveness of HIPEF treatments.

The input energy density supplied (Q, J/l) can be computed by Equation 1.

$$Q = \frac{V_0 \cdot I \cdot t}{v} \qquad \text{Equation 1}$$

where V_0 is the peak voltage (V), v the volume of the treatment chamber (l), I the intensity of the current (A) and t the treatment time (s).

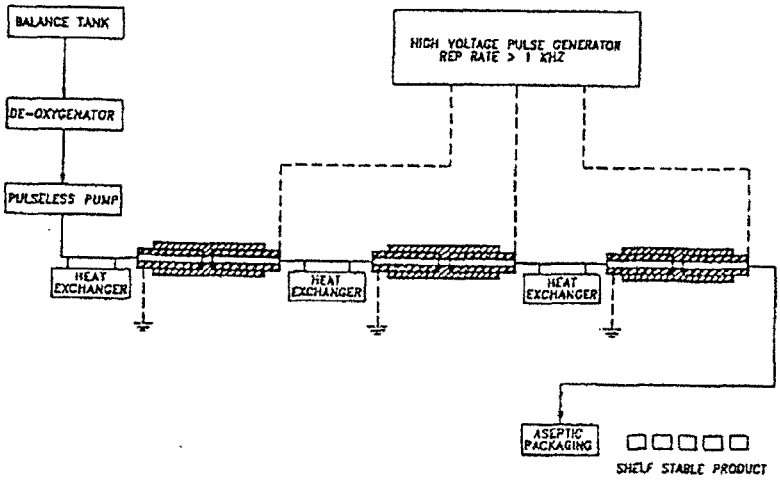

Figure 1. Scheme of the HIPEF continuous equipment of the University of Lleida (OSU-4F, Ohio State University, Columbus, OH, USA).

Statistics

Each processing condition was assayed in duplicate and enzyme determination was also performed in duplicate. Therefore, the results were averages of four measurements. Enzyme activity were expressed as residual relative activity [RA(%)] computed using Equation 2 and the percentage of enzyme inactivation I(%) can be calculated by Equation 3.

$$RA = 100 \cdot \frac{A_t}{A_o} \qquad \text{Equation 2}$$

$$I(\%) = 100 - RA \qquad \text{Equation 3}$$

where A_t is the enzyme activity in the sample after the treatment and A_o is the enzyme activity of the untreated sample, which was determined straight after processing, to avoid the effect of the storage time.

Analysis of variance was used to determine significant differences among the treatments ($P<0.05$) and was performed by Statgraphics plus version 3 for Windows package (Statistical Graphics Co., Rockville, MD, USA, 1997). All experimental values were adjusted to first order equations related to energy density (Equation 4), the treatment time (Equation 5) and also to the field strength (Equation 6).

$$RA = RA_0 \cdot e^{-k_1 \cdot Q} \qquad \text{Equation 4}$$

$$RA = RA_0 \cdot e^{-k_E \cdot t} \qquad \text{Equation 5}$$

$$RA = RA_0 \cdot e^{-k_t \cdot E} \qquad \text{Equation 6}$$

where RA_0 is the intercept of the curve (100%), k_I (l/kJ), k_E (μs^{-1}) and k_t (cm/kV) are first order constants, Q the input energy density (kJ/l), t the treatment time (μs) and E the field strength (kV/cm),

RESULTS

Protease activity decreased with any increase in the input energy density, treatment time, electric field intensity or pulse frequency ($P<0.05$). When SMUF samples were exposed to HIPEF treatments of up to 6786.8 kJ/l, it was observed that the enzyme activity lowered as the input energy density, treatment time and electric field intensity increased (Figure 2, 3 and 4). Maximum protease inhibition at 66.66 Hz was achieved after a 6786.8 kJ/l treatment that consisted on applying a HIPEF process of up to 895.8-μs at 35.5 kV/cm. Under these conditions, reductions up to 48% were attained (Figure 2, 3 and 4). Moreover, it was observed that the decrease of protease RA could be successfully related to the supplied energy densities (Q, Equation 1) using a first-order model (Equation 4) that led a k-value of $7x10^{-5}$ l/kJ (Equation 7, Figure 2).

$$RA = 100 \cdot e^{-7 \cdot 10\text{-}5 \cdot Q} \qquad (R^2 = 0.867) \qquad \text{Equation 7}$$

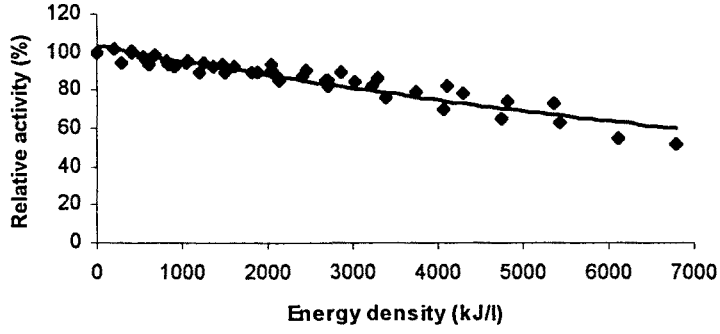

Figure 2. Relative protease activity (RA) in SMUF exposed to different inputs of electric energy densities (Q) supplied by high intensity pulsed electric field treatments. Plotted line is an exponential decay fit.

Figures 3 and 4 show the exponential decrease of the experimental RA values as a function of the treatment time (Equation 5) or field strength (Equation 6). The calculated rate constants (k_E and k_t) and the regression parameters of the fitted models ($P<0.05$) at the assayed electric field intensities or treatment times are reported in Table 3 and Table 4. The first order model related to the treatment time held R-squared statistic values from 0.821 to

0.979, indicating that the assayed model explains well enough the relationship between RA and the treatment time and the values of the resulting rate constants (k_E) increased from 0.00013 to 0.00068 μs^{-1}. In the case of the first-order model related to the field strength, it gave k_t values that raised from 0.0088 to 0.032 cm/kV, and their R-squared values were in a range from 0.729 to 0.967.

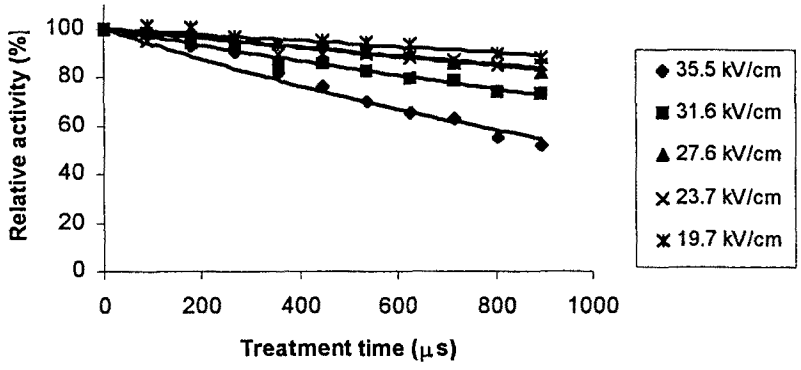

Figure 3. Inhibition of relative protease activity (RA) as a function of the treatment time after several continuous-flow high intensity pulsed electric field treatments. Protease was suspended in SMUF and the samples were exposed to field strengths from 19.7 to 35.5 kV/cm up to 895.8 μs at 66.66 Hz. Plotted lines correspond to the fit of experimental data to a first order model.

Table 3. Exponential decay model rate constants (k_E) for inhibition of protease activity in simulated milk ultrafiltrate by pulsed electric fields in continuous flow at different electric field intensities.

Electric field intensity (kV/cm)	k_E (μs^{-1})	R^2
19.7	0.00013	0.890
23.7	0.00021	0.821
27.6	0.00020	0.951
31.6	0.00036	0.979
35.5	0.00068	0.964

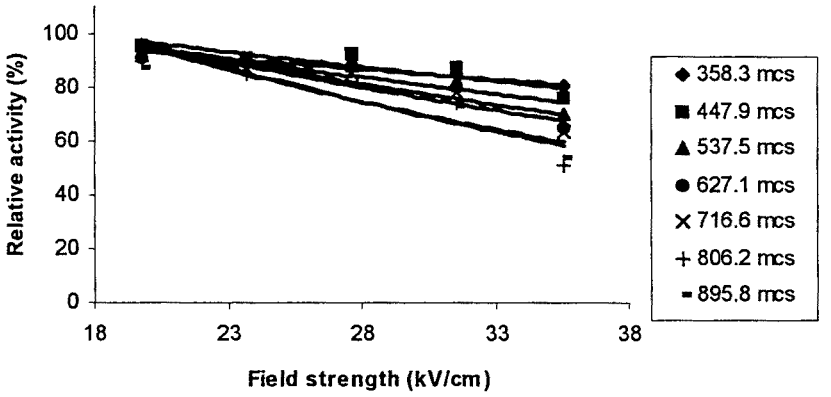

Figure 4. Inhibition of relative protease activity (RA) as a function of the field strength after several continuous-flow high intensity pulsed electric field treatments. Protease was suspended in SMUF and the samples were exposed to field strengths from 19.7 to 35.5 kV/cm up to 895.8 μs at 66.66 Hz. Plotted lines correspond to the fit of experimental data to a first order model.

Table 4. Exponential decay model rate constants (k_t) for inhibition of protease activity in simulated milk ultrafiltrate by pulsed electric fields in continuous flow at different treatment times.

Treatment times (μs)	RA_0	k_t (cm/kV)	R^2
358.3	110.88	0.0088	0.967
447.9	123.58	0.0124	0.793
537.5	129.27	0.0155	0.790
627.1	142.74	0.201	0.803
716.6	148.48	0.0222	0.867
806.2	193.4	0.032	0.729
895.8	171.07	0.0296	0.801

Keeping constant the electric field strength (35.5 kV/cm) and applying treatments of up to 895.8 μs, different frequencies and pulse width were tested. It was observed that when similar treatments in length and field strength were applied, the higher the frequency rate of pulse application, the greater the inactivation achieved. When treatments were applied at 111.11 Hz, inactivation up to 62.7% was reached, whereas at the same electrical conditions but at a frequency of 66.66 Hz just a reduction of 48% on enzyme activity was attained (Figure 5).

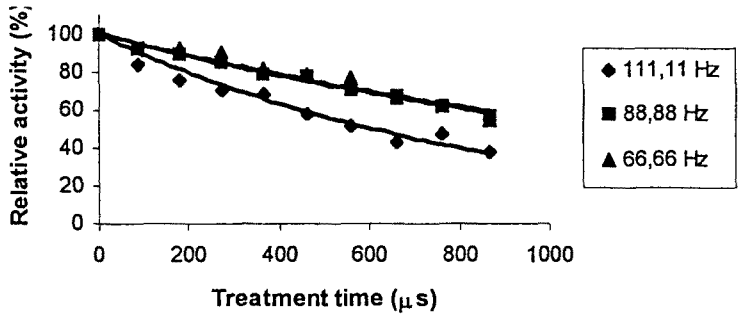

Figure 5. Inhibition of relative protease activity (RA) in SMUF exposed to 66.66, 88.88 and 111.11-Hz high intensity pulsed electric field treatments. Treatments were performed at 35.5 kV/cm.

As regards pulse width, no significant changes in the enzyme inactivation were observed if total treatment time is considered, whereas if the enzyme activity was related to the number of pulses there was a significant difference between the inactivation achieved using 4 or 7 μs pulse widths (Figure 6). It has to be noted that the 4 μs-pulse width process requires the application of a higher number of pulses than the 7 μs-pulse width process to achieve similar treatment times. So, the latter might be considered more effective because similar inactivation can be achieved with the application of less but longer pulses.

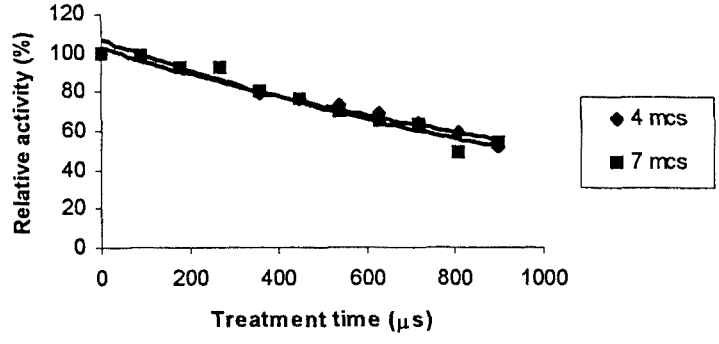

Figure 6. Inhibition of relative protease activity (RA) in SMUF exposed to high intensity pulsed electric field treatments of pulses of 4 and 7 μs of duration. Treatments were performed at 35.5 kV/cm.

DISCUSSION

HIPEF reduced the activity of this microbial protease, although the level of inactivation depended on the treatment applied. Protease activity decreased with an increase in the energy, treatment length, field strength and frequency. Activity was reduced a 48% after subjecting SMUF samples to 6786.8 kJ/l treatments (895.8 μs at 35.5 kV/cm) at a pulse repetition rate of 66.66 Hz. However, previous studies showed that when softer treatments (<500 kJ/l) were applied with the same in-continuous equipment, no significant inactivation was observed, neither using a batch mode equipment with a parallel plate treatment chamber (Bendicho et al., 2002b). However, up to 10-13% inactivation of this protease was achieved when it was suspended in SMUF after a 84-pulse treatment at 37.3 kV/cm using another in-continuous equipment whose treatment chamber had coaxial configuration (Bendicho et al., 2002b). Additional research about the effects of HIPEF on microbial enzymes was performed by Vega-Mercado et al., 1995a. In that case, when a protease from *Pseudomonas fluorescens* was suspended in skim milk, it could be inactivated up to a 60% with HIPEF treatments of 69.63 kJ/l. However, inactivation could be due to a thermal effect, since the treatment reached a temperature of up to 50°C. On the other hand, Bendicho et al. (2002c) studied the effects of HIPEF on another microbial dairy enzyme. In that case, a 62.1% of inactivation of a lipase from *Pseudomonas fluorescens* suspended in SMUF after a batch mode treatment of 504.97 kJ/l was repoted, whereas after a in-continuous process of 424.36 kJ/l lipase activity just decreased a 13%. In both batch and continuous processes, the treatment temperature never exceeded 35°C.

The activity of the evaluated protease decreased exponentially with the increase of the input energy densities applied, showing a similar pattern of inactivation than a lipase from *P. fluorescens* (Bendicho et al., 2002c). However, whereas the treatment of this protease with a continuous HIPEF treatment led a k-value of $7x10^{-5}$ l/kJ, the mentioned lipase gave lower k-values, that were $1.9x10^{-3}$ and $2x10^{-4}$, respectively, for a batch or a in-continuous mode HIPEF treatment. So the protease resulted more resistant to HIPEF than lipase since the lower the k-values of the first order equation the higher the energy densities required to reach a great extent of inactivation.

The depletion of protease also fitted to a first-order model related to the treatment time or the field strength. In the former case, the longer the treatment time the higher the inactivation achieved, and in the latter, the higher the field strength the higher the inactivation. The k-values (k_E and k_t) obtained from every adjustment increased with the processing field strength or with the treatment time, respectively. The inactivation of a lipase from *P. fluorescens* in SMUF using a batch HIPEF treatment also followed an exponential model with the treatment time or with the field strength, and as for the evaluated protease, the obtained k-values increased, respectively, with the field strength or the treatment time (Bendicho et al., 2002c). The activity of several vegetable enzymes also decreased exponentially with the treatment time or the field strength after HIPEF treatments (Giner et al., 2000 and 2001).

The frequency is another factor that is reported to affect on HIPEF effectiveness. In this case, among the frequencies tested (66.66, 88.88 and 111.11 Hz), the higher the pulse repetition rate the higher the inactivation achieved. Bendicho et al. (2002b) also reported a similar trend on the inactivation of the protease suspended in SMUF but applying treatments of much lower input energy densities using a continuous flow

equipment provided of a coaxial treatment chamber. In that case, treatments of up to 500 kJ/l and pulse repetition rates from 2 to 4 Hz were applied and it was observed that subjecting the samples to treatments of similar energy, the higher the frequency the higher the inactivation levels achieved. Other authors also reported differences on enzyme inactivation between treatments applied at different frequencies, Vega-Mercado et al. (2001) reported a reducton of up to 60% on a protease from *Pseudomonas fluorescens* suspended in milk using low field strengths (14-15 kV/cm) and frequencies of 1 and 2 Hz, whereas at lower frequencies (0.6 Hz) and higher field strengths (25 kV/cm) an enhancement of the proteolytic activity was observed. On a lipase from *P. fluorescens* suspended in SMUF, Bendicho et al. (2002c) observed that applying processes of similar energy, the higher the pulse repetition rate of the treatment the higher the level of enzyme inactivation. Since, a 80-pulse treatment at 37.3 kV/cm and 2 Hz led to near a 7% inactivation, whereas applying the same electrical conditions but at 3.5 Hz, the activity decreased up to a 13%.

Another factor that also could influence on protease inactivation was the pulse width. Considering the total treatment time, no significant changes in the enzyme inactivation were observed ($P<0.05$) between treatments of pulses of 4 or 7 μs of duration. However, it has to be noted that the longer the pulse width the lower the number of pulses required to reach the same inactivation levels, so the treatment of longer pulses may be considered more effective because with the application of less pulses, similar inactivation levels were achieved. Sensoy et al. (1997) also compared the effectiveness of treatments performed with pulses of different duration (2 and 8 μs) and noticed that similar inactivation levels were reached after treatments of a total duration of 127 μs using both 2 and 8 μs pulse width.

It is supposed that the effects on enzyme activity are due to the HIPEF treatment itself since the temperature did not increase enough to cause the decrease of enzyme activity and the influence of other media components is quite unlikely since SMUF is a very simple product. So, HIPEF might cause denaturation of the enzyme, probably by changing its conformational structure. Moreover, it is known that electric fields influence the conformational state of proteins through charge, dipole, or induced dipole chemical reactions (Tsong and Astunian, 1986). Charged groups and structures are highly susceptible to various types of electric field perturbations, and these changes cause the loss of its structure and consequently the loss of activity due to the difficulty of ensambling the substrate with the active site (Tsong, 1990).

CONCLUSIONS

In this paper it has been proved that the activity of this bacterial enzyme in SMUF can be reduced when increasing the input energy density of HIPEF treatments. HIPEF of up to 6786.8 kJ/l considerably reduced the activity of a protease from *B. subtilis*. The maximum inactivation (62.7%) was achieved after a 895.8-μs treatment at 35.5 kV/cm and 111.11 Hz. However, further investigation is required to know the effects of HIPEF on this enzyme when present in real food such as milk that would enable the study of the effects of food composition on enzyme inactivation. Moreover, biochemical studies are needed to

determine the three-dimensional structure of the enzyme and to evaluate how HIPEF affect on the internal configuration of the enzyme to decrease the enzymatic activity.

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Capítulo 7

Reduction of protease activity in milk by continuous flow high intensity pulsed electric field treatments

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ABSTRACT

High intensity pulsed electric fields (HIPEF) is a nonthermal food processing technology that is currently being investigated to inactivate microorganisms and certain enzymes with limited increase of food temperature. Promising results has been obtained on the inactivation of microbial enzymes from milk when suspended in SMUF.

The aim of this study was to evaluate the effectiveness of a continuous HIPEF equipment on inactivating a protease from *Bacillus subtilis* inoculated in milk. Samples were subjected to HIPEF treatments of up to 866 μs of squared wave pulses at field strengths from 19.7 to 35.5 kV/cm using a treatment chamber that consisted on 8 colinear chambers connected in series. Moreover the effects of different parameters such as pulse width (4 and 7 μs), pulse repetition rates (66.66, 88.88 and 111.11 Hz) and milk composition (skim and whole milk) were tested.

Protease activity decreased when increasing the treatment time or the field strength and the pulse repetition rate. As regards pulse width, no differences were observed between 4 and 7 μs pulses if total treatment time was considered. On the other hand, it was observed that milk composition affected on the results since higher inactivation levels were reached in skim than in whole milk. The maximum inactivation (81%) was attained in skim milk after a 866 μs treatment at 35.5 kV/cm and 111.11 Hz.

Keywords: High intensity pulsed electric fields, continuous flow system, protease, milk.

INTRODUCTION

The importance of the dairy industry involves that most of the HIPEF research is focused in studying the feasibility of using this technology to pasteurize milk and dairy products HIPEF process may achieve great levels of microorganism destruction (Dunn et al, 1987, Zhang et al, 1995, Pothakamury et al, 1995a, 1995b, Qin et al, 1998) maintaining the original flavour and nutrient content (Qin et al, 1995, Bendicho et al, 2002a, Grahl and Markl, 1996)

Most of the studies carried out on dairy products have been performed to evaluate HIPEF's effect on microorganisms The level of microbial inactivation achieved with HIPEF treatment mainly depends on the field strength and the treatment time (Qin et al, 1995, Pothakamury et al, 1997, Martín et al, 1997, Reina et al, 1998) As regards the effect of HIPEF on enzymes, some controversial results have been obtained In several cases, a large inactivation has been achieved, whereas in other cases no effect or even enhancement of the initial activity has been detected (Vega-Mercado et al, 1995, Vega-Mercado et al, 2001, Castro et al, 2001, Bendicho et al, 2002b, Bendicho et al, 2002c, Bendicho et al, 2002d) However, various process factors have been reported as influencing on enzyme activity The variation of enzyme activity after a HIPEF treatment depends on the field strength, treatment length, treatment temperature, equipment characteristics, type of enzyme and the media containing the enzyme (Castro et al, 2001, Vega-Mercado et al, 1995, Vega-Mercado et al, 2001, Bendicho et al, 2002b, Bendicho et al, 2002c, Bendicho et al, 2002d)

Some of the dairy enzymes whose behavior under HIPEF treatment has been evaluated suspended in SMUF or in milk itself are alkaline phospatase, plasmin, peroxidase and microbial enzymes such as proteases and lipases from *Pseudomonas fluorescens* or *Bacillus subtilis* (Castro et al, 2001, Grahl and Markl, 1996, Van Loey et al, 2002, Vega-Mercado et al, 1995, Vega-Mercado et al, 2001, Bendicho et al, 2002b, Bendicho et al, 2002c, Bendicho et al, 2002d)

Castro et al (2001) achieved up to 65% inactivation of alkaline phosphatase in SMUF or skim milk and up to a 59% when it was in 2% fat milk or in whole milk, although Grahl and Markl (1996) and Van Loey et al (2002) did not observe any significant activity depletion on this enzyme after a HIPEF process Grahl and Markl (1996) and Van Loey et al (2002) studied the effect of HIPEF on peroxidase, another milk enzyme, and no significant effects on its activity were observed after subjecting raw milk to different HIPEF treatments On the other hand, the activity of a dairy protease such as plasmin could be reduced up to a 90% in SMUF by applying continuous flow HIPEF treatments (Vega-Mercado et al, 1995)

Milk and dairy products may contain psychrotrophic microorganisms that can cause important problems in the dairy industry, since they can grow and maintain its activity even at refrigeration temperatures Among Gram-negative bacteria, the genera *Pseudomonas*, especially strains of *Pseudomonas fluorescens* are the most commonly encountered psychrotrophs in dairy products and *Bacillus* is representative of Gram-positive psychrotrophic bacteria (Muir et al, 1979, Richard, 1981, Cousin, 1982, Deeth and Fitz-Gerald, 1983, Driessen, 1983) These species may secrete enzymes such as lipases or proteases that may remain active even after pasteurization process The presence of lipases in milk causes a highly unpleasant rancid flavor mainly due to the liberation of butyric acid after the hydrolysis of triglycerides (Andersson et al, 1981) On the other hand, proteases degrade caseins, which implies losses on the yield of cheese, an increase of the nitrogen

content in the whey and technologically may cause diminution of milk thermal stability (Gebre-Egziabher et al., 1980; Veisseyre, 1988). Bendicho et al. (2002b) studied the effect of HIPEF processing on a lipase from *Pseudomonas fluorescens* suspended in SMUF and a maximum of 62.1% and 13% inactivation was reported for a batch and a continuous process, respectively, applying similar levels of energy densities. On the other hand, a protease also from *Pseudomonas fluorescens* was studied by Vega-Mercado et al. (2001) who achieved up to a 60% inactivation in skim milk but no significant effects were observed when the enzyme was suspended in a casein-Tris buffer.

The behaviour of a protease from *Bacillus subtilis* after exposure to HIPEF also was evaluated (Bendicho et al., 2002c; Bendicho et al., 2002d). Not very high inactivation levels were achieved on this enzyme on preliminary studies made in SMUF and milk applying HIPEF treatments in batch or continuous mode at energy densities of up to 500 kJ/l (Bendicho et al., 2002c). However, further investigations made with a continuous flow equipment but applying much higher input energy densities reached very promising results on the inactivation of this protease in SMUF (Bendicho et al., 2002d). So, the aim of this paper is to elucidate the effectiveness of high energy density continuous flow HIPEF treatments in inactivating this protease inoculated in skim or whole milk. The effects of media composition and PEF parameters (treatment time, field strength, pulse width and frequency) were also evaluated.

MATERIALS AND METHODS

Sample preparation

This study was performed in skim and whole milk provided by Granja Castelló S.A. (Mollerussa, Spain).

The evaluated protease from *Bacillus subtilis* was purchased in its liquid commercial form (Aldrich, Steinheim, Germany). According to the supplier information it contained 728 U/ml protease activity. Enzyme activity was expressed in mU/ml after verifying that there was a linear correlation between enzyme activity in mU/ml (from commercial information) and enzyme concentration in mg/ml.

Prior to apply the treatments, milk was added with 50 mU/ml of protease.

Enzyme determination

Reagents

Azocasein was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dihidrogenphosphate, disodium hidrogenphosphate and tricloracetic acid were from Prolabo (Fontenay S/Bois, France).

Solutions

Prior to run the analysis to determine protease activity, a pH 7.2 buffer solution was prepared mixing 36 ml of sodium hidrogenphosphate 5 mM and 14 ml of sodium dihidrogenphosphate 5 mM and diluting with distilled water until 1000 ml. This buffer was used to prepare a 1% azocasein solution. A 5% of tricloracetic acid aqueous solution was also required.

Protease activity determination

The assay for proteolytic activity using azocasein as substrate was described by Christen and Marshall (1984) and it has been validated for its use in SMUF, skim and whole milk by Bendicho et al. (2002e). It consisted on combining 1 ml of the azocasein solution with 100 µl of the sample that contains the enzyme. The contents of the tubes were mixed and incubated at 35.5 °C for 15 min. The reaction was stopped by the addition of 2 ml of 5% TCA. The absorbance of the supernatant fraction was read at 345 nm in a spectrophotometer UV/Visible (CECIL, CE 1021, England).

Pulsed electric field treatments

Pulse treatments were carried out using a in-continuous bench scale system (OSU-4F, Ohio State University), that held squared wave pulses. The treatment chamber device consisted on 8 colinear chambers disposed in series and everyone contained two stainless steel electrodes separated by a gap of 0.29 cm whose treatment volume was 0,012 cm^3. The flow rate of the process was adjusted to 60 ml/min and was controlled by a variable speed pump (model 75210-25, Cole Palmer, Vernon Hills, IL, USA). The product was refrigerated in the space provided between the chambers by means of iced water. The temperature of the entrance and exit of the treatment chamber was recorded by the equipment and it never exceeded 46°C.

Experiments were first carried out in skim milk to find out the conditions that held the maximum levels of enzyme inactivation. Samples were subjected to monopolar HIPEF treatments of up to 865.8 µs at field strengths from 19.7 to 35.5 kV/cm. The effectiveness of two different pulse widths (4 or 7-µs) and three pulse repetition rates (66.66, 88.88 and 111.11 Hz) was also tested. The effect of milk composition was evaluated comparing the results obtained with skim milk to those from exposing the samples of whole milk up to 865.8 µs at the maximum field strength (35.5 kV/cm) using 7-µs pulses at the three pulse repetition rates evaluated (66.66, 88.88 and 111.11 Hz).

The input energy density (Q, J/l) supplied to the samples can be computed by Equation 1.

$$Q = \frac{V_0 \cdot I \cdot t}{v} \qquad \text{Equation 1}$$

where V_0 is the peak voltage (V), I the intensity of the current (A), t the treatment time and v the volume of the total of treatment chambers (l).

Statistics

Each processing condition was assayed in duplicate and enzyme determination was also performed in duplicate. Therefore, the results were averages of four measurements. Enzyme activity were expressed as relative activity [RA(%)] computed using Equation 2 and the percentage of enzyme inactivation I(%) can be calculated by Equation 3.

$$RA = 100 \cdot \frac{A_t}{A_o} \qquad \text{Equation 2}$$

$$I(\%) = 100 - RA \qquad \text{Equation 3}$$

where A_t is the enzyme activity in the sample after the treatment and A_o is the enzyme activity of the untreated sample, which was determined straight after processing, to avoid the effect of the storage time
Analysis of variance was used to determine significant differences among the treatments (P<0 05) and was performed by Statgraphics plus version 3 for Windows package (Statistical Graphics Co , Rockville, MD, USA, 1997)

Experimental values were adjusted to a first order equation related to energy density (Equation 4)

$$RA = RA_0 \cdot e^{-k_1 Q} \qquad \text{Equation 4}$$

where RA_0 is the intercept of the curve (100%), k_1 (l/kJ) is a first order constant, and Q the input energy density (kJ/l)

RESULTS AND DISCUSSION

Studies to evaluate the influence of several HIPEF parameters (field strength, treatment time, treatment energy, pulse duration, and pulse frequency) on the effectiveness of HIPEF were initially performed in SM to avoid possible problems due to the fat presence. After having established the suitable conditions for inactivating the enzyme in skim milk, HIPEF treatments were applied to whole milk

The activity of the enzyme in skim milk decreased up to a 81 1% after applying the most severe evaluated treatment, a 865 8 μs treatment at 35 5 kV/cm and 111 11 Hz So, the evaluated HIPEF process conditions lead to considerable levels of enzyme inactivation It means that HIPEF caused enough changes on enzyme configuration to reach enzyme denaturation, and due to alteration of enzyme shape, the substrate cannot fit the active site, making them enable to convert substrates into products Changes on enzyme structure might be caused by changes on the conformational state of proteins due to the application of electric fields, since charged groups and structures are highly susceptible to various types of electric field perturbations, and these changes cause the loss of its structure and consequently the loss of activity due to the difficulty of ensambling the substrate with the active site (Tsong, 1990)

Effect of field strength, treatment time and input energy density

Protease activity varied with any change in the treatment time and electric field intensity (P<0 05) when skim milk samples were exposed to HIPEF treatments at 66 66 Hz Inactivation obtained at 19 7, 23 7 and 27 6 kV/cm showed analogous behavior patterns (P<0 05), reaching a maximum of 14 8% inactivation after a 865 8-μs treatment The maximum level of protease inactivation at 66 66 Hz was 37 9%, that was achieved after a 865 8 μs treatment at 35 5 kV/cm (Figure 1)

Bendicho et al. (2002d) studied the effects of similar HIPEF treatment on this protease but suspended in SMUF and they also observed that, in general, enzyme activity decreased with the field strength and the treatment time, although in that case, the maximum inactivation achieved at a pulse repetition rate of 66.66 Hz was of 48% after a treatment of 895.8 μs at 35.5 kV/cm. Other enzymes studied in milk or SMUF such as a lipase from *Pseudomonas fluorescens*, plasmin or phosphatase alkaline showed analogous patterns, since when subjected to HIPEF processing their activity decreased with the increase of the field strength and treatment time (Castro et al., 1994; Vega-Mercado et al., 1995; Bendicho et al., 2002b). However, the effect of HIPEF on other enzymes such as proteases from *Pseudomonas fluorescens* or from *Bacillus subtilis* did not show the same patterns, since depending on the HIPEF conditions, the effect varied from some decrease to no significant variation or even enhancement of the enzyme activity (Vega-Mercado et al., 2001; Bendicho et al., 2002c).

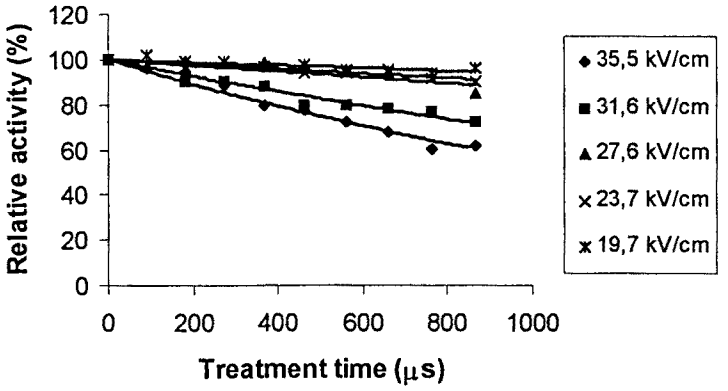

Figure 1. Inhibition of relative protease activity (RA) as a function of the treatment time after several continuous-flow high intensity pulsed electric field treatments. Protease was suspended in skim milk and the samples were exposed to field strengths from 19.7 to 35.5 kV/cm up to 865.8 μs at 66.66 Hz.

The most severe treatment evaluated at 66.66 Hz (35.5 kV/cm for 865.8 μs) delivered 6559.8 kJ/l to the samples, and lead to the maximum levels of enzyme inactivation (37.9%) (Figure 2), whereas a similar treatment of 6786.8 kJ/l in SMUF achieved up to a 48% inactivation (Bendicho et al. 2002d). However, treatments of lower energy (<500 kJ/l) did not held great levels of enzyme inactivation (0-13%) neither in milk nor in SMUF (Bendicho et al., 2002c). The activity of a lipase from *Pseudomonas fluorescens* also decreased with the increase of the input energy density. After a batch or a in-continuous mode treatment of 504.97 kJ/l and 424.36 kJ/l, respectively, up to 62.1% and 13% inactivation was achieved and in that case the treatment temperature never exceeded 35°C (Bendicho et al., 2002b).

The decrease of protease RA in skim milk could be successfully related to the supplied energy densities (Q) using a first-order kinetic model (Equation 4) leading a k-value of $6 \cdot 10^{-5}$ l/kJ (Equation 5, Figure 2).

$$RA = 100 \cdot e^{-6 \cdot 10^{-5} \cdot Q} \qquad (R^2 = 0.846) \qquad \text{Equation 5}$$

The decrease of protease activity with the increase of input energy when the enzyme was suspended in SMUF was also studied by Bendicho et al. (2002d). In that case, the activity also lowered exponentially with the applied energy density leading a k value of $7 \cdot 10^{-5}$ l/kJ, value that resulted similar to the obtained in this study when protease was suspended in skim milk. The decrease of enzyme activity with the input energy density after HIPEF treatments of another microbial enzyme such a lipase from *Pseudomonas* also fitted to a first-order model (Bendicho et al., 2002b); in that case, the k-values obtained were 1.9×10^{-3} l/kJ for a batch mode treatment and 2×10^{-4} l/kJ for the continuous one, indicating that protease is more resistant to HIPEF treatment since it requires the deliver of higher values of energy density to achieve the same levels of inactivation than lipase.

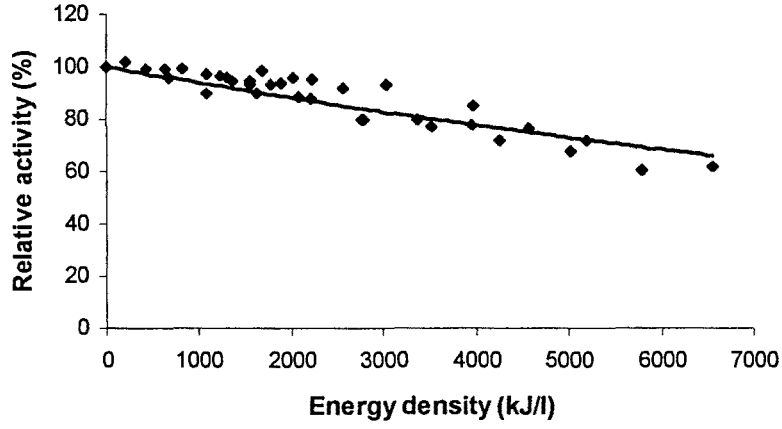

Figure 2. Relative protease activity (RA) in milk exposed to different inputs of electric energy densities (Q) supplied by high intensity pulsed electric field treatments. Plotted line is an exponential decay fit.

Effects of pulse duration

To evaluate the influence of the pulse duration on HIPEF effectiveness, treatments at 35.5 kV/cm up to 865.8 μs and 66.66 Hz using pulses of 4 or 7 μs were carried out. No significant changes in enzyme inactivation were observed if total treatment time was considered, whereas if the enzyme activity was related to the number of pulses, there was a significant difference between the inactivation achieved using 4 or 7-μs pulses. It has to be noted that the 4 μs-pulse width process requires the application of much more pulses than

the 7 μs-pulse width one to achieve similar cumulated treatment time. So, longer pulses might be considered more effective because similar inactivation can be reached with the application of less pulses (Figure 3). Bendicho et al (2002d) neither observed any significant difference between protease inactivation achieved subjecting the samples to processes of 4 or 7-μs pulses.

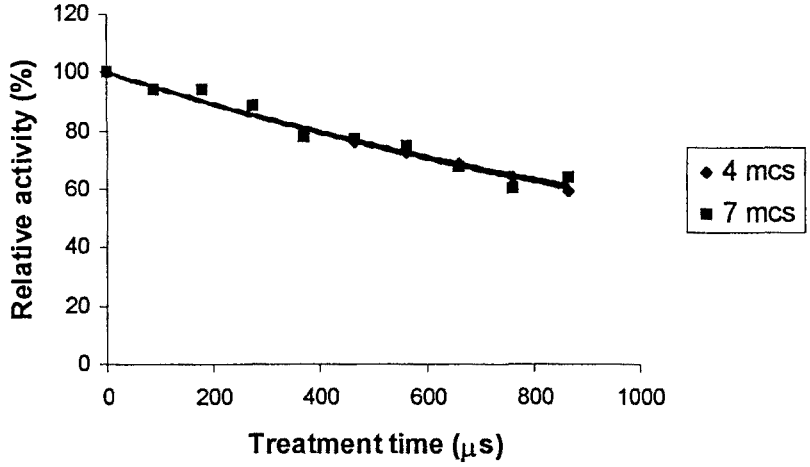

Figure 3. Inhibition of relative protease activity (RA) in skim milk exposed to high intensity pulsed electric field treatments of pulses of 4 and 7 μs of duration. Treatments were performed at 35.5 kV/cm.

Effects of pulse frequency

The effectiveness of HIPEF at different frequencies was studied in skim and whole milk. Maintaining constant the electric field strength (35.5 kV/cm) and applying the same treatment times, different pulse repetition rates (66.66, 88,88 and 111.11) were tested. For both skim and whole milk, it was observed that, when similar treatments in cumulated time and field strength were applied, the higher the frequency pulse rate the higher the inactivation achieved (Figure 4).

In skim milk, inactivation levels varied from 37.9% to 81.1% depending if treatments of 35.5 kV/cm for 865.8 μs where performed at 66.66 Hz or 111.11 Hz. In whole milk, both mentioned treatments led to 37.9% and 57.1% inactivation, respectively.

Temperature might not be the cause of inactivation increase, as other authors suggested (Van Loey et al., 2002) since maximum treatment temperature achieved at 66.66 Hz treatments was 34-37°C, and for 88.88 and 111.11 Hz was 39-44 and 41-46°C, respectively. These increases are not enough to justify the raise on enzyme inactivation, since previous studies on thermal inactivation of protease in skim milk showed that treatments of up to 30 min at 50°C only reached inactivation levels of about 12-13% (Bendicho et al., 2002c).

A similar study performed in SMUF held a similar trend, since the higher the frequency rate of pulse application the higher the inactivation obtained for treatments of equal field strength and treatment time, in that case, the maximum inactivation was reached after a 111.11 Hz treatment at 35.5 kV/cm and 865.8 μs that was 62.7% (Bendicho et al., 2002d).

Bendicho et al. (2002c) also reported a similar pattern of inactivation of the protease suspended in SMUF but applying treatments of much lower input energy densities using a continuous flow equipment provided of a coaxial treatment chamber. In that case, treatments of up to 500 kJ/l and pulse repetition rates from 2 to 4 Hz were applied and it was observed that subjecting the samples to treatments of similar energy, the higher the frequency the higher the inactivation levels achieved. Other authors also reported differences on enzyme inactivation between treatments applied at different frequencies, Vega-Mercado et al. (2001) reported a reduction of up to 60% on a protease from *Pseudomonas fluorescens* suspended in milk using low field strengths (14-15 kV/cm) and frequencies of 1 and 2 Hz, whereas at lower frequencies (0.6 Hz) and higher field strengths (25 kV/cm) an enhancement of the proteolytic activity was observed. On a lipase from *P. fluorescens* suspended in SMUF, Bendicho et al. (2002b) observed that applying processes of similar energy, the higher the pulse repetition rate of the treatment the higher the level of enzyme inactivation. Since, a 80-pulse treatment at 37.3 kV/cm and 2 Hz led to near a 7% inactivation, whereas applying the same electrical conditions but at 3.5 Hz, the activity decreased up to a 13%.

Effects of milk composition

The effectiveness of HIPEF on inactivating this protease was evaluated in skim and whole milk applying treatments of up to 865.8 μs at 35.5 kV/cm at three different frequencies (66.66, 88.88 and 111.11 Hz).

At the lower frequency (66.66 Hz), no significant differences were observed among the results of inactivation attained with SM or WM, ($P<0.05$). This similarity among the inactivation results of the two different media might be due to the low levels of inactivation achieved at this HIPEF treatment conditions (35-38%). On previous studies performed on this enzyme suspended in SMUF, Bendicho et al. (2002d) reported a similar inactivation pattern on inactivation after treatments of similar conditions of treatment time and field strength, although the maximum inactivation achieved in SMUF was slightly higher than the obtained in milk ($P<0.05$).

When HIPEF were applied at the intermediate (88.88 Hz) and the highest frequencies (111.11 Hz), more difference among the effectiveness of the treatments were observed, in those cases the inactivation obtained with WM was lower than that attained with skim milk, it should be due to the fat presence that interfere on the process protecting the enzyme and making its structure more stable. Whereas in WM up to a 38.9% inactivation was attained after a 865.8-μs treatment at 88.88 Hz, with SM up to 64.4% inactivation was reached. The highest levels of enzyme inactivation for both media were achieved when samples were subjected to 111.11 Hz treatments. In that case, samples of WM led to inactivation levels of about 57.1%, whereas in SM up to 81.1% inactivation was observed (Figure 4).

Bendicho et al. (2002c) also studied the effect the media composition on the effectiveness of HIPEF on inactivating this protease suspending it in SMUF or in skim milk. In that case, when HIPEF treatments were applied with a continuous equipment

provided by a coaxial chamber, enzyme activity varied from a slight inactivation when suspended in SMUF to some enhancement of enzyme activity when suspended in milk.

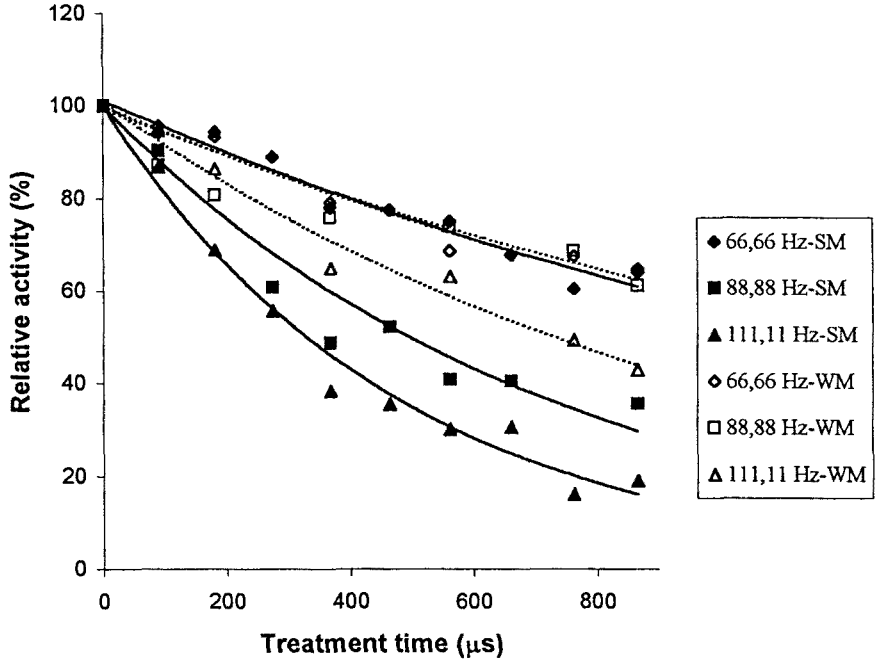

Figure 4. Inhibition of relative protease activity (RA) in skim milk (black) and whole milk (white) exposed to 66.66, 88.88 and 111.11-Hz high intensity pulsed electric field treatments. Treatments were performed at 35.5 kV/cm.

Other authors also reported differences among enzyme inactivation in different media. Castro et al. (1994) studied the HIPEF inactivation of ALP in SMUF, nonfat milk, 2% fat milk, and whole milk. HIPEF treatment was able to reduce up to 65% of ALP activity after 70 pulses at 22 kV/cm in SMUF, and at 18.8 kV/cm in skim milk. When 2% milk and whole milk were treated, ALP activity was reduced up to 59% after a 70-pulse treatment at 18.8 kV/cm. For microbial proteases, Vega-Mercado et al. (2001) achieved up to 80% inactivation of an extracellular protease from *Pseudomonas fluorescens* when the enzyme was suspended in tryptic soy broth enriched with a yeast extract after a HIPEF treatment of 20 pulses at 18 kV/cm at 0.25 Hz. Inactivation levels changed significantly when the media was skim milk; in that case, low field strengths (14 and 15 kV/cm) and frequencies of 1 and 2 Hz reached up to 40 and 60% reductions after 32 and 98 pulses, respectively. It is interesting to note that no significant inactivation of protease or any significant change in susceptibility of the casein to proteolysis was obtained in the casein-Tris buffer medium. This indicates that casein has a protective effect on protease against HIPEF treatment.

For microorganism destruction, proteins has been reported to have a protective effect (Martín et al., 1997). However, protease inactivation resulted more effective in a medium

with proteins, since among SMUF (fat and protein free solution) and SM (fat free emulsion) the highest levels of enzyme inactivation after similar treatments were achieved in SM. So, the presence of proteins may enhance the effect of HIPEF treatment (Bendicho et al., 2002d). However, the presence of fat has demonstrated to diminish the effect of HIPEF, since in WM less reduction of enzyme activity was observed than in SM after the same HIPEF treatments. Temperature cannot be the responsible of these changes on enzyme inactivation since the maximum temperatures reached treating WM or SM were similar (41-46 °C).

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Capítulo 8

Effect of high intensity pulsed electric fields and heat treatments on vitamins of milk

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ABSTRACT

The effects of high intensity pulsed electric field (HIPEF) treatments at room or moderate temperature on water-soluble (thiamine, riboflavin, ascorbic acid) and fat-soluble vitamins (cholecalciferol and tocopherol) were evaluated and compared with conventional thermal treatments. Vitamin retention was determined in two different substrates, milk and simulated skim milk ultrafiltrate (SMUF). Samples were subjected to HIPEF treatments of up to 400 μs at field strengths from 18·3 to 27·1 kV/cm and to heat treatments of up to 60 min at temperatures from 50 to 90 °C. No changes in vitamin content were observed after HIPEF or thermal treatments except for ascorbic acid. Milk retained more ascorbic acid after a 400 μs-treatment at 22·6 kV/cm (93·4%) than after low (63 °C-30 min; 49·7 % retained) or high (75 °C-15 s; 86·7% retained) heat pasteurisation treatments. Retention of ascorbic acid fitted a first order kinetic model for both HIPEF and thermal processes. First-order constant values varied from $1{\cdot}8 \times 10^{-4}$ to 127×10^{-3} μs^{-1} for the HIPEF treatments (18·3-27·1 kV/cm) and, for thermal processing ranged from 5×10^{-3} to 8×10^{-2} min^{-1} (50 °C-90 °C). No significant differences were found between the results obtained after applying HIPEF treatments at room or moderate temperature. However, results depended on the treatment media. A beneficial effect of natural skim milk components, mainly proteins, was observed on the preservation of ascorbic acid, since skim milk retained more ascorbic acid than SMUF after HIPEF treatments.

Keywords: Milk, vitamins, thiamine, riboflavin, ascorbic acid, tocopherol, cholecalciferol, pulsed electric fields.

INTRODUCTION

Milk is the most complete single food but, unfortunately, it has to be treated to be marketable. Treatments currently used consist on applying heat as low (63 °C, 30 min) or high pasteurisation (72-80 °C, 10-20 s), in-bottle sterilisation (110-120 °C, 20-30 min) or UHT sterilisation (135-140 °C, 2-4 s). However, all these thermal processes affect the nutrients originally present in raw milk.

Some of the vitamins present in raw milk are ascorbic acid, thiamine and riboflavin as water-soluble and cholecalciferol and tocopherol as fat-soluble.

Water-soluble vitamins are generally more sensitive to heat treatments than fat-soluble (Rechcigl, 1982). Among them, riboflavin is the most thermostable, only a 10 % destruction has been found when milk was sterilised (Ford *et al.* 1969; Haddad & Loewenstein, 1983; Lavigne, 1989). Thiamine may suffer a decrease of 25-50 % after in-bottle sterilisation. Haddad and Loewenstein (1983) reported a maximum destruction of 12 % after a heat treatment at 72 °C for 16 s. Ascorbic acid is the most heat sensitive vitamin. Losses of 40-60 % ascorbic acid in milk after an in-bottle sterilisation and about 20-40 % after UHT treatments have been reported (Van Eeklen & Heijne, 1965; Ford *et al.* 1969; Mottar & Noudts, 1979; Haddad & Loewenstein, 1983; Lavigne, 1989). After a pasteurisation treatment, Mottar and Noudts (1979) found a drop of 15 % and Rechcigl (1982) reported a reduction of 25 %.

Fat-soluble vitamins are, in general, thermostable. No losses of cholecalciferol have been observed after pasteurisation or sterilisation treatments of milk (Rechcigl, 1982). Tocopherols, are also heat-stable, but they are vulnerable to aggressive forms of oxygen. Their oxidation is accelerated at high temperatures (Macrae *et al.* 1993).

Since some milk components are unstable to heat, non-thermal technologies could be suitable to process milk avoiding adverse effects on flavour and nutrients. Among them, high intensity pulsed electric fields (HIPEF) might be adequate because it can achieve microbial stability of food similar to heat pasteurisation and preserves its original composition (Barbosa-Cánovas *et al.* 1999).

HIPEF treatment consists on applying very short electric pulses (μs) at high electric field intensities (10-50 kV/cm) keeping moderate temperatures during the treatment.

Some studies have been performed on skim milk and simulated skim milk ultrafiltrate (SMUF) in order know the effect of HIPEF on microorganisms. A 9-log reduction of *Escherichia coli* was achieved after 80 pulses of 40 kV/cm when it was inoculated in SMUF (Zhang *et al.* 1994), and 4-5 log reductions of *Lactobacillus delbrueckii*, *Bacillus subtilis*, *Staphylococcus aureus* and *Esch. coli* were reached after several pulses at a 16 kV/cm field intensity (Pothakamury *et al.* 1995*a*, *b*). Damaged cells of *Staph. aureus* produced by the electric treatment were observed by electronic microscopy (Pothakamury *et al.* 1996). Dunn and Pearlman (1987) achieved a 99·9 % inactivation of *Esch. coli* in milk after 23 pulses at 43 kV/cm and complete inactivation of *Salmonella dublin* after a 40-pulse treatment of 36·7 kV/cm. Martín *et al.* (1997) found that HIPEF treatment inactivated *Esch. coli* in skim milk following a first order kinetics for both electric field intensity and number of pulses. Michalac *et al.* (1999) also found a first order kinetics for the reduction of *Pseudomonas fluorescens*, *Lactococcus lactis*, and *Bacillus cereus*. Moreover, shelf-life of milk could be extended up to 14 days with a 40 kV- HIPEF treatment (Raso *et al.* 1999).

A synergistic effect between the temperature of the test medium and HIPEF treatment has been observed on microbial destruction. In SMUF, Zhang *et al.* (1995) observed that increasing the initial medium temperature from 7 to 20 °C, significantly increased microbial

inactivation of *Esch. coli* by HIPEF treatment. Pothakamury *et al.* (1996) subjected similar samples to HIPEF at selected temperatures ranging between 3 and 40 °C and got higher rates of inactivation of *Esch. coli* at high processing temperatures.

Having established that HIPEF treatment can destroy microorganisms in milk, it is important to evaluate the effects of this process on the nutritional quality of the treated product. The purpose of this work was to study the effects of this nonthermal process on the destruction of water-soluble (riboflavin, thiamine and ascorbic acid) and fat-soluble (cholecalciferol and tocopherol) vitamins in SMUF and milk at room or moderate temperature and also to compare the effect of HIPEF with thermal treatments. Experiments were performed in skim milk and SMUF to evaluate the effect of proteins and other minor natural components of milk on vitamin depletion.

MATERIALS AND METHODS

Sample preparation

This study was carried out in skim milk supplied by Granja Castelló, S.A. (Mollerusa, Lleida, Spain) and in SMUF (Jeness & Koops, 1962). The physico-chemical characteristics of milk were analysed according to A.O.A.C. (1990) and it held pH 6·86 ± 0·01, a conductivity of 5·19 ± 0·01 mS/cm and it had a composition of 48·08 ± 0·20 g lactose/l, 30·93 ± 0·22 g protein/l and 4·67 ± 0·19 g fat/l. SMUF is an aqueous solution composed by lactose and several salts and unlike milk it has no proteins. SMUF conductivity was adjusted to that of milk (5·2 mS/cm at 20 °C), in order to make equal their electrical properties. Conductivity increased up to 6·1 mS/cm for both media when samples were at 50 °C.

Before each treatment, multivitamin model systems of skim milk or SMUF were prepared. They contained 100 μg/ml of thiamine, riboflavin, ascorbic acid and tocopherol and 10 μg/ml of cholecalciferol (Sigma-Aldrich Química S.A., 28100 Alcobendas, Spain) to enable the evaluation of the effect of HIPEF or thermal treatments on both water and fat-soluble vitamins.

Vitamin determination

Determination of vitamins was performed by an HPLC system, consisting of a TM 600 controller pump, an in-line degasser and a 996 photodiode array detector (Waters Cromatografia S.A., Barcelona, Spain). Methods used in this study were modifications of the methods reported by Albalá Hurtado *et al.* (1997*a*) for water-soluble vitamins and by Albalá Hurtado *et al.* (1997*b*) for fat-soluble vitamins.

Water-soluble vitamins determination. Water-soluble vitamins were extracted using the method described and validated by Albalá Hurtado *et al.* (1997*a*) using 5 ml of milk. Chromatographic determination was performed using a Novapack C_{18} 3·9 × 150 mm column (Waters Cromatografia S.A.) and the mobile phase was composed by 30 % methanol and 70 % an aqueous solution.

Fat-soluble vitamins determination. Fat-soluble vitamins were extracted using the method described and validated by Albalá Hurtado *et al.* (1997*b*) using 10 ml of milk. HPLC quantification of vitamins was performed using a Waters Spherisorb S5 ODS2 C18 column (Waters Cromatografia S.A.) and methanol as mobile phase.

Linearity, sensitivity, precision and accuracy of both methods were validated (ISO 30, 1992) and were reliable for measuring water and fat-soluble vitamins in milk or SMUF. They were linear in the working range, that was from 0 to 10 μg/ml for cholecalciferol and up to 100 μg/ml for the other evaluated vitamins. Colechalciferol had a detection (DL) and a quantification limit (QL) of 0·216 μg/ml and 0·719 μg/ml and the other vitamins held DL and QL of 2·84-3·64 μg/ml and 9·48-12·07 μg/ml, respectively. Precision, measured as relative standard deviation, was between 2·35 and 5·7 2% and mean recoveries (accuracy) ranged between 80·26 and 99·17 %.

Pulsed electric field and thermal treatments

Pulse treatment was carried out using an exponential decay pulse TG-70 multiple trigger generator (Maxwell Technologies Physics International, San Leandro, California, USA). This generator has a capacitance of 0·1 μF. A stainless steel static parallel plate chamber with a 1·5 cm electrode gap and a volume of 12·4 ml was used. Pulses were applied manually and the chamber was refrigerated by air during the treatment.

The HIPEF treatments were performed at 20-25 °C and 50-55 °C to compare the effect of HIPEF at room temperature with the combination of HIPEF with moderate heating. It is known that transmittance decreases with increase of temperature. So, when treatments were performed at 50-55 °C, higher input voltages were applied (31·2, 38·5 and 46·2 kV) than at 20-25 °C HIPEF treatments (30, 37·5 and 45 kV) in order to reach equal electric field intensities for both HIPEF treatment conditions. These selected input voltages led to electric field intensities of 18·3, 22·6 and 27·1 kV/cm at both temperatures

Treatment time (t) was calculated as the product of the number of pulses (n) and pulse width (ι) (Equation 1). This was related to the capacitance of the generator (C) and the resistance of the sample in the treatment chamber (R) according to Equation 2. The resistance was 47 Ω at 20-25 °C and 38 Ω at 50-55 °C, so the number of applied pulses were in a range from 10 to 100 at 20-25 °C and from 14 to 118 at 50-55 °C in order to reach equal treatment times of 47, 91, 174, 252, 327, 400 μs.

$$t = \Sigma (\tau_i \cdot n_i) \qquad \text{Equation 1}$$

$$\tau = R \cdot C \qquad \text{Equation 2}$$

Several heat treatments including high (75 °C-15 s) and low (63 °C-30min) pasteurisation were applied to skim milk and SMUF to be used as reference values. Various temperatures and treatment times were studied (Table 1) to study the kinetics of vitamin retention when exposed to thermal treatments.

Table 1. Experimental conditions on heat treatment

Temperature (°C)	Time (min)
50 & 63	10, 20, 30, 45, 60
75 & 90	0.25, 1, 3, 5, 10

Data analysis

Each processing condition was assayed in duplicate and vitamin determination was also performed in duplicate. Therefore, the results were averages of four measurements. Vitamins contents were expressed as vitamins retention [VR (%)] computed using the following expression:

$$VR = 100 \cdot \frac{C_t}{C_o}$$

where C_t is the vitamin content in the sample after a treatment and C_o is the vitamin content of the untreated sample, which was determined immediately after processing, to avoid the effect of the storage time.

Analysis of variance was used to determine significant differences among the treatments (P = 0·05) and was performed by Statgraphics plus version 2.1 (Statistical Graphics Co., Rockville, MD, USA; 1996). Experimental values were fitted to linear (Equation 3), and first order kinetics (Equation 4) to determine the model that best described vitamin retention after treatments.

$$VR = VR_0 + k_0.t \qquad \text{Equation 3}$$

$$VR = VR_0 \cdot \exp(-k_1 \cdot t) \qquad \text{Equation 4}$$

where VR_0 is the intercept of the curve, t is the treatment time, k_0 the linear constant and k_1 the first order kinetic constant.

k-Values obtained from thermal treatments were fitted to the Arrhenius Equation (Equation 5).

$$k_1 = k_a \cdot \exp(-E_a/RT) \qquad \text{Equation 5}$$

where k_1 is the first order constant for each evaluated temperature (min^{-1}), k_a is the intercept of the curve, E_a the energy of activation (J/mol), R the constant of the ideal gases (8·31 J/°K·mol) and T the treatment temperature (°K).

All estimated parameters are given with their respective standard deviation, calculated as the product of the standard error of the estimates by the t-Student adjusted to the degrees of freedom.

RESULTS AND DISCUSSION

Neither HIPEF nor thermal treatments affected thiamine, riboflavin, cholecalciferol and tocopherol content of either SMUF or skim milk, since no significant differences (P > 0·05) were found between treated and untreated samples. However, Haddad and Loewenstein (1983) reported 9·4 % and 1·5 % destruction of thiamine and riboflavin in milk, respectively, applying a 80 °C-16 s treatment.

Ascorbic acid was the only vitamin that suffered some destruction. Reduction depended significantly on field strength (for HIPEF processing) or the treatment time, media and temperature (for thermal treatments). Treatment temperature had no influence on the inactivation of ascorbic acid by HIPEF, since there were no significant differences between the destruction attained at 20-25 °C or 50-55 °C HIPEF treatments (Table 2).

Table 2. ANOVA test for the relative ascorbic acid content in milk or skim milk ultrafiltrate treated by high intensity pulsed electric field.

Effect/Interaction[†]	Sum of Squares	Df	F-Value	p-Value
S	1645.65	1	91.09	0.00001
E	3840.57	2	113.30	0.00001
T	3.018	1	0.18	0.6740
t	9566.1	6	94.07	0.00001
S – E	397.18	2	11.72	0.00001
S – T	0.0655	1	0.00	0.9505
S – t	522.361	6	5.14	0.0001
E – T	31.7556	2	0.94	0.3954
E – t	1202.06	2	5.91	0.00001
T – t	27.3827	6	0.27	0.9500
S – E – T	20.9392	2	0.62	0.5413
S – E – t	141.968	12	0.70	0.7498
S – T – t	49.6338	6	0.49	0.8158
E – T – t	68.899	12	0.34	0.9799

[†]S, substrate; T, treatment temperature; E, electric field intensity; t, treatment time.

Retention of this vitamin after a 400-μs HIPEF treatment was always higher than 72·4 % or 61·0 % in skim milk or SMUF, respectively (Fig. 1). However, Grahl and Märkl (1996) reported only a 10 % retention after processing milk by HIPEF (non-specified conditions). Milk held the highest rates of ascorbic acid retention after applying the intermediate electric field strength evaluated (22·6 kV/cm) (Fig. 1). After a 400 μs-treatment at 22·6 kV/cm, milk retained more ascorbic acid (93·4 %) than after low (63 °C-30 min; 49·7% retained) or high (75 °C-15 s; 86·7 % retained) heat pasteurisation treatments. When applying the highest electric field intensity (27·1 kV/cm), which destroys microorganisms and enzymes more effectively (Martín *et al.* 1997; Bendicho *et al.* in press), samples subjected up to 252 μs, retained similar ascorbic acid levels than that observed after processing milk by high traditional heat pasteurisation (75 °C–15 s).

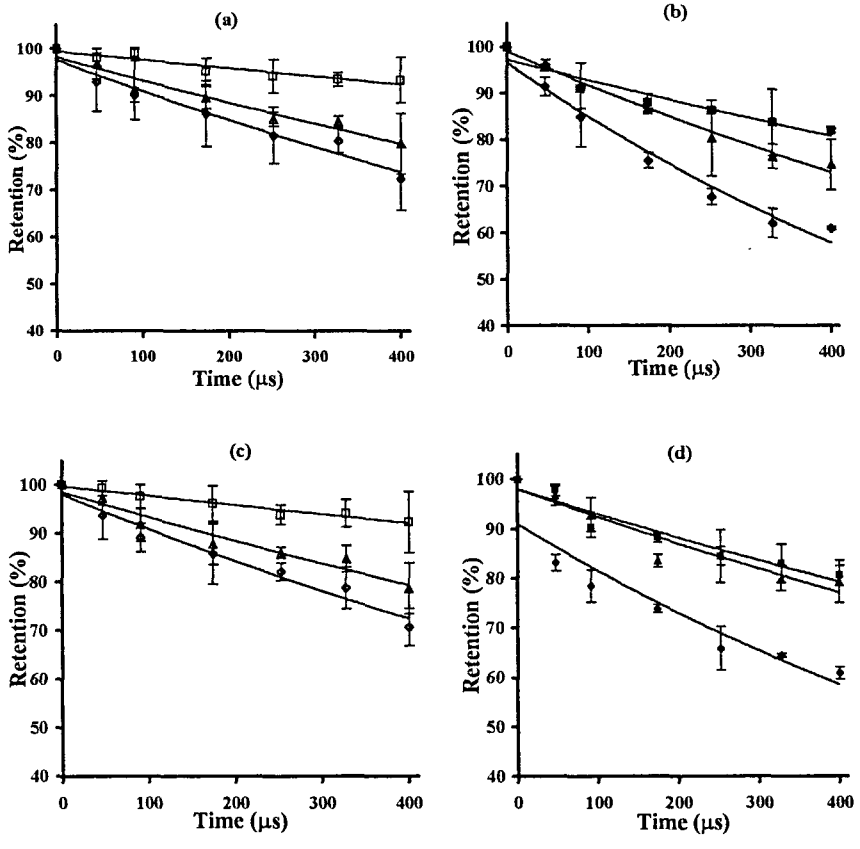

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Fig. 1. Retention of ascorbic acid in milk or simulated milk ultrafiltrate (SMUF) exposed to pulsed electric field treatments (HIPEF) at room or moderate temperature. a) milk exposed to HIPEF at 20-25°C; b) SMUF exposed to HIPEF at 20-25°C; c) milk exposed to HIPEF at 50-55°C; d) SMUF exposed to HIPEF at 50-55°C. Treatments were performed at 18.3 (○,●), 22.6 (□,■) and 27.1 kV/cm (△,▲). Plotted lines correspond to first order kinetic fits.
White symbols are for milk and black symbols are for simulated milk ultrafiltrate.

The maximum HIPEF conditions selected for this study were similar to that used for high enzyme inactivation (Bendicho *et al.* in press) that are more severe than those effective in the destruction of microorganisms in skim milk and SMUF (Sobrino *et al.* 2000). Several authors achieved significant inactivation levels on microorganisms after similar or softer treatments to those evaluated in this study. Sensoy *et al.* (1997) reported near 4 log reductions in *Salmonella dublin* after a treatment of 30 kV/cm and 163·9 μs. Reina *et al.* (1998) and Calderón-Miranda *et al.* (1999) reported reductions from 1·5 to 2 D in *Listeria monocytogenes* and *Listeria innocua* in skim milk with similar treatment conditions. The former subjected the samples to a treatment of 25 kV/cm during 600 μs and the latter processed the milk at 30 kV/cm with just 32 pulses of 2 μs. Martín *et al.* (1997) found that HIPEF treatment inactivated *Esch. coli* in skim milk up to 2 log reductions after 25 pulses at 25 kV/cm. Moreover, Raso *et al.* (1999) extended the shelf-life of milk up to 14 days with a HIPEF at 40 kV (field strength not reported). As regards treatments for enzyme inhibition, similar conditions to those used in this study were carried out to evaluate the effects of heat and HIPEF on a thermoresistant lipase from *Pseudomonas fluorescens* (Bendicho *et al.*, in press). It was observed that lipase inactivation achieved after thermal high (75 °C-20 s) or low (63 °C-30 min) pasteurisation was similar to that obtained after a HIPEF treatment of 80 μs at field strengths from 18·4 to 27·4 kV/cm (Bendicho *et al.*, in press). When applying the latter treatment conditions that resulted in equivalent enzyme inactivation levels to those achieved by heat, residual ascorbic acid levels remained higher in HIPEF treated samples. Most enzymes require severe HIPEF treatments to reach high levels of inactivation (Giner *et al.* 2000; Giner *et al.* 2001; Bendicho *et al.* in press); even under these severe conditions, HIPEF processed samples may retain more ascorbic acid than the heat treated.

Thermally processed SMUF retained more vitamins than milk; on the contrary, HIPEF allowed more retention of vitamins in milk than in SMUF, which is a more simple media. This difference gives HIPEF a real advantage over thermal treatments when products with complex matrices have to be processed. It was observed that after similar HIPEF treatments, milk retained more ascorbic acid than SMUF (Fig. 1). This could be due to its higher complexity and to a protective effect of natural milk components such as proteins, mainly casein. A protective role of casein for microorganisms and enzymes, against inactivation has been described (Goff & Hill, 1993; Vega-Mercado *et al.* 1996; Martín *et al.* 1997). However, when thermal treatments were applied, SMUF retained higher levels of this vitamin than skim milk, in all the cases. A standard pasteurisation (75 °C-15 s) treatment of SMUF maintained 98·1 % while in milk the initial content decreased to 86·7 % (Fig. 2). This retention rate was similar to that reported by Mottar and Noudts (1979) and Haddad and Loewenstein (1983), but lower than the 75 % found by Rechcigl (1982). This higher ascorbic acid retention in SMUF than in skim milk after thermal treatments may be due to the formation of more new reactive compounds in milk than in SMUF during the heat treatment. These compounds may oxidise the ascorbic acid, reducing the ascorbic acid content more in milk than in SMUF.

In both cases HIPEF and thermal treatments, the concentration of ascorbic acid decreased significantly in milk and SMUF when the treatment time rose following a first order kinetic model. Several authors, also proposed first-order models to describe microorganism inactivation by HIPEF (Martín *et al.* 1997; Michalac *et al.* 1999). The HIPEF first order rate constant (k_p) for ascorbic acid took values from $1{\cdot}8 \times 10^{-4}$ to $1{\cdot}27 \times 10^{-3}$ μs^{-1} (Table 3). The values of the constant in SMUF were higher than in skim milk and the maximum k_p were obtained applying the lower electric field intensity. On the other

hand, the resultant k_p were similar at different treatment temperatures (Table 3). Unfortunately, k_p could not be adjusted to any model as a function of the electric field intensity. Results at higher electric field intensity were tried, but it was no possible to carry out because at more severe working conditions, dielectric sample breakdown occurred (arching phenomenon).

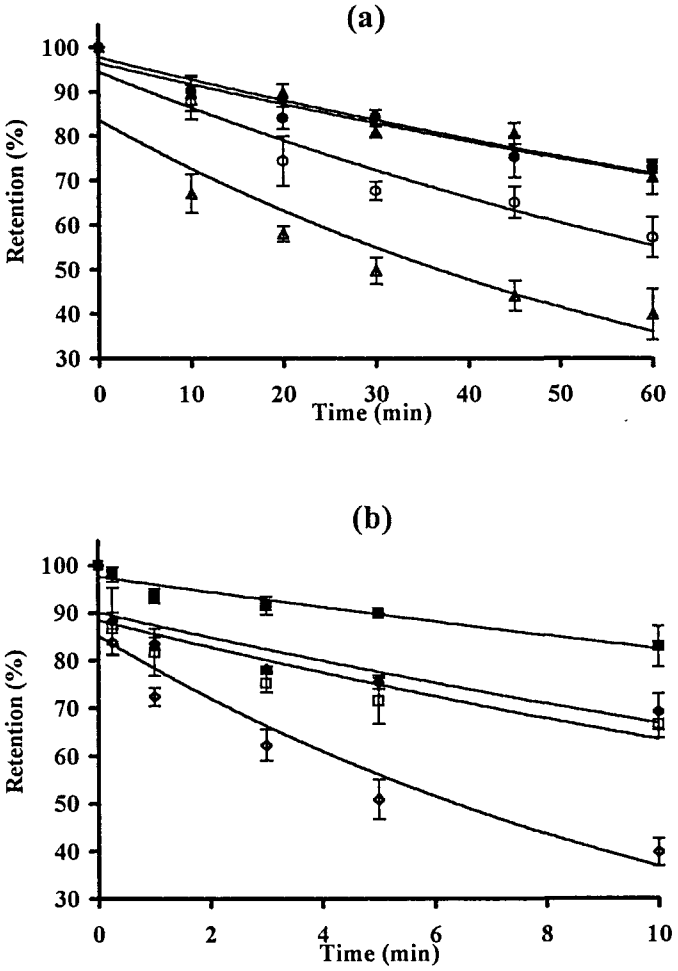

Fig. 2. Retention of ascorbic acid exposed to different heat treatments: a) 50 (○,●) and 63°C (△, ▲); b) 75 (□, ■) and 90 °C (◇, ◆). Plotted lines correspond to first order kinetic fits. White symbols are for milk and black symbols are for simulated skim milk ultrafiltrate.

Table 3. First order rate constant (k_P) and correlation coefficient (R^2) of the ascorbic retention as a function of the time of the pulsed electric field treatment.

Product†	Treatment temperature (°C)	Field strength (kV/cm)	k_P (μs^{-1})	R^2
SMUF	20-25	18.3	0.00127 ± 0.00021	0.972
		22.6	0.00046 ± 0.00012	0.937
		27.1	0.00076 ± 0.00009	0.985
Milk	20-25	18.3	0.00070 ± 0.00014	0.960
		22.6	0.00018 ± 0.00006	0.900
		27.1	0.00052 ± 0.00011	0.958
SMUF	50-55	18.3	0.0011 ± 0.0004	0.905
		22.6	0.00053 ± 0.00015	0.929
		27.1	0.00060 ± 0.00018	0.911
Milk	50-55	18.3	0.00075 ± 0.00014	0.965
		22.6	0.00020 ± 0.00004	0.959
		27.1	0.00054 ± 0.00012	0.949

†SMUF, simulated skim milk ultrafiltrate (Jeness & Koops, 1962).

For the thermal treatment, k_T obtained in milk was always higher than k_T for SMUF, for the same conditions (Table 4). The first order kinetic constant values (k_T) for SMUF ranged from 5×10^{-3} to $2{\cdot}9 \times 10^{-2}$ min^{-1} and for milk between 9×10^{-3} and 8×10^{-2} min^{-1} (Table 4). At the evaluated conditions, the influence of temperature (T) on k_T of skim milk [$k_{T(SM)}$] and k_T of SMUF [$k_{T(SMUF)}$] could be described by the Arrhenius equation (Equations 6 and 7), respectively.

$$k_{T(SM)} = 1.22 \cdot 10^{8} \cdot \exp\left(\frac{-63665.5}{8.31 \cdot T}\right) \qquad (R^2=0{\cdot}996) \qquad \text{Equation 6}$$

$$k_{T(SMUF)} = 44.93 \cdot 10^{4} \cdot \exp\left(\frac{-49817.7}{8.31 \cdot T}\right) \qquad (R^2=0{\cdot}951) \qquad \text{Equation 7}$$

Interactions between different factors (electric field intensity, treatment time and substrate) that could affect the results of inactivation were also studied for the HIPEF process and it was observed that there were some significant interactions. The most significant was between the electric field intensity and substrate (milk or SMUF), followed by electric field intensity and treatment time and, finally, by treatment time and substrate

(Table 2). The decrease of ascorbic acid content after applying a HIPEF treatment was more significantly affected by the increase of electric field intensity in SMUF than in skim milk. At 18·3 kV/cm retention rates of 72·4 % and 61·0 % were observed in skim milk and SMUF, respectively. While after a 27·1 kV/cm treatment, skim milk retained 79·9% and SMUF 74·7%. An increase of the treatment time had more influence on the vitamin retention in SMUF than in milk. The effect of electric field was also related to treatment time. At any electric field applied, a higher treatment time resulted in greater reduction of ascorbic acid, although treatment time had more influence at 18·3 kV/cm than at 22·6 or 27·1 kV/cm.

Table 4. First order rate constant (k_T) and determination coefficient (R^2) of the ascorbic acid retention as a function of the time of heat treatment.

Substrate†	Treatment temperature (°C)	k_T (min^{-1})	R^2
SMUF	50	0.0050 ± 0.0014	0.938
SMUF	63	0.0052 ± 0.0016	0.930
SMUF	75	0.017 ± 0.005	0.931
SMUF	90	0.029 ± 0.017	0.787
Milk	50	0.009 ± 0.003	0.936
Milk	63	0.014 ± 0.006	0.880
Milk	75	0.033 ± 0.021	0.760
Milk	90	0.08 ± 0.03	0.910

†SMUF, simulated skim milk ultrafiltrate (Jeness & Koops, 1962).

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DISCUSIÓN GENERAL

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Los pulsos eléctricos de alta intensidad de campo (PEAIC) son una nueva tecnología no térmica de procesado de alimentos. Su implantación en la industria láctea permitiría la estabilización de la leche evitando los cambios en el gusto, aroma y contenido en nutrientes que provoca el calor.
Con este trabajo se pretendía, principalmente, estudiar la efectividad de los PEAIC para inactivar dos enzimas producidos por bacterias psicrótrofas de la leche (lipasa de *Pseudomonas fluorescens* y proteasa de *Bacillus subtilis*) y también evaluar el efecto de los tratamientos mediante PEAIC en varias vitaminas hidro y liposolubles, ambos campos de estudio poco desarrollados en las investigaciones existentes sobre PEAIC.
Para ello, en primer lugar, se eligieron y validaron los métodos para la cuantificación tanto de las actividades de los dos enzimas como de las vitaminas en los medios a estudiar para así corroborar la confianza en los métodos y asegurar los resultados que proporcionen.

Validación de métodos de cuantificación

Los métodos elegidos para determinar la actividad lipásica y proteásica y cuantificar el contenido en vitaminas tanto en leche como en solución modelo de ultrafiltrado de leche (SMUL) se validaron en términos de linealidad, sensibilidad, precisión y exactitud.

Lipasa

El método para cuantificar la actividad de la lipasa de *Pseudomonas fluorescens* en leche desnatada y entera se eligió después de validar y comparar la fiabilidad de tres métodos diferentes. Los tres métodos estaban basados en el uso de p-nitrofenil caprilato como sustrato (Blake et al., 1996; Humbert et at., 1997), ya que permitía una determinación sencilla, rápida y sensible de la actividad lipásica, sin requerir los pasos previos de centrifugación y extracción necesarios en otros métodos de valoración (Castberg et al., 1975; McKellar y Cholette, 1986).

Uno de los métodos (A), basado en el descrito por Blake et al. (1996), consistía en medir el incremento de color amarillo (parámetro b*) mediante colorimetría (Blake et al., 1996) después que el enzima hubiese reaccionado con el sustrato durante un tiempo determinado liberando p-nitrofenol (compuesto amarillo). Los otros métodos (B y C), basados en el método descrito por Humbert et at. (1997), permitían cuantificar la actividad por espectrofotometría después de la reacción, transparentizando la muestra mediante un reactivo clarificante. El método C únicamente se diferenciaba del B por el uso de una mezcla inhibidora para detener la reacción.

Los 3 métodos resultaron lineales en el rango de 0 a 300 mU/ml ($p<0.001$) dando coeficientes de correlación (R) superiores a 0.974 en todos los casos. La sensibilidad se estudió mediante los parámetros límite de detección (LD) y cuantificación (LC) (Long y Winefordner, 1983) dando valores de 9.31-28.83 mU/m y 31.03-96.10 mU/ml, respectivamente. En términos de precisión todos los métodos dieron un coeficiente de variación menor del 5%, satisfaciendo el criterio de Horwitz (Horwitz, 1982), por tanto,

todos ellos se consideraron precisos. La exactitud se determinó mediante el estudio de la recuperación a 2 niveles diferentes de adición, observándose que los resultados no dependieron del nivel de actividad lipásica de las muestras excepto para el método colorimétrico (A) en leche entera.
Los métodos espectrofotométricos (B y C) presentaron mejor linealidad (R:0.987-0.997) y sensibilidad (LC: 33.90-68.00 mU/ml) que el método colorimétrico (A) (R:0.974-0.980; LC: 83.91-96.10 mU/ml), dando los menores valores de LD y LC en leche desnatada. En leche desnatada, los tres métodos dieron resultados de recuperación similares (81%-90%); contrariamente, en leche entera, el método colorimétrico tuvo peor comportamiento, ya que sus resultados de recuperación dependieron del nivel de adición (nivel I: 104%; nivel II: 89.2%), mientras que los métodos espectrofotométricos B y C presentaron recuperaciones medias del 83 y 81%, respectivamente. En general, ambos métodos espectrofotométricos (B y C) mostraron resultados similares en cuanto a los términos estadísticos estudiados y en la mayoría de los casos dieron mejores resultados que el método colorimétrico (A). Además, los métodos espectrofotométricos permitían la detección de menores concentraciones de enzima; consecuentemente, eran más adecuados. Asimismo, el método espectrofotométrico que requería el uso de una mezcla inhibidora para detener la reacción enzimática (C) dió recuperaciones más bajas que el otro método (B). El uso de la mezcla inhibidora no mejoró los resultados, además de hacer el procedimiento más tedioso y más caro. Por tanto, el método B fue el de elección para su uso como análisis de rutina para la determinación de la actividad lipásica.
Una vez determinado el método de elección (B), éste se validó para su uso en SMUL, resultando lineal ($p<0.001$, $R=0.992$), preciso (CV=5%), sensible (LD:10.98 mU/ml y LC:36.59 mU/ml) y con una recuperación similar al 100% teórico. Por tanto este método también se consideró aceptable para determinar la actividad lipásica en SMUL después de observar los resultados obtenidos en los parámetros evaluados.

Proteasa

Para la determinación de la actividad proteásica tanto en solución modelo de ultrafiltrado de leche (SMUL) como en leche con diferente contenido en grasa (denatada y entera) se eligió un método espectrofotométrico descrito por Christen y Marshall (1984) basado en el uso de azocaseína como sustrato. La azocaseína es un compuesto artificial que libera productos coloreados al hidrolizarse por lo que permite la valoración de la actividad enzimática específicamente. Además, el uso de este compuesto permitía la posibilidad de desarrollar un método sencillo y de fácil operabilidad.
El método evaluado resultó lineal ($p<0.001$) en el rango de concentraciones estudiado (0-50 mU/ml) tanto en SMUL como en leche con diferente contenido en grasa (entera y desnatada), dando valores de R superiores a 0.991 en todos los casos. Mediante los coeficientes de correlación de las rectas de calibración obtenidas se comparó la bondad de ajuste para cada tipo de muestra, observándose que no habían diferencias significativas entre los valores de R obtenidos.
La sensibilidad se estudió mediante los parámetros LD y LC (Long y Winefordner, 1983) cuyos valores fueron 2.29-3.26 mU/ml y 7.64-10.9 mU/ml, respectivamente. Después de comparar los resultados de sensibilidad se observó que los LD y LC no dependían del tipo de muestra estudiado.

En cuanto a la precisión, el método pudo considerarse preciso según el criterio de Horwitz (Horwitz, 1982) para su uso en cualquiera de los medios, con diferente contenido en proteína o grasa, evaluados.
La exactitud se estudió como porcentaje de recuperación para dos niveles diferentes de adición. Los resultados no dependieron de la actividad proteásica de las muestras, por tanto, se pudo tomar como valor de recuperación la media de las recuperaciones obtenidas a los dos niveles de adición y además resultó similar al 100% teórico.
Finalmente, se pudo considerar, que el método podía ser usado para la cuantificación de la actividad proteolítica en los diferentes medios evaluados ya que la bondad del método no dependía del contenido en grasa o proteína de las muestras.

Vitaminas

Los métodos elegidos para la determinación de vitaminas hidrosolubles (tiamina, riboflavina y ácido ascórbico) y liposolubles (colecalciferol y tocoferol) en leche estaban basados en los descritos por Albalá-Hurtado et al., 1997a y Albalá-Hurtado et al., 1997b. En ambos métodos se cuantificó cromatográficamente el contenido en vitaminas después de una extracción, que en el caso de las vitaminas hidrosolubles se hizo mediante el uso de ácido tricloracético para separar la fase acuosa de la leche y en el caso de las liposolubles mediante n-hexano para obtener la parte lipídica de la leche.
Los dos métodos resultaron lineales ($p<0.001$) en los rangos de concentración que se iban a usar, que eran de 0 a 10 μg/ml para el colecalciferol y de 0 a 100 μg/ml para el resto de las vitaminas. Los valores de R fueron superiores a 0.9965 en todos los casos.
Los límites de determinación y cuantificación (Long y Winefordner, 1983) para el colecalciferol fueron 0.216 y 0.719 μg/ml, respectivamente. En las otras vitaminas estudiadas el límite de determinación varió de 2.84 a 3.62 μg/ml y el límite de cuantificación de 9.48 a 12.07 μg/ml. Los resultados de precisión dieron siempre inferiores al 4% (CV), manteniéndose siempre por debajo del intervalo fijado por Horwitz (Horwitz, 1982). Por tanto, los métodos presentaron una precisión aceptable para la determinación de todas las vitaminas evaluadas. La exactitud se estudió mediante la determinación de la recuperación a dos niveles de adición de vitamina diferentes, observándose que en el caso de la tiamina y el ácido ascórbico la recuperación obtenida dependía del nivel de adición.

Efectos de los PEAIC en una lipasa y una proteasa bacterianas

Gran parte de este estudio ha consistido en profundizar en el estudio de los parámetros eléctricos y de las características del medio de tratamiento que pueden influenciar en la efectividad de los PEAIC para inactivar una lipasa de *Pseudomonas fluorescens* y una proteasa de *Bacillus subtilis*.

Lipasa

El efecto de los PEAIC en una lipasa de *Pseudomonas fluorescens* comercial se estudió en SMUL, ya que por su composición simple se consideró un medio ideal para iniciar estos estudios. La SMUL se procesó usando 2 sistemas diferentes de tratamiento mediante

PEAIC; uno de flujo continuo y otro por tandas. Se aplicaron tratamientos de diferentes condiciones de intensidad de campo, duración y frecuencia de aplicación de pulsos (sistema continuo). Por otro lado, las muestras se procesaron mediante calor, incluyendo tratamientos de pasteurización alta y baja, para así poder realizar estudios de comparación entre los dos tipos de procesado.

La lipasa evaluada resultó resistente a los tratamientos térmicos de pasteurización ya que después de aplicar condiciones de pasteurización alta (75°C-15 s) y baja (63°C-30 min) sólo se consiguieron niveles de inactivación de un 5 y 20%, respectivamente. Griffiths et al. (1981) estudió el comportamiento de una lipasa producida por otra cepa de *P. fluorescens* y también observó que el enzima era bastante resistente a la pasteurización, ya que después de un tratamiento de 17 s a 77°C la actividad sólo se redujo un 35%. Kishonti (1975) observó que varias lipasas producidas por bacterias psicrótrofas podían mantener más del 75% de su actividad inicial después de un tratamiento de 30 min a 63°C.

La reducción de la actividad enzimática por la aplicación de tratamientos térmicos se ajustó a un modelo exponencial. Las constantes de primer orden que resultaron para cada temperatura (50 a 90°C) se pudieron ajustar al modelo de Arrhenius dando una energía de activación (E_a) de 134.48 kJ/mol. Estos resultados coinciden con los de Driessen (1983) que después de procesar diversas lipasas termoresistentes producidas por bacterias Gram-negativas a temperaturas de 50 a 130°C también pudieron ajustar la reducción de la actividad enzimática a modelos exponenciales y las constantes resultantes al modelo de Arrhenius.

El proceso de PEAIC afectó a la actividad de esta lipasa termoresistente, aunque el nivel de inactivación dependió del tratamiento aplicado. En el equipo de tratamiento en continuo después de aplicar hasta 80 pulsos a 37.3 kV/cm y 3.5 Hz únicamente se consiguió reducir la actividad del enzima en un 13%. Mientras que al aplicar el tratamiento por tandas se llegó hasta una inactivación del 62.1% después de un aplicar a las muestras 80 pulsos de 27.4 kV/cm. Para conseguir niveles de inactivación similares, aplicando calor, se requirieron 10 min de tratamiento a 75°C o de 1 min a 90°C.
En todos los casos, el nivel de inactivación del enzima aumentó con el número de pulsos, la intensidad de campo y la densidad de energía aplicados; mostrando un comportamiento similar a otros enzimas (Giner et al., 2000; Giner et al., 2001). También se observó, en el caso del tratamiento en flujo continuo, que al aplicar los pulsos a frecuencias más altas, se obtuvieron niveles más altos de inactivación.

La efectividad de ambos sistemas de tratamiento se comparó mediante la densidad de energía aplicada en los procesos observándose que, para el proceso continuo, con la aplicación de 424.36 kJ/l se consiguió una inactivación máxima del 13% mientras que, en el tratamiento por tandas, con la aplicación de una densidad de energía similar (504.97 kJ/l) la actividad se redujo hasta un 62.1%. El tratamiento aplicado en tandas resultó más efectivo que el de flujo continuo al aplicar densidades de energía similares. Este efecto no era el esperado ya que en el tratamiento por tandas las intensidades de campo aplicadas eran más bajas y los pulsos más cortos. Por tanto, en las condiciones evaluadas, se observó que la respuesta de este enzima a los PEAIC no dependió tanto de la intensidad del tratamiento como en el caso de los microorganismos. Martín et al. (1997) observó que un tratamiento en continuo resultaba más efectivo para la inactivación de *E. coli* que un tratamiento por

tandas. Estas diferencias en la efectividad de los tratamientos pueden ser debidas a la propia configuración de los equipos, ya que difieren en el tipo de tratamiento (continuo y por tandas), el tipo de cámara (electrodos planos paralelos y forma coaxial), la anchura de pulso y la frecuencia de aplicación de los pulsos. También puede afectar en los resultados obtenidos, la propia estructura y mecanismo de acción del enzima (Jaeger, 1994; Wong et al., 1995), ya que puede resultar más sensible a un tipo de tratamiento que a otro.
De todas formas, otros autores también observaron que los enzimas presentaban un comportamiento diferente a los microorganismos cuando eran expuestos a PEAIC ya que dependiendo del propio enzima y de las condiciones de tratamiento, el efecto podía ser muy diferente. Ho et al. (1997) observó que, dependiendo del tipo de enzima, el efecto a los PEAIC podía variar desde una alta inactivación como el 85% conseguido en peroxidasa, hasta un aumento de la actividad inicial de hasta el 250% como en el caso de la pepsina.

Se sabe que el calor inactiva los enzimas porque provoca cambios en su estructura, llegando a la desnaturalización. Si el enzima pierde su configuración, sobretodo del centro activo, se dificulta el ensamblaje con el sustrato y por tanto se limita su actividad (Tsong, 1990). Se supone que el tratamiento mediante PEAIC también provoca la desnaturalización del enzima pero sin requerir un incremento de temperatura, ya que la aplicación de un campo eléctrico también puede causar modificaciones en la conformación de las proteínas debido a alteraciones en las cargas, dipolos o reacciones químicas (Tsong y Astunian, 1986).

La reducción de la actividad lipásica se pudo ajustar a modelos exponenciales respecto al número de pulsos aplicados para cada intensidad de campo (Ecuación 1) y también respecto a la densidad de energía descargada (Ecuación 2).

$$RA = RA_0 \cdot e^{-k_E \cdot N} \qquad \text{Ecuación 1}$$

$$RA = RA_0 \cdot e^{-k \cdot Q} \qquad \text{Ecuación 2}$$

donde RA es la actividad relativa, RA_0 la actividad relativa inicial (100%), k_E y k son constantes de primer orden, N el número de pulsos y Q la densidad de energía.

El ajuste de los resultados a la Ecuación 1 dio valores de R^2 entre 0.702 y 0.967 y las constantes (k_E) obtenidas variaron de $6.2 \cdot 10^{-3}$ a $1.28 \cdot 10^{-2}$ pulsos^{-1} para el tratamiento por tandas (16.4-27.4 kV/cm); mientras que para el tratamiento en continuo los valores de k_E estuvieron en un rango de $3 \cdot 10^{-4}$ a $1.6 \cdot 10^{-4}$ pulsos^{-1} para intensidades de campo de 26.1 a 37.3 kV/cm. Cuando los resultados se ajustaron a la Ecuación 2 se obtuvieron valores de k de $1.9 \cdot 10^{-3}$ l/kJ y $2 \cdot 10^{-4}$ para el tratamiento en por tandas y en continuo, respectivamente. Giner et al. (2000) y Giner et al. (2001) también propusieron un modelo exponencial para describir la inactivación de diversos enzimas vegetales respecto al número de pulsos o a la densidad de energía.

Los resultados de inactivación enzimática obtenidos después de aplicar el proceso por tandas, además de ajustarse a modelos exponenciales simples (Ecuación 1 y 2) también se pudieron ajustar a diversos modelos que contienen los dos parámetros de más influencia en

el efecto del tratamiento mediante PEAIC que son el tiempo de tratamiento (t) y la intensidad de campo (E)
La disminución de la actividad lipásica se pudo ajustar a modelos de primer orden respecto al tiempo o a la intensidad de campo cuya constante de primer orden incrementaba linealmente con la intensidad de campo o con el tiempo de tratamiento, respectivamente, dando las Ecuaciones 3 y 4 cuyas R^2 son 0 889 y 0 863, respectivamente

$$RA = RA_0 \cdot e^{-(0\,0003\,e^{(0\,0839\,E)})\cdot t} \qquad \text{Ecuación 3}$$

$$RA = RA_0 \cdot e^{-(0\,0002\,t - 0\,0182)\,E} \qquad \text{Ecuación 4}$$

Los resultados también se ajustaron a los modelos de Hulsheger (Hulsheger et al , 1983, Giner et al , 2000) y Fermi (Peleg, 1995, Giner et al , 2000) modificados Ambos modelos fueron inicialmente concebidos para describir la inactivación microbiana, aunque en este caso, también sirvieron para describir la reducción de la actividad enzimática (Ecuación 5 y Ecuación 6) mostrando un buen ajuste, R^2 de 0 867 y 0 833, respectivamente

$$RA = 100 \cdot \left(\frac{t}{39\,32}\right)^{\frac{(E-2\,88)}{191\,38}} \qquad \text{Ecuación 5}$$

$$RA = \frac{100}{1 + e^{\frac{E-(297\,97\,e^{(-0\,0092\,t)})}{413\,27\,e^{(-0\,0135\,t)}}}} \qquad \text{Ecuación 6}$$

Se puede decir que la actividad de esta lipasa bacteriana termoresistente se puede reducir considerablemente con la aplicación de PEAIC y además esta inactivación se puede ajustar a diversos modelos cinéticos ya descritos en la literatura Desde el punto de vista cuantitativo todos los modelos propuestos pueden ser útiles para predecir el nivel de inactivación enzimática, aunque cada uno proporciona una información cualitativamente diferente

Proteasa

El efecto de los PEAIC en una proteasa de *Bacillus subtilis* se estudió usando 3 equipos de procesado diferentes, uno de flujo discontinuo (cámara de electrodos planos paralelos) (UdL-B) y dos de flujo continuo cuyas cámaras de tratamiento eran de forma coaxial (WSU-C) y colineal (UdL-C) La efectividad de los tratamientos se estudió aplicando densidades similares de energía (hasta 500 kJ/l) en los 3 equipos para así poder realizar comparaciones entre los tratamientos Además el efecto del tratamiento en el enzima se evaluó en SMUL y en leche para poder determinar la influencia de la composición del medio Para poder comparar la efectividad del tratamiento mediante PEAIC respecto a los

tratamientos convencionales también se procesaron las muestras mediante diversos tratamientos térmicos entre los que se incluía la pasteurización alta y baja.

Después de someter las muestras de SMUL o de leche a tratamientos térmicos (50-75°C hasta 30 min) se observó que la actividad del enzima disminuyó con el incremento del tiempo de tratamiento y con la temperatura aplicada. En las muestras procesadas a 50 y 55°C se observó poca variación de la actividad enzimática, tanto en leche como en SMUL. Al aplicar condiciones de pasteurización baja (63°C-30 min) se consiguió casi la completa inactivación del enzima en ambos medios de tratamiento; mientras que al procesar las muestras mediante pasteurización alta (75°C-15 s) en SMUL sólo se consiguió reducir la actividad un 7%, observándose la alta resistencia del enzima en este medio, aunque en leche la actividad se redujo hasta el 96%. Por tanto se pudo observar que la influencia del medio resultaba importante en cuanto al nivel de efectividad de los tratamientos, ya que el enzima resultó mucho más susceptible al calor en leche que en SMUL.
En ambos casos (SMUL y leche), la actividad disminuyó exponencialmente con el tiempo de tratamiento para cada temperatura, observándose además que las constantes de primer orden obtenidas se pudieron ajustar a la ecuación de Arrhenius que dio E_a de 209.5 y 296.8 kJ/mol en SMUL y en leche, respectivamente, indicando que en el caso de la proteasa las constantes de inactivación aumentaron mucho más rápidamente con la temperatura que en el caso de la lipasa (E_a=134.48 kJ/mol), o sea que la proteasa es mucho más sensible al incremento de la temperatura de tratamiento.

Las muestras procesadas mediante tratamientos por PEAIC de hasta 500 kJ/l usando los equipos UdL-B y UdL-C no sufrieron una variación significativa de la actividad proteásica ni con el número de pulsos ni con la intensidad de campo en ninguno de los medios evaluados (SMUL y leche). Únicamente, las muestras de SMUL o de leche procesadas con el equipo WSU-C sufrieron una ligera variación de la actividad enzimática que dependió de la intensidad de campo, el número de pulsos, la frecuencia de aplicación de los pulsos, la densidad de energía y el medio de tratamiento. En SMUL el enzima sufrió una reducción de la actividad del 10-13%; mientras que en leche la actividad se llegó a incrementar un 10-15% dependiendo de las condiciones aplicadas.
Los resultados dependieron principalmente de la configuración del equipo usado y del medio de tratamiento. Un comportamiento similar se observó en la lipasa de *Pseudomonas fluorescens*, ya que el nivel de inactivación obtenido también dependió del sistema usado para su procesado; aunque la lipasa mostró un comportamiento opuesto, ya que el tratamiento por tandas fue el que resultó más efectivo. En lo que se refiere al medio de tratamiento, Vega-Mercado et al. (2001) también observaron diferencias entre los resultados obtenidos de aplicar tratamientos en diferentes medios, ya que mientras que en leche la actividad de una proteasa de *P. fluorescens* se pudo reducir hasta un 60% después de un tratamiento de 98 pulsos a 14-15 kV/cm, al aplicar el mismo tratamiento en una solución de caseína no se observaron cambios significativos en la actividad del enzima.

En este caso, también se estudió la relación entre la densidad de energía aplicada y la reducción de la actividad enzimática, observándose que la disminución de actividad únicamente pudo ajustarse a un modelo exponencial cuando el enzima se trataba con el equipo WSU-C en SMUL ya que en los otros casos no se producía inactivación significativa. Anteriormente, también se propuso este modelo tanto para describir la

inactivación de lipasa como de diversos enzimas vegetales (Giner et al., 2000; Giner et al., 2001).

Como se observó que aplicando estos niveles de densidad de energía (<500 kJ/l) la reducción de actividad enzimática era mínima se continuó el estudio del efecto de los PEAIC en este enzima con el equipo de flujo continuo que consta de cámaras colineales (UdL-C) aplicando tratamientos de hasta 6786.8 kJ/l en diferentes medios (SMUL, leche desnatada y entera).

El efecto del tiempo de tratamiento, la intensidad de campo y la densidad de energía se estudió aplicando tratamientos de hasta 895.8 μs a una intensidad de campo de 19.7 a 35.5 kV/cm y a una frecuencia de 66.66 Hz. En todos los casos, la actividad disminuyó con el incremento del tiempo de tratamiento, la intensidad de campo o la densidad de energía. Otros autores también han descrito comportamientos similares (Vega-Mercado et al., 1995; Castro et al., 2001; Giner et al., 2000; Giner et al., 2001)

La actividad del enzima disminuyó exponencialmente con el aumento de la densidad de energía aplicada tanto en SMUL como en leche desnatada dando constantes similares en ambos medios ($7 \cdot 10^{-5}$ y $6 \cdot 10^{-5}$ l/kJ) cuando se trató de describir esta variación mediante la Ecuación 2. La inactivación de lipasa de *P. fluorescens* también se ajustó al mismo modelo, aunque los valores de la constante de primer orden para la lipasa fueron mucho mayores tanto en el equipo continuo como en el discontinuo ($2 \cdot 10^{-4}$ y $1.9 \cdot 10^{-3}$ L/kJ) indicando que la lipasa de *P. fluorescens* es más sensible al tratamiento mediante PEAIC que la proteasa, ya que con la aplicación de densidades mucho más bajas de energía se pueden conseguir niveles considerables de inactivación. Giner et al. (2000) y Giner et al. (2001) también observaron que la actividad de diversos enzimas vegetales disminuía exponencialmente con el aumento de la densidad de energía aplicada.

La frecuencia es otro factor que intervino en la efectividad del tratamiento, ya que al aplicar los tratamientos a frecuencia más elevada, también se obtuvieron niveles más altos de inactivación para tratamientos de igual intensidad. En SMUL, mientras que después de un tratamiento de 895.8 μs a 35.5 kV/cm y 66.66 Hz la actividad se redujo un 48%, al aplicar el mismo tratamiento pero a 111.11 Hz la actividad disminuyó un 62.7%. En leche desnatada, un tratamiento de 865.8 μs a 35.5 kV/cm y 66.66 Hz inactivó un 37.9% el enzima y con un tratamiento a las mismas condiciones pero a frecuencia más alta (111.11 Hz) se inactivó un 81.1%; mientras que en leche entera, la aplicación de los mismos tratamientos dio lugar a reducciones del 35 y 57.1%. Algunos autores han relacionado la efectividad de la frecuencia con el incremento de la temperatura de tratamiento, pero en este caso, la temperatura final del tratamiento varió de 34 a 46°C, condiciones que no son suficientes para causar estas variaciones de actividad, ya que cuando se realizó el estudio del efecto del calor en el enzima, un tratamiento de hasta 30 min a 50°C únicamente causó una reducción de la actividad del 12-13%. Para la lipasa de *P. fluorescens* en SMUL también se observó que a mayor frecuencia de tratamiento mayor inactivación ya que un tratamiento de 80 pulsos a 37.3 kV/cm y 2 Hz se llegó a un 7% de inactivación, mientras que aplicando el tratamiento a 3.5 Hz la actividad disminuyó un 13%.

El efecto de la anchura de pulso se estudió aplicando tratamientos con pulsos de 4 y 7 μs. En este caso, para tiempos de tratamiento iguales el nivel máximo de inactivación fue el mismo para cada tipo de producto. Sensoy et al. (1997) también comparó la efectividad de dos tratamientos realizados con pulsos de 2 y 8 μs y observó que para un tratamiento de 127 μs los niveles de inactivación que consiguieron fueron similares.

Los niveles de inactivación del enzima en SMUL, leche desnatada y entera difirieron significativamente aún aplicando condiciones similares de tratamiento. La inactivación del enzima resultó más efectiva cuando éste estaba suspendido en el propio sustrato (leche desnatada) que no en la solución modelo (SMUL), pudiéndose decir por lo tanto que la presencia de proteínas no interfiere en la efectividad del tratamiento; sin embargo, la presencia de grasa redujo el efecto de los PEAIC ya que en leche desnatada se pudieron conseguir niveles de inactivación mucho más altos que en leche entera. Otros autores también han descrito diferencias entre la inactivación conseguida según el medio de tratamiento. Castro et al. (2001) estudió la inactivación de fosfatasa alcalina en SMUL, leche 2% grasa y leche entera y consiguió reducir la actividad un 65% con un tratamiento de 70 pulsos a 22 kV/cm en SMUL y a 18.8 kV/cm en leche desnatada, o sea que la inactivación del enzima fue más fácil en leche desnatada que en la solución modelo indicando que al estar el enzima en su sustrato original su inactivación es más fácil. Y al igual que sucedió para la proteasa, también concluyó que la presencia de grasa interfería en la efectividad del tratamiento, ya que en leche 2% grasa y en leche entera después de un tratamiento de 70 pulsos a 18.8 kV/cm redujo la actividad un 59%.

Los resultados de inactivación de proteasa en SMUL se ajustaron a modelos exponenciales relacionados con el tiempo de tratamiento (Ecuación 7), la intensidad de campo (Ecuación 8) o la densidad de energía (Ecuación 2).

$$RA = RA_0 \cdot e^{-k_E \cdot t} \qquad \text{Ecuación 7}$$

$$RA = RA_0 \cdot e^{-k_t \cdot E} \qquad \text{Ecuación 8}$$

donde RA es la actividad relativa, RA_0 la actividad relativa inicial (100%), k_E y k_t son constantes de primer orden, t el tiempo de tratamiento y E la intensidad de campo.

El modelo de primer orden respecto al tiempo de tratamiento (Ecuación 7) se ajustó bien a los resultados dando R^2 de 0.821 a 0.979 y las k_E resultantes aumentaron desde $1.3 \cdot 10^{-4}$ a $6.8 \cdot 10^{-4}$ μs^{-1} con el incremento de la intensidad de campo aplicada (19.7-35.5 kV/cm). En el caso del modelo de primer orden respecto a la intensidad de campo, éste dio valores de k_t que incrementaron de $8.8 \cdot 10^{-3}$ a $3.2 \cdot 10^{-2}$ cm/kV con el incremento del tiempo de tratamiento (358.3 a 895.8 μs) y sus R^2 estuvieron en un rango de 0.729 a 0.967. En el apartado anterior, también se ha visto que la inactivación de la lipasa de *P. fluorescens* se ajustaba bien a un modelo de primer orden, aunque en aquel caso se relacionó con el número de pulsos, parámetro que es proporcional al tiempo de tratamiento. La inactivación de enzimas

vegetales también se ha descrito con modelos exponenciales relacionados con el tiempo de tratamiento o la intensidad de campo (Giner et al., 2000; Giner et al., 2001)

La causa de la variación en la actividad enzimática no puede ser debida a un efecto térmico, porque la temperatura durante los diversos procesos aumentó muy poco; ni tampoco a un efecto de los componentes del medio ya que, además de en leche, los estudios también se realizaron en SMUL que es un medio muy simple. Por tanto, los PEAIC deben causar la desnaturalización de los enzimas probablemente por los cambios que provoca en su estructura. El campo eléctrico inactiva los enzimas debido a que modifica su conformación por causar alteraciones en las cargas, dipolos o reacciones químicas (Tsong y Astunian, 1986). La estructura de las proteínas es muy sensible a las perturbaciones por campos eléctricos, ya que éstos provocan cambios en la molécula y consecuentemente provocan la pérdida de actividad debido a que se dificulta el ensamblaje entre el sustrato y el centro activo (Tsong, 1990). Ambos enzimas (lipasa y proteasa) se han llegado a inactivar a niveles considerables, aunque la proteasa resultó mucho más resistente al tratamiento mediante PEAIC que la lipasa. Esto puede ser debido a las diferencias en su estructura (Bugg, 1994; Wong, 1995), lo que hace que la sensibilidad del enzima a la influencia de un campo eléctrico o del calor sea diferente para cada uno.

Efecto de los PEAIC en las vitaminas de la leche

Después de haber demostrado que utilizando PEAIC se podían conseguir niveles elevados de inactivación de enzimas que incluso eran termoresistentes, se continuó la investigación para llegar a determinar si este tratamiento no térmico mantenía las propiedades nutricionales de los alimentos, mejor que los tratamientos térmicos. Por tanto se realizó esta última parte del trabajo para evaluar el efecto del tratamiento mediante PEAIC en diversas vitaminas hidro y liposolubles tanto en SMUL como en leche propiamente.

Se aplicaron a las muestras diversos tratamientos mediante PEAIC (18.3-27.1 kV/cm hasta 400 μs) y además a 2 temperaturas de tratamiento diferentes (20-25°C y 50-55°C) para poder comparar el efecto de los PEAIC a temperatura ambiente y combinado con temperaturas moderadas. Además se aplicaron tratamientos térmicos (50-90°C hasta 60 min) para comparar los resultados de ambos tipos de procesado.

Ni el tratamiento de PEAIC ni de calor afectaron al contenido en tiamina, riboflavina, colecalciferol y tocoferol, ni en SMUL ni el leche, ya que no se observaron diferencias significativas entre las muestras tratadas y sin tratar. Autores como Haddad y Loewenstein (1983) encontraron disminución de tiamina y riboflavina después de un tratamiento de 80°C-16 s aunque fueron variaciones muy bajas: 9.4% y 1.5%, respectivamente.

La única vitamina que sufrió destrucción, en ambos casos, fue el ácido ascórbico. La reducción del contenido en ácido ascórbico dependió de la intensidad de campo (PEAIC) o la temperatura (térmico), tiempo de tratamiento y medio que contenía el enzima. En el caso del tratamiento mediante PEAIC la temperatura de procesado (20-25°C y 50-55°C) no influyó en la destrucción del ácido ascórbico.
La destrucción de la vitamina después de 400 μs de tratamiento mediante PEAIC fue siempre menor de 27.6 y 39% en leche y SMUL, respectivamente. Las condiciones de

PEAIC más severas usadas en este estudio, no produjeron los niveles más altos de destrucción de la vitamina, hecho que resulta ventajoso, ya que, en general, a intensidades de campo más altas el nivel de destrucción microbiana y enzimática también es más alto (Martín et al., 1997; Giner et al., 2000; Giner et al., 2001). Entre las 3 intensidades de campo evaluadas (18.3, 22.6 y 27.1 kV/cm), el campo que dio los menores niveles de destrucción fue el intermedio (22.6 kV/cm) ya que después de 400 μs únicamente se redujo el contenido un 6.6% mientras que después de un tratamiento de pasteurización baja (63°C-30 min) el contenido en vitamina bajó hasta un 50.3% y después de procesar las muestras por pasteurización alta (75°C-15 s) se redujo un 13.3%. Este nivel de reducción por pasteurización de la leche fue similar a los observados por Mottar y Noudts (1979) y Haddad y Loewenstein (1983), y menores que el 25% encontrado por Rechcigl (1982).

En las muestras procesadas térmicamente, la destrucción de vitamina fue menor en SMUL que en leche, por el contrario, los PEAIC provocaron mayor destrucción en SMUL. Esto supone una ventaja de los PEAIC frente al calor cuando se tienen que tratar productos con matrices complejas. Para tratamientos similares mediante PEAIC, la leche retuvo mayor cantidad de ácido ascórbico que la SMUL, esto puede ser debido a la mayor complejidad del medio y al efecto protector de los componentes naturales de la leche como las proteínas. En otros casos ya se ha visto el efecto protector de las caseínas frente a la inactivación de microorganismos y enzimas (Goff y Hill, 1993; Vega-Mercado et al., 2001; Martín et al., 1997). Al aplicar los tratamientos térmicos, la mayor destrucción en leche que en SMUL puede ser debida a que durante la aplicación de calor se formen más compuestos reactivos en la propia leche que en la solución modelo, compuestos que puedan oxidar la vitamina causando una mayor reducción de su contenido en la propia leche.

La concentración de ácido ascórbico, tanto en leche como en SMUL, disminuyó significativamente con el aumento del tiempo de tratamiento al aplicar PEAIC siguiendo una cinética de primer orden (Ecuación 9).

$$VR = VR_0 \cdot e^{-k_0 \cdot t} \qquad \text{Ecuación 9}$$

donde VR es el porcentaje vitamina retenida, VR_0 la concentración de vitamina inicial, k_0 una constante de primer orden y t el tiempo de tratamiento.

También para microorganismos y enzimas se han propuesto cinéticas de inactivación de primer orden (Martín et al., 1997; Michalac et al., 1999; Giner et al., 2000; Giner et al., 2001). Los resultados de disminución del contenido en ácido ascórbico producidos por el tratamiento mediante PEAIC se ajustaron bien al modelo exponencial (Ecuación 9, R^2=0.900-0.985) del que resultaron constantes (k_0) de valores entre $1.8 \cdot 10^{-4}$ y $1.27 \cdot 10^{-3}$ μs^{-1}. Los valores de las constantes en SMUL fueron más altos que en leche corroborando que la inactivación en el medio más simple fue mayor. La intensidad de campo más baja, fue la que dio una constante mayor y por otra parte, los valores de las constantes fueron similares aún a temperaturas de tratamiento diferentes, observándose una vez más que la temperatura del proceso por PEAIC no influyó en la destrucción de vitamina.

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CONCLUSIONES

Las conclusiones que se deducen del presente estudio son las siguientes:

- Los métodos evaluados para la cuantificación de la actividad lipásica y proteásica y para la determinación de vitaminas resultaron satisfactorios en cuanto a la linealidad, sensibilidad, precisión y exactitud en solución modelo de ultrafiltrado de leche (SMUL) y en leche con diferente contenido en grasa (desnatada y entera) para las condiciones de trabajo establecidas.

- El enzima lipasa de *Pseudomonas fluorescens* suspendido en SMUL resultó resistente a la pasteurización térmica, ya que después de aplicar tratamientos de pasteurización alta (75°C-15 s) o baja (63°C-30 min), la actividad enzimática sólo se redujo un 5% y un 20%, respectivamente. Sin embargo, el enzima se pudo inactivar considerablemente mediante PEAIC, observándose que la inactivación aumentó con el número de pulsos y la intensidad de campo aunque resultó mucho más sensible a un tratamiento mediante PEAIC en flujo continuo (cámara electrodos planos paralelos) que en discontinuo (cámara coaxial). En el primer caso, la actividad del enzima se redujo hasta un 62.1% al aplicar 80 pulsos a 27.4 kV/cm (504.97 kJ/l), mientras que en el segundo y después de aplicar 80 pulsos a 37.3 kV/cm (424.36 kJ/l) la actividad únicamente disminuyó un 13%.

- El enzima proteasa de *Bacillus subtilis* presentó menos resistencia a los tratamientos de pasteurización térmica que la lipasa de *P. fluorescens*, ya que al aplicar condiciones de pasteurización baja (63°C-30 min) se consiguió casi la completa inactivación del enzima tanto en SMUL como en leche; resultados similares se obtuvieron al procesar leche mediante un tratamiento de pasteurización alta (75°C-15 s), aunque en SMUL este último tratamiento sólo consiguió reducir la actividad de la proteasa un 7%. Por otro lado resultó mucho más resistente al tratamiento mediante PEAIC ya que al aplicar procesos de hasta 500 kJ/l en tres equipos diferentes (uno de flujo discontinuo y dos de flujo continuo) se observó poca efectividad en la inactivación de la proteasa tanto en SMUL como en leche, requiriéndose tratamientos superiores a 6000 kJ/l para observar reducciones considerables de la actividad enzimática.

- Al aplicar tratamientos de hasta 6786.8 kJ/l a muestras de SMUL o leche con distinto contenido en grasa que contenían el enzima proteasa de *Bacillus subtilis* se observó que en todos los casos la inactivación aumentó con el tiempo de tratamiento, la intensidad de campo, la densidad de energía y la frecuencia. Asimismo se observó que en las condiciones evaluadas, la anchura de pulso no afectó al nivel de inactivación conseguido si se consideraba el tiempo total de tratamiento.

- La leche desnatada fue el medio en que los tratamientos resultaron más efectivos para la inactivación de la proteasa de *Bacillus subtilis*. Al aplicar las condiciones más severas evaluadas (6559.8 kJ/l a 111.11 Hz) la actividad se llegó a reducir hasta un 81.1% mientras que aplicando condiciones similares, en SMUL y en leche entera la actividad disminuyó un 62.7% y un 57.1%, respectivamente.

- La reducción de la actividad de ambos enzimas (lipasa y protesa) por PEAIC se pudo ajustar a diversos modelos matemáticos respecto a la densidad de energía, número de pulsos o tiempo de tratamiento y/o intensidad de campo.

- Las vitaminas estudiadas, en leche y SMUL, resistieron a los tratamientos térmicos y por PEAIC sin pérdidas significativas a excepción del ácido ascórbico. La disminución del contenido en ácido ascórbico siguió una tendencia de tipo exponencial respecto al tiempo de tratamiento en ambos procesos. La leche retuvo mayor cantidad de ácido ascórbico después de un tratamiento de 400 μs a 22.6 kV/cm (93.4%) que después de aplicar procesos térmicos de pasteurización baja (50.3%) o alta (13.3%). La retención de vitamina después del tratamiento mediante PEAIC fue mayor en leche que en SMUL.

- Los PEAIC pueden llegar a inactivar en un elevado porcentaje los enzimas termoresistentes estudiados, siendo un proceso cuidadoso con las vitaminas. Este tratamiento puede considerarse como alternativa a los tratamientos térmicos para obtener productos de alta calidad nutricional, aunque se requiere seguir investigando para llegar a establecer los parámetros importantes para reducir la actividad de los enzimas indeseables sin afectar a las propiedades nutritivas de los alimentos.