UNIVERSIDAD POLITÉCNICA DE VALENCIA

Departamento de Tecnología de Alimentos

Impacto del tratamiento por pulsos eléctricos de alta intensidad y altas presiones hidrostáticas sobre la calidad y seguridad microbiológica de un alimento mezcla de zumo de naranja y leche

TESIS DOCTORAL EUROPEA

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La presente tesis doctoral europea se engloba en los siguientes proyectos de investigación:

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- "*Optimización y validación de procesos mínimos de conservación de un nuevo alimento funcional líquido, mediante aplicación de pulsos eléctricos de alta intensidad (PEAI), solos o con calor*" del Ministerio de Ciencia y Tecnología-CICYT (2004-2006).

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RESUMEN

La creciente demanda de alimentos con características lo más parecidas al producto fresco, está impulsando el desarrollo de nuevas tecnologías "no térmicas" de conservación. Dentro de las más prometedoras se encuentran el tratamiento por Pulsos Eléctricos de Alta Intensidad (PEF) y la tecnología de Altas Presiones Hidrostáticas (HHP). Estas tecnologías permiten conservar, en mayor medida que los tratamientos térmicos, la calidad (sabor, aroma, color y vitaminas) de determinados alimentos frescos e inactivar microorganismos y enzimas, incrementando su vida útil en refrigeración y facilitando su comercialización. El objetivo general de la presente tesis doctoral ha sido el procesado por PEF y HHP solos o combinados con calor de una nueva bebida mezcla de zumo de naranja y leche incluyendo aspectos microbiológicos y de calidad. El plan de trabajo comenzó con la elaboración y caracterización físico-química y sensorial del nuevo producto eligiendo la formulación adecuada para desarrollar los estudios cinéticos y de vida útil. Los parámetros de calidad más importantes en el nuevo producto fueron la actividad enzimática y el contenido en aroma (concentración de compuestos volátiles). En el caso del zumo de naranja la pectin metil esterasa (PME) es una de las enzimas de mayor importancia. Se evaluó el efecto del tratamiento por PEF, HHP y calor en la inactivación de PME. Todas las tecnologías estudiadas lograron un nivel de inactivación enzimática del 90%. Se observó la aparición de dos fracciones con diferente resistencia al tratamiento, por ello, el modelo bifásico fue el que mejor describió las curvas de inactivación de PME mediante tratamiento combinado de HHP y calor en el producto. Posteriormente se estudió la variación en el contenido en aroma (concentración de compuestos volátiles) tras el tratamiento de HHP, PEF y calor en el producto siendo la tecnología por PEF la que mejor preservó el aroma original del producto fresco. Una vez establecidos los estudios relacionados con aspectos de calidad en el producto se realizaron los estudios microbiológicos. Se estudió la influencia de las variables del proceso por PEF en la inactivación de un microorganismo alterador (*Lactobacillus plantarum*) en el producto. La intensidad de campo eléctrica, tiempo de tratamiento y temperatura fueron las variables que más influyeron produciendo un aumento de la inactivación con un menor gasto energético. Más tarde, se estudiaron las cinéticas de inactivación de *L. plantarum* mediante la combinación de la tecnología de PEF y calor. A su vez, se eligió el modelo matemático de Weibull como el que mejor describía las curvas de supervivencia del microorganismo tras el tratamiento por PEF siendo el parámetro $\overline{tcw}$ un índice de resistencia del microorganismo al tratamiento. Entre los microorganismos patógenos, *Salmonella typhimurium* se ha demostrado como un problema de seguridad alimentaria en alimentos ácidos como el zumo de naranja por lo que se decidió estudiar

sus cinéticas de inactivación por PEF en función de las características del alimento, pH y concentración de estabilizante (pectina). Una disminución del pH favoreció la inactivación mientras que el porcentaje de pectina no tuvo un efecto significativo. Finalmente se realizó un estudio de vida útil del producto en refrigeración tras el tratamiento de PEF y calor demostrando la viabilidad de la tecnología de PEF para obtener alimentos con similar vida útil que el tratamiento térmico pero con mejor calidad final.

SUMMARY

The growing demand of food with characteristics similar to the fresh product is enhancing the development of nonthermal preservation technologies. Among them High Hydrostatic Pressure (HHP) and Pulsed Electric Field (PEF) processing are the most promising. These technologies allow the preservation of food quality (flavor, aroma, color and vitamins) in a greater extent than thermal technology. The microbial and enzyme inactivation degree obtained through these technologies allow extending the shelf-life of the product at chilled storage facilitating its commercialization. The general objective of the present doctoral thesis was to study the PEF and HHP processing alone or combined with thermal treatment in a new beverage based on the mixture of orange juice and milk including microbiological and quality aspects. The work plan began with the preparation and physicochemical and sensorial characterization of the new product, choosing the adequate formulation for developing the kinetic and shelf-life studies. The main quality parameters in an orange juice based product were the enzyme activity and the aroma content (volatile compounds concentration). In an orange juice, pectin methyl esterase (PME) is one of the most relevant enzymes. A study of the influence of PEF, HHP and thermal processing on PME inactivation was developed. The different technologies could inactivate 90% of PME. The appearance of two fractions with different resistance to the treatment was observed and a biphasic model was used to describe PME inactivation curves by a combined HHP and thermal treatment. The study of the effect of the different processing technologies on aroma content (volatile compounds concentration) was also performed. The results revealed PEF technology as the best process to preserve the original aroma of the product. Once studies related to quality parameters were established, the microbiological aspects related to the new product were also performed. The influence of PEF processing variables on the inactivation of a spoilage microorganism (*Lactobacillus plantarum*) was carried out in the new product. Electric field, treatment time and temperature were the most influential variables, producing higher inactivation with lesser energy consumption. A kinetic study of PEF inactivation of *L. plantarum* was also developed. Weibull model was chosen as the one which best described microorganism survival curves after PEF treatment and the $\overline{tcw}$ parameter could be considered as an index of the microorganism treatment resistance. Within the pathogen microorganisms, it has been demonstrated that *Salmonella typhimurium* could originate a food safety problem in acid foods such as orange juice. In order to study the microorganism behavior against PEF treatment a kinetic study was done based on the food characteristics such as pH and stabilizer concentration (pectin). It was found that any pH diminish favored inactivation while pectin concentration did not have a significant effect. Finally a shelf-life

study after PEF and thermal treatment was carried out in the beverage stored at refrigeration temperature. The study showed the viability of PEF technology to obtain food with similar shelf-life than thermal treatment but better final product quality.

RESUM

La creixent demanda d'aliments amb unes característiques pròximes als productes frescos, està impulsant el desenvolupament de noves tecnologies "no tèrmiques" de conservació. Entre les que ofereixen més opcions de futur, es troben el tractament amb Polsos Elèctrics d'Alta Intensitat (PEF) i la tecnologia d'Altes Pressions Hidrostàtiques (HHP). Aquestes noves tecnologies permeten conservar en un alt grau la qualitat de determinats aliments frescos (sabor, aroma, color i vitamines) i, a més, inactivar microorganismes i enzimes, que incrementen la seua vida útil en refrigeració i faciliten la seua comercialització. L'objectiu general d'aquesta tesi doctoral ha estat l'estudi del processat d'una nova beguda (un barreig de suc de taronja i llet) per PEF i HHP sols o combinats amb calor, incloent-hi aspectes microbiològics i de qualitat. El pla de treball va començar amb l'elaboració i la caracterització físico-química i sensorial del nou producte i l'elecció de la formulació adequada per desenvolupar els estudis pertinents. Els paràmetres de qualitat més importants en els sucs de fruita son l'activitat enzimàtica i el contingut en aroma (concentració de compostos volàtils). En el cas del suc de taronja, la pectin metil esterasa (PME) és una de las enzimes de major importància. Per això es va realitzar un estudi per avaluar la influència de les diferents tecnologies de conservació (PEF, HHP i calor) en la inactivació enzimàtica. Les tecnologies estudiades van aconseguir un grau d'inactivació del 90%. Després de l'estudi es va observar l'aparició de dos fraccions amb diferent resistència al tractament. Així mateix, el model bifàsic va ser el que millor va descriure les corbes d'inactivació de PME, mitjançant el tractament combinat de HHP i calor en el producte. Es va estudiar també la variació en la concentració de compostos volàtils després del tractament de HHP, PEF i calor en el producte. La tecnologia de PEF va ser la que millor va preservar l'aroma original del producte fresc. Una vegada es van establir el estudis relacionats amb el paràmetres de qualitat es van estudiar els aspectes microbiològics. El inici va consistir en la realització d'un estudi per determinar la influència de les variables del procés per PEF en la inactivació d'un microorganisme alterador en el producte (*Lactobacillus plantarum*). La intensitat de camp elèctric, el temps de tractament i la temperatura, van ser les variables que més van influir, produint un augment de la inactivació i alhora una menor despesa energètica. A continuació es van estudiar las cinètiques d'inactivació de *L. plantarum* mitjançant la combinació de la tecnologia de PEF i calor. Al mateix temps, es va elegir el model matemàtic de Weibull per ser el que millor descrivia les corbes de supervivència del microorganisme després del tractament amb PEF. Es va considerar el paràmetre $\overline{tcw}$ com a l'índex de resistència del microorganisme al tractament. Entre els microorganismes patògens, s'ha demostrat últimament que *Salmonella typhimurium* pot

ocasionar un problema de seguretat alimentària en aliments àcids, com és el cas del suc de taronja. Per aquest motiu, es va decidir estudiar les cinètiques d'inactivació d'aquest microorganisme per PEF, en funció de les característiques de l'aliment (pH i concentració d'estabilitzant). Una disminució del pH va afavorir la inactivació, mentre que el percentatge de pectina no va tenir un efecte significatiu. Finalment, es va realitzar un estudi de la vida útil del producte en refrigeració després del tractament de PEF i calor. El resultat d'aquesta última experiència va demostrar la viabilitat de la tecnologia de PEF per obtenir aliments amb una vida útil similar, però amb millor qualitat final.

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1.-FRAMEWORK

One of the main duties of any national sanitary authority is to guarantee a food supply in safety conditions and recommend those with high nutritional value. In that sense, there is a scientific evidence that vegetables and fruit consumption can lessen the impact of illnesses such as cancer, cardiovascular and brain illnesses or diabetes. Nowadays fruit and vegetable consumption is considered the most important strategy to prevent the appearing of cancer after reducing tobacco. The HWO recommends at least 400 grams or five rations of fruit and vegetable consumption per day, including 30 grams of legumes, seeds and nuts (Southon and Faulks, 2002).

A growing rhythm of life is generating a higher demand of "ready to eat" food. On the other hand there is also a higher interest on food quality (low-preservative content food and products with characteristics near to the fresh product) and biofood. It is supposed that such food has all food safety guaranties and an adequate shelf-life in refrigeration conditions. The industry is conscious about the changes in the consumer tendencies. A significant fact is the appearance in the market of a great variety of minimally processed food that need chilled chain for its storage and distribution (fruit juices, sauces, precooked products, fresh pasta, among others). Within these products, refrigerated juices and the combination of fruit juice and milk have been on the increase lately. They offer an interesting flavor mixture and provide essential nutrients to the daily intake such as vitamin-C, vitamin-D, calcium and natural antioxidants, among others.

The majority of such products are processed by a mild pasteurization treatment. However, thermal treatment alters the original food characteristics such as vitamins, color and flavor, among others. In that sense, since twenty years ago, scientists have begun to develop alternative technologies that could guaranty food safety and improve the overall product quality. These technologies have been called "Emerging preservation technologies" or "Nonthermal technologies" because are based in other aspects different from heat (Raso and Barbosa, 2003).

Within these technologies, Pulsed Electric Field (PEF) and High Hydrostatic Pressure (HHP) processes are the most important due to their

application and promising results. In Spain, HHP technology is present in the industry and several companies have recently marketed different sliced-jam and precooked products treated by HHP. In the rest of Europe, commerce of HHP treated food is growing including a wide range of products. It is believed that the technology would expand to other industrial fields (Trujillo et al., 2002).

PEF is a more recent technology and several industrial-scale equipments have been developed in USA and a great number of bench-scale equipments are spread in different researching groups, three of them in Spain. Nowadays a company has marketed mixtures of different fruit juices processed by combined PEF and thermal treatment in USA. It is also believed that the introduction of PEF technology as a method for liquid food pasteurization would increase in the future (Clark, 2006).

2.-INTRODUCTION

2.1 HIGH NUTRITIONAL FOOD: FRUIT JUICE-MILK BASED PRODUCTS

Nowadays, new technologies, new food production practices and new food processes are being developed to satisfy the needs of a changing society. At the same time, new products are marketed with the purpose to provide to the consumer improved nutritional food with functional properties. The global market of functional food is estimated in approximately 33 billions US$, from which 2 billion US$ belongs to the European market. The key-sector of functional food is dairy food reaching sales for approximately 1.35 billion US$ in 2003 (Menrad, 2004). Germany is the most dynamic European country in the development and sales of functional food. The market volume of functional dairy food has passed from 5 million US$ in 1995 to 419 million US$ in 2002 (Hilliam, 2003a). Other important category of functional products is the non-alcoholic beverages fortified with vitamins A, C or E and other functional ingredients. The market volume for these beverages was 89 million US$ in 2002 against 15 million US$ in 1996 (Hilliam, 2003b).

Regarding to Spain, the total juice consumption was 18.4 1/ *per capita* in 2005 belonging 10% to short-term life products (Artiach, 2005). There is no data related to the consumption of juice and milk based products in Spain but during last years a great variety of new products of such nature have appeared in the market showing a consumer growing interest.

2.2 THERMAL TREATMENT

Since decades ago, thermal treatment has been the most widespread method for food preservation. Through the years microbiologically safe food with low enzyme activity have been produced but at the expense of the final product quality. Nowadays, sterilization and pasteurization are the most common methods for food preservation.

In order to design the optimum thermal treatment (intensity and type of treatment), it is necessary to clearly identify the food physicochemical and microbiological characteristics that most influence on the process such as: pH (acid and basic food), viscosity (particulate food), food composition (fat content, proteins, sugar and additives), natural flora, endogenous enzymes and thermophysical properties (specific heat capacity and thermal conductivity) (Ramaswamy and Chen, 2002).

2.2.1 Influence of thermal treatment on fruit juices quality

Fruit juices are products susceptible to the loss of their organoleptic characteristics such as color (luminosity loss), water-soluble vitamins, carotenoids, changes in the physical properties (viscosity), flavor and volatile compounds content loss (aroma). At the same time, the temperature increases the rate of non-enzymatic browning reactions (Maillard reaction, sugar caramelization and ascorbic acid oxidative reaction) and as a result, a reduction in the sensorial quality and nutritional value of the juice is produced (Primo, 1979; Gerardi et al., 1983; Lafuente, 1985). The characteristic juice flavor is also altered appearing a defect known as “boiled taste”.

The extent of loss after the thermal treatment depends on its nature. On that basis, the highest loss of nutrients is produced in the sterilization process due to the severity of the treatment. In addition, food can be deteriorated during the storage by enzyme or microorganism fractions resistant to the treatment, increasing the rate as the storage temperature rises. The type of packaging and oxygen concentration in the headspace will also influence the degradation of the juice (Martín et al., 1995).

2.2.2 Methods for thermoresistance measurement

Different methods to measure the microorganism and enzyme thermoresistance as well as quality factor degradation are well established depending on treatment characteristics (time-temperature combination) and food.

In the present doctoral thesis, the TDT (Thermal Death Time) disk/tube method and capillary tubes method have been developed.

2.2.2.1 TDT disk/tube

This method consists in enclosing the sample in a stainless steel tube (13 mm inner diameter) or disk (3x3 cm) closing it with a sealed screw top. Thermal treatment is carried out in a water bath with temperature control and immediately cooled in ice-water after the preset times. To measure the heating time or come-up time (CUT), a thermocouple connected to a data logger is introduced in the inner side of the tube/disk containing the sample measuring the time to reach the different treatment temperatures (Figure 2.2.1). Generally the sample is pre-heated to 40ºC to avoid a long come-up time. The come-up time in the disk method is shorter due to the minor sample volume used in the analysis.

The method with tubes is usually used in quality studies when higher sample volume is needed for the analysis (~20 mL). The method with disks is more convenient when the quantity of the sample needed is relatively small (1 mL) or when using high viscosity foods. The main advantage of this method is the low cost and simplicity. However, the failure of sealing may produce the contamination of the sample.

(A) **(B)**

Figure 2.2.1-TDT tube with an inner thermocouple (A). Instituto de Agroquímica y Tecnología de Alimentos, Valencia (Spain). TDT disk (B). Eastern Regional Research Center, USDA, PA, (USA).

2.2.2.2 Capillary tubes

This method is based on a modification of that proposed by Stern and Proctor, (1954). This method consists in introducing the sample in a ring-marked microhematocrit capillary tube with an inner diameter of 0.7-2.5 mm and a volume ranging from 50-1000 μL (Figure 2.2.2). Tubes are sealed by pulling off both ends in an oxygen gas flame and then heated in a stirred oil or water bath. After the preset times, the sample is withdrawn from the bath and immediately cooled in ice-water. Tubes are opened in aseptic conditions.

The main advantages of this method are the short come-up (6-8 seconds, depending on the temperature) and its suitability to temperatures up to 130ºC. Nevertheless, this method is more laborious.

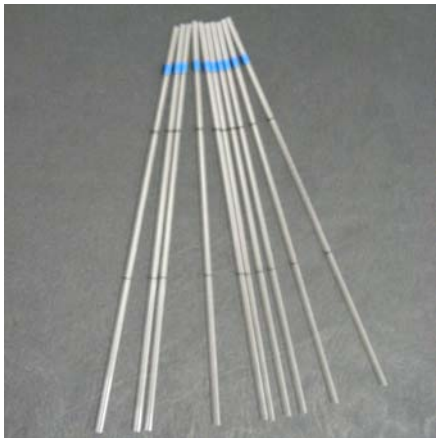

Figure 2.2.2: Capillary tubes (200 μL). Instituto de Agroquímica y Tecnología de Alimentos, Valencia (Spain).

2.2.3.3 Industrial scale-up

Once thermoresistance studies have been performed and optimum treatments have been established, it is important to scale them up into a semi-industrial equipment. In the present doctoral thesis, a plate heat exchanger has been used for such purpose (Figure 2.2.3).

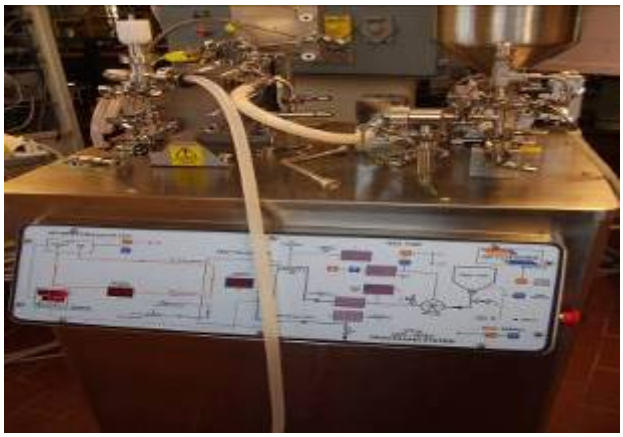

Figure 2.2.3: Plate heat exchanger. Eastern Regional Research Center, USDA, PA, (USA).

2.3 NONTHERMAL PRESERVATION TECHNOLOGIES

Nonthermal preservation technologies are defined as those that do not use temperature as the main factor in microbial and enzyme inactivation. In the majority of these technologies a moderate temperature rise is produced, however, it does not reach the level of a thermal treatment (Raso and Barbosa, 2003).

The main goal of the nonthermal preservation technologies is to obtain microbiologically safe food with a low enzyme activity keeping the original sensorial properties such as flavor, aroma and color and nutritional properties such as vitamins. In essence preserving the characteristics of the fresh food. Within these technologies is worthy to remark the use of natural antimicrobials, irradiation, high intensity luminic pulses, high intensity magnetic pulses, high and low intensity ultrasounds, UV radiation and radiofrequency electric fields, among others (Barbosa et al., 1998). Nowadays the most promising and applicable technologies are High Hydrostatic Pressure (HHP) and Pulsed Electric Field (PEF). These are the subject matter of this doctoral thesis and are described in the following lines.

2.4 HIGH HYDROSTATIC PRESSURE (HHP)

HHP technology is lately acquiring high relevancy as an alternative of thermal treatment in the preservation of certain type of products (meat products, fruit and vegetable juices and sauces). The first product treated by HHP appeared at the beginning of the 90's in Japan. The company Heidi-ya began to distribute pasteurized products by HHP such as marmalades, sauces and fruit juices, among others (Rovere, 2002). Nowadays, great number of companies commercialize HHP treated products (Figure 2.4.1). In Spain, several meat and canned industries commercialize products treated with this technology and it is believed that this tendency will increase in the future (Norton and Sun, 2007).

HHP technology is based in two fundamental principles. The Chatelier principle that establishes that pressure helps all the reactions and structural changes that lead to a volume diminish. On the other hand, the isostatic principle that establishes that pressure distribution is proportional in all food parts independently of its shape and size (Heremans, 2002).

Van der Berg et al., (2002) estimated the cost of processing a litre of a product by HHP in 10-20 euro-centimes against 2-4 euro-centimes with heat. Rastogi et al., (2007) observed the same energy proportion to comprise a litre of water by HHP (19.2 kJ at 400 MPa) and heat a litre of water (20.9 kJ from 20 to 25ºC). A better design of the process and materials along with the combination with other technologies (moderate temperature, antimicrobials and CO_2) will permit in a near future talking about a HHP sterilization process (Smelt et al., 2002).

2.4.1 HHP treatment system

The main components of the HHP equipment are as follow:

- Vessel and yoke
- Hydraulics (generation pressure system)
- Temperature control system

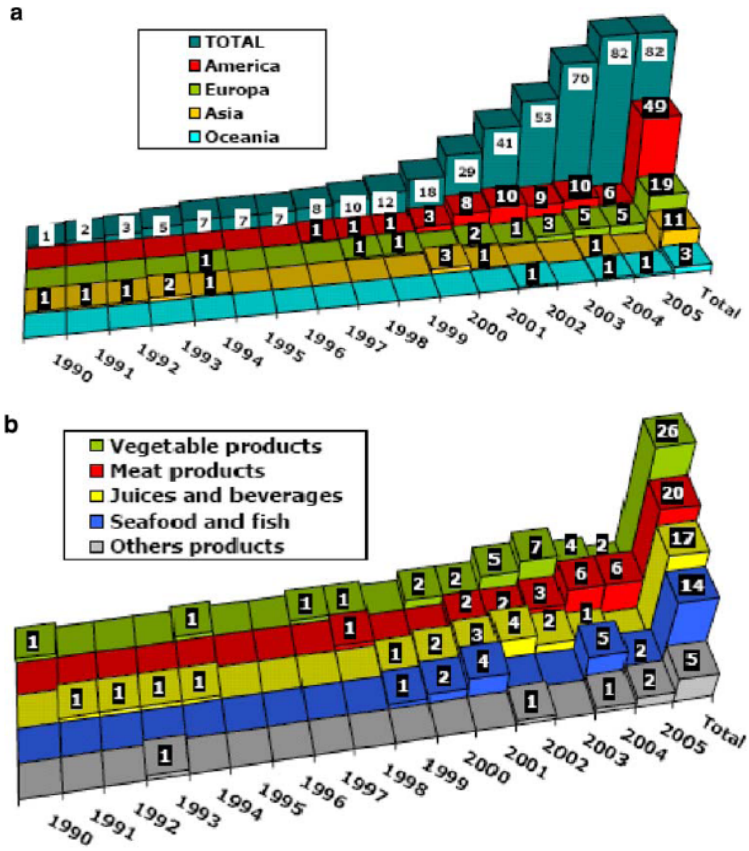

Figure 2.4.1: Number of HHP equipment installed in Europe by Hyperbaric® versus (A) year of instalment and (B) industrial sector for the instalment (Urrutia-Benet, 2005).

In general term, the operation mechanism of HHP equipment consists in pumping a pressurizing fluid (water or oil) from a deposit where it is contained to the treatment vessel. Once the vessel is filled with the liquid and no air is inside, pressure is built-up by a hydraulic pump. Pressure is transmitted through the liquid to the vessel up to reaching the desired pressure value. This method is called "indirect pumping" and it requires static seals (Figure 2.4.4-B). A "direct pumping" method has been also designed consisting in a piston that directly compresses the

vessel increasing the pressure. In this system dynamic seals are required (Figure 2.4.4-A).

(A) **(B)**

Figure 2.4.2: HHP lab-scale equipment: (A)-Laboratory of Food Process, Katholieke Universiteit Leuven, (Belgium), (B)-Instituto de Agroquímica y Tecnología de Alimentos, Valencia, (Spain).

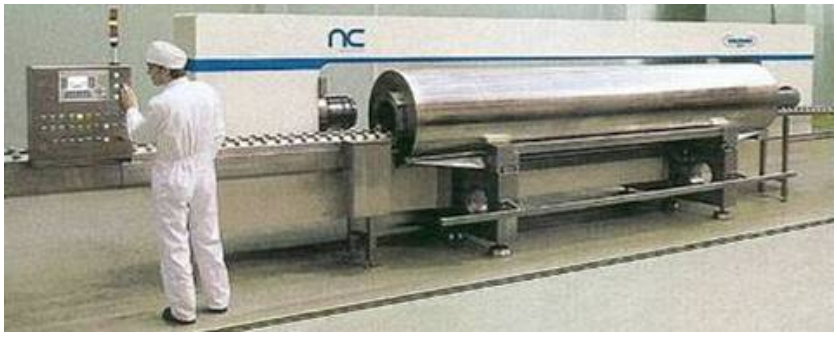

Figure 2.4.3: HHP industrial-scale equipment (Hyperbaric S.A., Burgos, Spain).

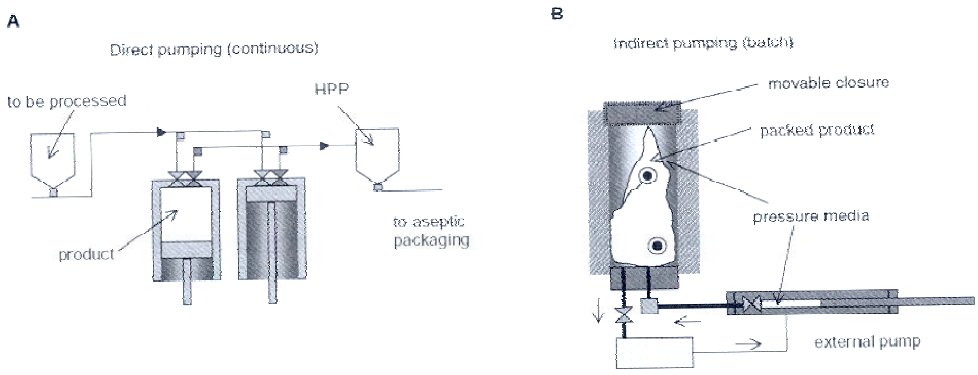

Figure 2.4.4: Illustration of a direct pressurization (A) and indirect pressurization (B) (Rovere, 2002).

Temperature is controlled by a thermostated mantle connected to a cryostat that surrounds the vessel. This method is adequate in an isothermal treatment. However, in a nonisothermal treatment, the above temperature control is very slow and the use of a heat exchanger is preferred. The measure of the temperature is usually carried out by thermocouples placed inside the vessel in contact with the pressure medium. Improvement of the temperature control by measuring the temperature of food during the HHP treatment should be considered (van den Berg et al., 2002).

Several companies in Japan, USA and Europe (one of them in Spain) design and commerce HHP industrial and laboratory scale equipments. The equipments are design with a capacity up to 500 L and provide pressures up to 800 MPa. For technical reasons the majority of the equipments are static. The pressure medium is regularly water or glycol-oil mixture and food is packaged in a flexible material with no headspace to resist the volume variation during the treatment. HHP treatment of fruit juice is based on a pressure range of 400-500 MPa during 1-5 min under room temperature. Lately, different continuous equipments have appeared. The principle of its functioning is pumping the product to the vessel and then pressurized by a floating piston that separates the product from the pressure

medium. Other continuous equipments have lastly been marketed based on various consecutive treatment vessels (van der Berg et al., 2002).

2.4.1.1 One step treatment

This is the most widespread HHP treatment for both industries and laboratories. It is based on building up the pressure up to the working pressure level, keeping it during the holding time and depressurization the system (Figure 2.4.5).

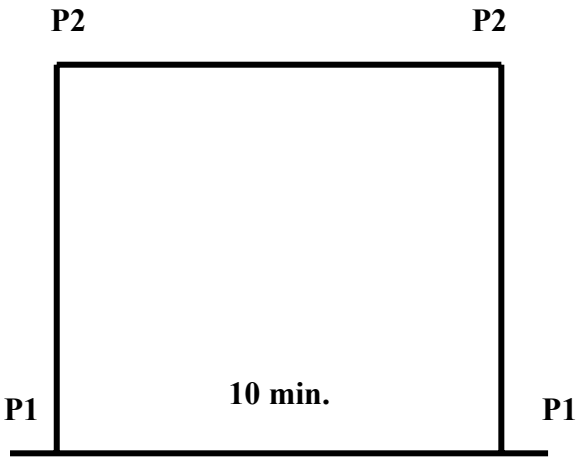

Figure 2.4.5: One step treatment.

2.4.1.2 Multi-step treatment

The treatment is based on the combination of consecutive one-step treatments. The total treatment time will be the sum of the single time of every treatment (Figure 2.4.6). It is believed that the multi-step treatment produces an increase in the microorganism inactivation due to higher cellular stress by the successive compressing and decompressing.

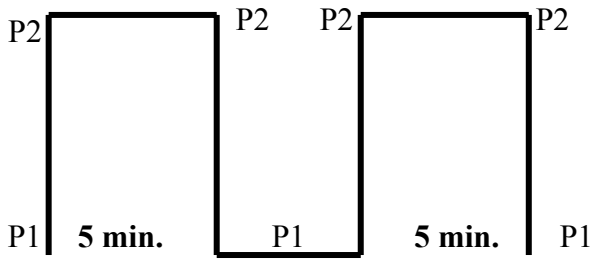

Figure 2.4.6: Multi-step treatment (2 steps).

2.4.1.3 Step-wise treatment

This treatment is a nonisobaric process and is used when the pressure value during the treatment is wanted to vary. A direct application of this type of treatment is the HHP spore germination (phenomenon explained later).

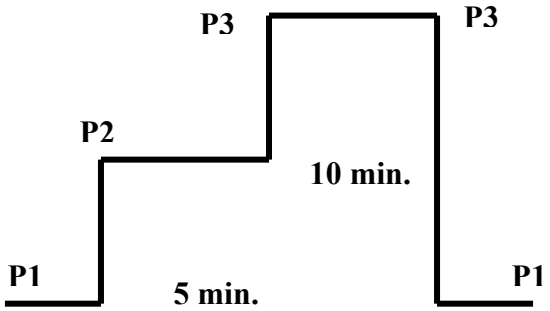

Figure 2.4.7: Step-wise treatment

2.4.2 HHP technology related factors

2.4.2.1 Pressure level and treatment time

Pressure level and treatment time are the two most important variables in the HHP technology. As general rule an increase in the pressure value produces higher inactivation shorten the treatment time. In the equipment design is important to know the relation between P-t to reach the required inactivation degree. Generally a treatment time more than 20 min would not produce a significant

inactivation increase being 5 min the optimum value (Balasubramaniam et al., 2004). Vegetative cells need pressure values between 200-400 MPa while enzymes and bacterial spores need higher pressure values (500-1000 MPa) and combination with moderate temperature (30-60ºC). Increasing the required pressure value will increase the cost of the equipment as well the total operating time. These circumstances will make the final product more expensive. A way to reduce the required pressure level is increase the initial temperature of the product (Figure 2.4.8).

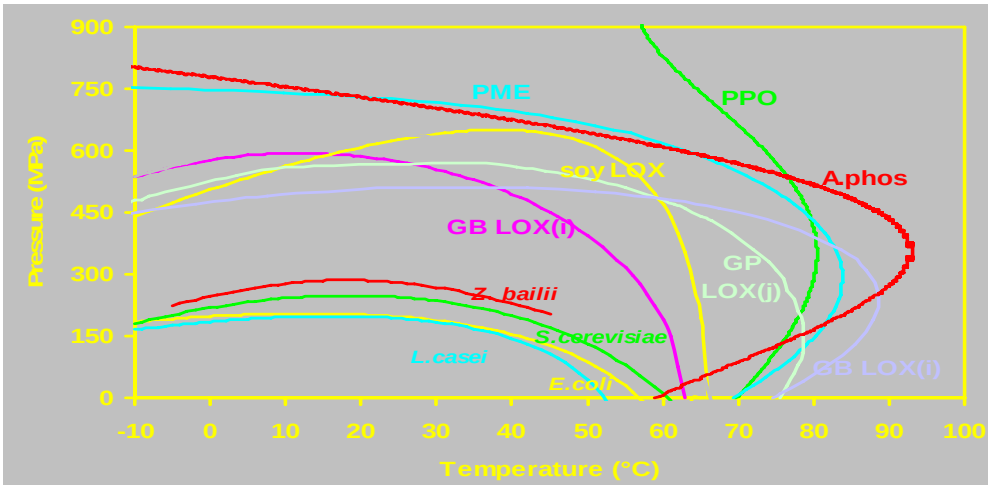

Figure 2.4.8: T-P combination to obtain 1 log reduction of different microorganisms and enzymes (Ludikhuyze et al., 2002).

2.4.2.2 Temperature: adiabatic heating

During the compression the temperature of all compressible materials increases due to the adiabatic heating. The general expression used to illustrate the temperature rise during the compression in adiabatic-isentropic conditions is as follow (Toepfl et al., 2006):

$$\frac{dT}{dp} = \frac{\beta \cdot T}{\rho \cdot C_p}$$ **Equation 2.4.1**

where β, ρ and C_p are thermal expansivity, density and specific heat capacity of the pressurizing fluid, respectively. The thermophysical properties are pressure and temperature dependent. When these properties are known, it is possible to estimate the temperature profile during the compression. Different pressure transmitting media show different adiabatic heating curves that can be obtained from the mixtures of pure substances. The main ingredient in many products is water, allowing estimating the temperature rise during the treatment using its thermodynamic properties. Water has a low compression heating value while the temperature rise in fat-containing food can be three times higher (Table 2.4.1). The heat transfer of the pressure medium can be used to increase the food temperature during and after the adiabatic heating.

Figure 2.4.9 shows the pressure and temperature variation during the HHP treatment. Ps and Pf represent the atmospheric pressure (0.1 MPa). Tm is the maximum process temperature. The difference in room temperature before and after HHP treatment (Ts y Tf) generally indicates the heat loss produced during the treatment (with the presumption that the depressurizing time only takes few seconds) (Balasubramaniam et al., 2004).

Table 2.4.1: Adiabatic heating in different food (Toepfl, et al., 2006).

Substance	T increase / 100 MPa (ºC)
Water	~3.0
Potato purée	~3.0
Orange juice	~3.0
Tomato sauce	~3.0
Milk (2% fat)	~3.0
Salmon	~3.2
Chicken fat	~4.5
Beef fat	~6.3
Olive oil	6.3-8.7
Soya oil	6.2-9.1

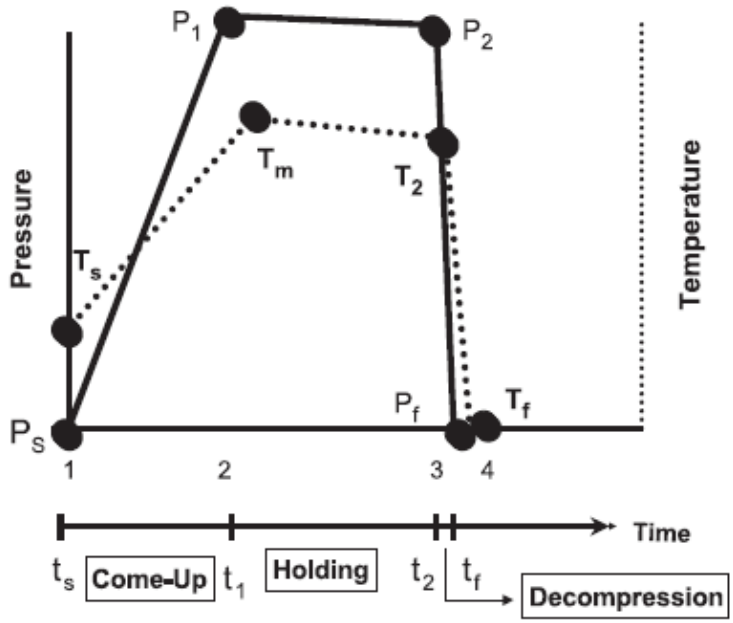

Figure 2.4.9: HHP common variables: temperature, pressure and treatment time (Balasubramaniam et al., 2004).

2.4.2.3 Pressurization/depressurization time

The temperature rise during compression depends on the required pressure value and come-up time (CUT). Higher CUT will increase the temperature that follows the pressure rising. The pressurizing/depressurizing time will depend on the equipment used. Automatic equipments where the pressurizing/depressurizing time will depend on the equipment facilities and manual equipments where the pressure is building-up manually being this variable able to control (Rovere, 2002).

2.4.3 Food and packaging related factors

2.4.3.1 Type of packaging

One of the most important factors in HHP treatment is the use of an appropriate packaging material. Their physical and mechanical properties will

influence the treatment effectiveness. In general, the packaging must be resistant to high pressure levels maintaining their sealing properties to prevent quality losses during the treatment. During HHP treatment the volume loss suffered by the packaging can reach 15% turning back to its original volume during decompression. For that reason, at least a packaging inter-phase must be flexible to transmit the pressure adequately. According to that, metal, glass or rigid plastic materials are not suitable for the treatment. Flexible plastic (bags, tubes or trays) are up to now the best options for HHP treatment, however, systematic studies are necessary to improve the design of packaging and seals to acquire resistance to higher pressure values. The type of packaging used for some fruit juices, sauces and milk, among others, are good examples where packaging and sealing should be redesign for a HHP treatment purpose (Rastogi, et al., 2007).

2.4.3.2 Presence of air

The presence of air is other important factor intimately related with the food and packaging. As stated before, the packaging volume tends to be reduced during HHP treatment. The presence of air (headspace) which is more compressible than water could produce the packaging breakage. The texture loss produced by the air compression in food such as strawberries, lettuces or prefrozen bread is other of the HHP main effects.

2.4.3.3 Water activity

In general by diminishing the food water activity the treatment efficacy decreases due to a less pressure transmission. In the enzyme denaturation process it is also necessary the presence of water (Van den Broeck et al., 1999 b). As a consequence, dried or dehydrated food should be evaluated for being treated by HHP.

2.4.3.4 pH

A decrease in the pH value generally improves the effectiveness of the treatment. Gram- bacteria seem to be more acid-tolerant and thus baroresistant than Gram+ bacteria (Alpas et al., 2000).

2.4.4 HHP microbial inactivation

2.4.4.1 Spore inactivation

Spore formation is a surviving strategy of some microorganisms to adverse conditions being *Bacillus* and *Clostridium* genus the most important in food. Despite that applying high pressure levels spore counts in food can be reduced, in order to reach an sterilization process, it is necessary the combination with other technologies. A combined HHP and thermal treatment has been used in numerous studies with promising results in spore inactivation (3 log reductions in *Clostridium sporogenes* and *Bacillus coagulans* after 500 MPa combined with 60-70ºC) (Roberts and Hoover, 1996; Mills et al., 1998).

Different spore germination methods have been proposed by the combination of a germinant, pressure and moderate temperature (Sojka y Ludwig., 1994 and 1997; Raso et al., 1998; Wuytack et al., 1998; Black et al., 2006). It is based on a step-wise treatment (Figure 2.4.7) applying a treatment at low pressure levels (200-300 MPa) combined with the use of a germinant to induce spore germination, followed by a combined HHP and thermal treatment (500-600 MPa, 30-50ºC) to inactivate vegetative cells. Up to now, this type of treatment is under study due to the variability in the germination of a spore population and differences among different microorganisms. The absence of spore germination kinetic studies is also remarkable. In spite of the good results obtained, it is still not possible to talk about a sterilization process (Wuytack, 1999).

2.4.4.2 Vegetative cells inactivation

The mechanism of microorganism inactivation by HHP is a combination of different reactions, such as noncovalent bonds breakdown and cell membrane permeability increase that modifies the ions efflux leading to a decrease in the intracellular pH. At low pressure levels the morphological damage produced in the cellular membrane is often reversible while at higher levels the death of the microorganism is produced by an irreversible damage (Rastogi, et al., 2007). Generally the microorganism baroresistance is inversely proportional to the membrane rigidity. Pressure values between 200-600 MPa at room temperature are often enough to inactivate the majority of vegetative cells including molds and yeasts. An initial temperature rise between 45 and 50ºC increase the inactivation level producing a synergetic effect (Smelt et al., 2002).

The microorganism baroresistance also depends on the species. Molds and yeasts are less baroresistant and are frequently inactivated at 200-300 MPa. Gram- bacteria are often more sensitive to the treatment than Gram+ bacteria and a treatment at 400 MPa is generally enough to inactivate them. However, there are numerous exceptions to this affirmation. It has been demonstrated that some *E. coli* O157:H7 strains are more baroresistant. The combination of HHP with other technologies such as acidification or antimicrobials (ascorbic acid, nisin o lysozyme) seems to reduce microorganism baroresistance (Smelt et al., 2002).

2.4.5 HHP enzyme inactivation

Enzymes are a special type of proteins with an enormous catalytic power and a great specificity. Their biological activity arises from active sites brought together by a three-dimensional configuration. They have two important regions; one that recognizes the substrate and other that catalyzes the reaction once the substrate has been bound (Ludikhuyze et al., 2002). These two are called the active site and take place in a small part of the enzyme total volume. Changes in the active site or protein denaturation can produce an activity loss or functionality variations (Tsou, 1986). In general, covalent bonds are not affected by HHP

treatment because the primary structure of the enzyme will not be damage. The hydrogen bonds are also relatively baroresistant and secondary structure will not be affected up to pressure values around 700 MPa. However, HHP treatment affects electrostatic and hydrophobic interactions that maintain the tertiary and quaternary structures stability (Ludikhuyze et al., 2002).

Within the enzymes, the most important in fruit juices are the following:

- *Polyphenol oxidase* (*PPO*): it is responsible of the enzymatic browning.
- *Lipoxygenase* (*LOX*): It induces changes in flavor, color and nutritional value.
- *Pectin methyl esterase* (*PME*): it is responsible of cloud loss and consistency changes.
- *Peroxidase* (*POD*): it increases the production of undesirable flavors.

HPP treatment also produces structure damage in the active site interfering in the enzyme-substrate union. As for the microorganism, the baroresistance varies among different enzymes. In fruit juices enzyme baroresistance is generally higher than the majority founded microorganisms. For that reason, fruit juice preservation treatment is based on the inactivation of the enzymes responsible for its deterioration (PME in orange juice or PPO in apple juice, among others). However, there is no relation between enzyme baroresistance and thermoresistance among different enzymes.

Enzyme baroresistance also depends greatly on its origin. Depending on the fruit and variety, enzyme structure can differ varying the resistance to the treatment. As illustration, orange PME presents different fractions with different treatment resistance and orange variety (Navel and Valencia var., among others) seems to modify the behavior against the treatment. Other conditions such as harvest season could also affect enzyme baroresistance.

Food characteristics also affect the extent of enzyme baroresistance. As same as occurs to the microorganisms, pH diminishes enzyme baroresistance in an acid environment. However, exceptions to this affirmation have been found. Orange PME presents greater baroresistance at pH values of orange juice (Van den

Broeck et al., 1999 a). Sugar content also seems to protect the enzyme against the treatment due to a decrease in water activity content (Marshall et al., 1985; Seyderhelm et al., 1996; Van den Broeck et al., 1999 b). In overall, the influence of food composition is not clear and further discussion is presented in the results section.

Other important fact in the HHP treatment of the enzyme is the enzyme activation phenomenon at low pressure levels. Several studies state that by applying a treatment around 100-400 MPa at room temperature an enzyme activation phenomenon (PPO and PME) is observed, increasing its activity after the treatment (Asaka and Hayashi, 1991; Asaka et al., 1994). An increase in the cellular membrane permeabilization by the HHP treatment has been proposed as the principal reason. Part of the enzyme bounded to the juice pulp could be released after the treatment producing an increase in the enzyme activity. Different changes that increase the enzyme-substrate union also seems to be one of the other main causes.

2.4.6 HHP advantages and disadvantages

2.4.6.1 Advantages

Within the main advantages of HHP technology, it can be remarked:

- Pressure transference in the whole system is instantaneous and independent of the food size and geometry.
- Microbial death and enzyme inactivation is produced at room temperature improving the overall food quality.
- Creation of new food textures.

2.4.6.2 Disadvantages

Within the main disadvantages of HHP technology, it can be remarked:

- Continuous food processing equipment has not been well established.
- High equipment cost.

- It is necessary to have qualified staff and adequate facilities.
- Low efficacy in food with occluded air and low water activity.
- Food sterilization by HHP has not been established.

2.5 PULSED ELECTRIC FIELD (PEF)

The main basic principle of this technology consists in the generation of a high voltage to a food situated between two electrodes separated by an insulator. Treatment time range from 1-2500 μs and electric field strength between 10-80 kV/cm. Either PEF equipment design and microorganism and enzyme inactivation kinetics are the basic principles to obtain microbiologically safe food with a long shelf-life keeping the sensorial and nutritional properties of fresh product.

PEF technology is a promising alternative specially indicated to pasteurize liquid food such as fruit and vegetables juices, milk and liquid egg with thermolabile nutritional components. Nowadays good expectations for using such technology to an industrial level are expected. Various companies manufacturing PEF processing units have appeared in recent years, among them, Diversified Technologies Corp. in USA and ScandiNova Systems AB in Sweden are the most important. In addition, Genesis Juice Corp. in Oregon State (USA), commercialize different fruit juice mixtures processed by a combined PEF and thermal treatment (60ºC) (Figure 2.5.1). The PEF equipment was designed at Ohio State University (OSU, USA) and is combined with a plate heat exchanger (Figures 2.5.2 and 2.5.3). Besides, there are serious expectations in using PEF in other countries such as Brazil to export fresh orange juice to USA.

PEF technology applications are mainly focused on food preservation, however, PEF treatment has also been applied in different studies to improve the extraction of different components by increasing the permeability of plant cells from diverse foodstuff (Ade-Omawaye, et al., 2001) and by improving the juice yield and quality parameters (Guderjan et al., 2007, Schilling et al., 2007).

Figure 2.5.1-Fruit juices treated by PEF. Genesis Juice Corp. (Prof. H. Zhang).

(A) **(B)**

Figure 2.5.2- Treatment chambers-(A). PEF industrial-scale equipment-(B). Ohio State University, OH, (USA). (Prof. H. Zhang).

Figure 2.5.3-PEF industrial-scale equipment combined with a heat exchanger (Prof. H. Zhang).

2.5.1 PEF treatment system

PEF processing system is mainly constituted by:

- Power source
- Switches
- Resistors
- Capacitor bank
- Transformer
- Pump system
- Treatment chambers
- Voltage, current and temperature probes
- Oscilloscope
- Cooling system
- Aseptic packaging equipment (optional)

A power source is used to charge the capacitor bank and a switch to discharge energy to the treatment chambers. The different disposition and number of the components (resistors, power source and switches) give rise to different pulse waveforms. An oscilloscope is used to monitor voltage, current and pulse

width. The cooling system (thermostated bath) avoids an excessive temperature rise due to the ohmic heating (Barbosa et al., 1998).

A restricting factor in a PEF treatment is the dielectric breakdown. The phenomenon is produced when electric field exceeds the dielectric strength of the food, causing the interruption of the treatment. Main causes such as high treatment intensity, high product electrical conductivity or the presence of air bubbles are discussed. The dielectric breakdown is also known as "arc" or "spark" due to the formation of a spark inside of the treatment chamber (Barbosa et al., 1998). During latest years the practice of including an overpressure valve at the entrance of the treatment chambers has been adopted. This valve increases the internal pressure diminishing the presence of air bubbles. Normally 25 psi (1.72 bar) values are adequate to avoid problems with the presence of air.

Main components of the PEF equipment used in this doctoral thesis are shown in Figure 2.5.4.

(A) **(B)**

Figure 2.5.4: PEF laboratory equipment-(A). Main components of PEF equipment: insulator, treatment chambers and thermocouples-(B). Instituto de Agroquímica y Tecnología de Alimentos, Valencia, (Spain).

2.5.1.1 Treatment chambers

Two principle types of chambers are used in the PEF technology depending on the working system. Continuous chambers used for pasteurization of liquid food and static chambers working in batch mode more suitable for studies in solid food (components extraction). Nowadays, the majority of laboratories use continuous chambers (Barbosa et al., 1999).

According to the chamber distribution and geometry, it can be distinguished (Figure 2.5.5):

- *Parallel-plate chamber*: static chamber with a lesser electrode distance than the surface among them.
- *Coaxial chamber:* continuous chamber constituted by two cylindrical electrodes where the food is flowing between them.
- *Co-field chamber*: continuous chamber where the direction of the electric field is parallel to the food flowing. This type of chamber configuration has been used in the present work (Figure 2.5.6).

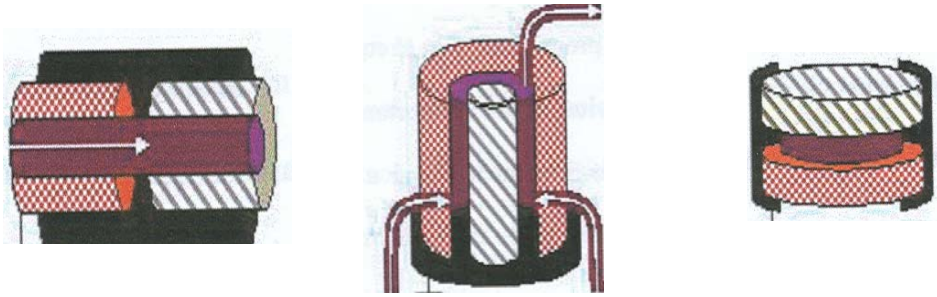

Figure 2.5.5: Scheme of treatment chambers configuration: co-field, coaxial and parallel (Barbosa et al., 1998).

Chambers should be designed to obtain a uniform electric field that improve treatment effectiveness, energy saving and avoid dielectric breakdown phenomenon. Within three studies published in recent years (Evrendilek et al., 2004; Roodenburg et al., 2005 a and b) it was observed that PEF treatment in

orange juice and beer produced the dissolution of the main components that composed the treatment chamber (nickel, chrome, manganese, iron and zinc). The analysis of the metal concentration in the PEF treated samples showed that they were under the maximum standards stated by the European legislation for fruit juices and water for human consumption. However, the flavor of the PEF treated samples was significantly different from the fresh ones containing a certain "metallic" taste. It has been recently adopted to include designing materials such as titanium much more resistant to metal migration.

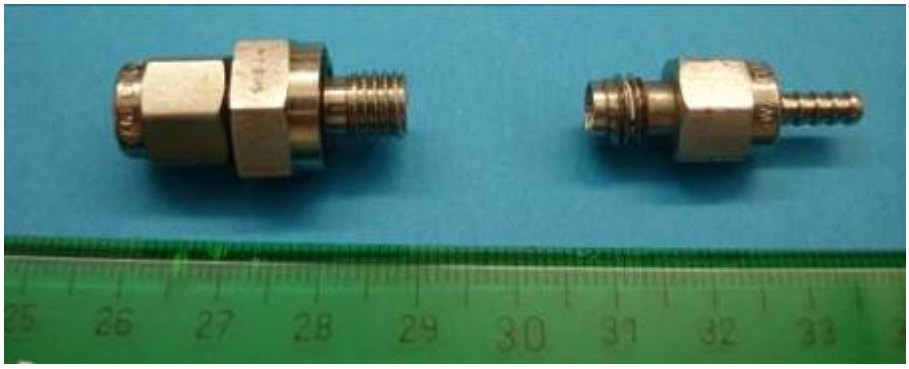

Figure 2.5.6: Components of a co-field treatment chamber.). Instituto de Agroquímica y Tecnología de Alimentos, Valencia, (Spain).

2.5.2 PEF technology related factors

2.5.2.1 Electric field strength (E)

The electric field applied inside the chambers is defined by the relation between the electrical potential difference applied in two electrodes and the distance between them. The electric field for a flat geometry and a co-field design is defined as:

$$E = \frac{V}{d}$$

Equation 2.5.1

where E, is the electric field (kV/cm), V is the potential difference (kV) and d is the distance between electrodes (cm).

Electric field is the most influential factor in PEF treatment. The external electric field induces a potential difference across the cellular membrane. Maximum value that the membrane could withstand is known as critical transmembrane potential or critical electric field (E_c). When the external electric field exceeds E_c value, membrane breakdown is produced Such value principally depends on the microorganism and environment and differences in the electric field value affect the treatment effectiveness (Qin et al., 1998).

2.5.2.2 Pulse waveform and polarity

The main pulse waveforms used in a PEF treatment are:

- *Exponential decay wave*: is defined by a voltage increase up to the selected peak value, decreasing exponentially to its initial value (Figure 2.5.7).
- *Square wave*: is defined by a rapid voltage increase up to the selected peak value during a period of time and rapidly descending to its initial value (Figure 2.5.8).

Various studies state that greater microbial inactivation is produced by applying square wave pulses comparing to exponential decay wave with the same energy applied (Zhang et al., 1994 b; Rodrigo et al., 2003 b).

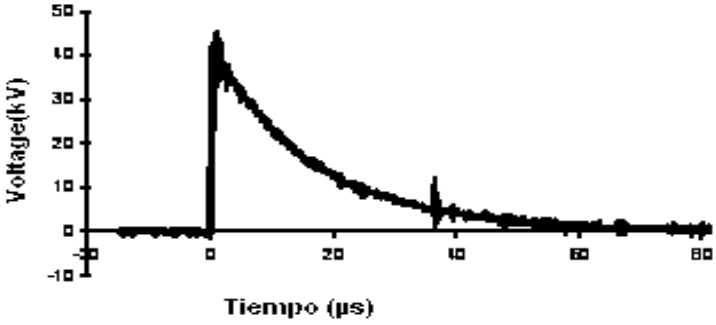

Figure 2.5.7: Exponential decay wave (Barbosa et al., 1999).

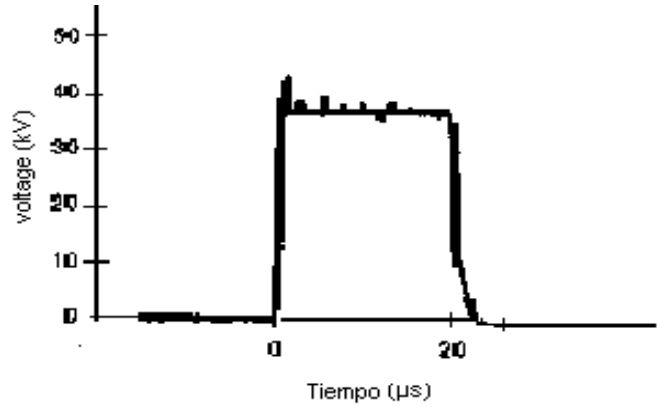

Figure 2.5.8: Square wave (Barbosa et al., 1999).

Pulses can also be classified in monopolar or bipolar depending on their polarity. Bipolar pulses apply a positive pulse followed by a negative or the opposite way causing a reversal of the electric charge. The application of bipolar pulses changes the direction of movement of charged ions in the cell membrane causing a structural fatigue, increasing the susceptibility to electrical breakdown. This phenomenon will promote the microorganism death (Ho et al., 1995) (Figure 2.5.9). In the case of monopolar pulses (Figure 2.5.10), same polarity is maintained and seem to be less effective on microorganism inactivation (Barbosa et al., 1998). In turn, monopolar pulses separate charged particles forming a deposit on the electrode, distorting the electric field. In the enzyme inactivation the relation between pulse polarity and the reached inactivation has not been established yet (Giner et al., 2001; Élez et al., 2006 a).

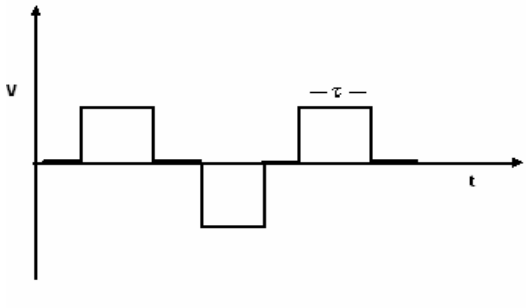

Figure 2.5.9: Bipolar square wave (Barbosa et al., 1999).

2.5.2.3 Temperature (T)

PEF treatment produces a temperature rise due to the ohmic heating. Temperature rise increases when the treatment intensity also increases (electric field and treatment time). Such rise generally favors enzyme and microorganism inactivation, producing a synergetic effect (pulses-temperature) at 35-60ºC. An increase in the membrane permeability and fluidity and subsequent structural fatigue seem to be the main effects of T on microorganism inactivation (Jayaram et al., 1992). Sepulveda, et al., (2005) argued that at low temperatures the phospholipids of the microorganism membrane are attached creating a gel-shaped rigid structure. At greater temperatures the membrane adopts a crystalline liquid appearance that diminishes its physical stability being more susceptible to PEF treatment. In the enzyme inactivation, a temperature rise would favor structural changes and charges alteration leading to enzyme denaturation and activity loss.

2.5.2.4 Treatment time (t)

Treatment time is defined as:

$$t = n \times w \qquad \textbf{Equation 2.5.2}$$

where t is the treatment time (μs), n is the number of pulses and w the pulse width (μs). In general, a treatment time increase causes a temperature rise and greater inactivation. Nevertheless, the relation between the survivors number or enzyme activity and treatment time is not linear observing the appearance of shoulders and tailing phenomena. In that sense, once certain inactivation is produced, a rise in the treatment time and thus in the energy applied, do not produce any significant increase in the inactivation (Raso et al., 2000, Aronsson et al., 2001).

2.5.2.5 Pulse width (w)

Pulse width is defined as the pulse duration in microseconds (μs) (Figure 2.5.10). In an exponential decay wave, it is defined as the elapsing time from the maximum peak value to a value 37% lesser. Several authors have tried to determine the optimum pulse width based on microbial inactivation obtained (Raso et al., 2000). It has been proved that a value close to 2 μs obtains a greater microbial reduction within a lesser energy consumption. A pulse width increase does not produce in many cases any significant effect on the inactivation degree. However, several studies conclude that a pulse width increase produces higher microbial inactivation (Aronsson et al., 2001; Abram et al., 2003, Élez et al., 2005), but only under low field intensities (25-28 kV/cm). In the enzyme inactivation by PEF, Giner et al., (2001) and Élez et al., (2006 b) observed a greater POD and PPO inactivation by increasing pulse width. As a conclusion, it seems that pulse width effectiveness depends on the treatment conditions and type of study.

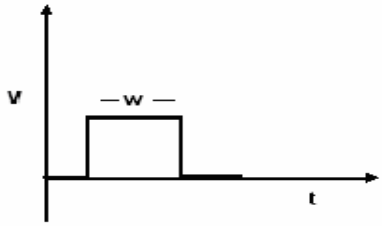

Figure 2.5.10: Pulse width (w) (Barbosa et al., 1999).

2.5.3 Product related factors

Product physicochemical characterization is essential since it directly affects PEF treatment effectiveness. Electrical conductivity, pH, water activity, particle size or viscosity seem to be the most important. Several studies have observed that factors such as the presence of fat and sugar seem to protect the microorganism and enzyme against treatment (Bendicho et al., 2003 a). On the contrary, other studies do not show any significant effect of food composition on PEF microbial inactivation (Reina et al., 1998; Dutreux et al., 2000; Mañas et al.,

2001). It seems that the different food constituents and target microorganism do not affect equally on the inactivation degree. Further discussion is provide in the present doctoral thesis

2.5.3.1 Electrical conductivity

In general high electrical conductivity foods are more difficult to be processed by PEF due to a less electrical resistivity (Barbosa et al., 1999). In turn, lesser microbial inactivation is produced due to a greater solutes concentration that increases membrane rigidity turning it more stable to the treatment (Wouters et al., 2001).

A decrease in the electrical conductivity will produce higher conductivity difference between the microorganism and the environment, creating a flux of ionic substances through the membrane. This would increase cell membrane weakening and permeability making it more susceptible to the treatment (Wouters et al., 1999; Dutreux et al., 2000). However, Álvarez et al., (2000 and 2003) observed that by increasing the medium conductivity (2, 3 and 4 mS/cm) the inactivation of *S. senftenberg* and *L. monocytogenes* also increased. The fact that by increasing conductivity it was necessary to apply greater voltage to obtain the same electric field could be the main reason.

2.5.3.2 pH

Generally microorganisms have a greater treatment resistance at the optimum growth pH. When pH varies from the optimum value, treatment resistance decreases. Various studies demonstrate that any food pH diminishing favors PEF microbial inactivation due to an additional cell stress. In an acid environment, a greater H^+ ions transporting to cell is produced and due to the higher membrane permeability after PEF treatment a cytoplasm pH reduction is produce leading to cellular death. Intracellular pH modification can also unchain chemical variation in compounds such as DNA and ATP (Vega-Mercado et al., 1996, Wouters et al., 2001).

pH resistance differs among different microorganisms. Differences in the optimum growth pH seem to be the most probable cause of the observed differences (Álvarez et al., 2000; Aronsson and Rönner, 2005). Evrendilek and Zhang, (2003) demonstrated that pre-adapting *E. coli* 0157:H7 to the acid environment the pulsoresistance increased. This circumstance could occur in fruit juice processing where any adaptation to the acid environment could cause greater resistance to the subsequent preservation treatment.

2.5.3.3 Viscosity (particulate food)

Viscosity is considered a limiting factor in the PEF treatment. The presence of particles could induce to a sudden change in electrical conductivity, causing an increase in the electric field and the subsequent treatment interruption. An increase in food viscosity would also difficult to obtain an adequate flow-regime (laminar-turbulent). This fact confirms that the use of an adequate pump is essential.

2.5.3.4 Water activity

Food water activity could also affect PEF treatment effectiveness. Álvarez et al., (2002) observed lesser PEF inactivation of *L. monocytogenes* reducing food water activity from 0.99 to 0.93. Such authors hypothesized about an increase in membrane rigidity that would lead to a wall cell greater PEF resistance. In turn, cellular wall contraction would decrease the cellular size affecting PEF treatment effectiveness (Aronsson and Rönner, 2001).

2.5.4 PEF microbial inactivation

Development of new food preservation technologies demands the knowledge of the different mechanisms of microorganism inactivation. The majority of studies agree to remark that PEF treatment produces a series of structural and functional changes in the cellular membrane that lead to a microorganism death (Mañas and Pagán, 2005).

Zimmermann, (1986) observed that charged particles of a cell tend to accumulate in the inner and outer membrane surface generating 10 mV transmembrane potential. When an external electric field is applied, a great quantity of charged particles is accumulated on both sides compressing the membrane. As commented earlier, cellular membrane can hold an Ec maximum value. When the applied electric field exceeds Ec value, an electrocompression effect by the accumulation of charges is produced leading to the formation of pores in the membrane. Size and quantity of pores will depend on the applied electric field and treatment time. In general it can be divided in "reversible" and "irreversible" pores. "Reversible" pores are those formed at low treatment conditions returning to the membrane initial form after the treatment. This could cause certain cell injury known as sublethal damage leading to cellular death in stress conditions such as low pH and refrigeration temperature. On the contrary, if the electric field is higher than Ec, "irreversible" pores are produced leading to the death of the microorganism (Figure 2.5.11). Besides structural changes in the membrane, other changes have been observed inside the cell indicating that pores formation is not the only PEF microbial inactivation mechanism; however, it is the most important (Harrison et al., 1997).

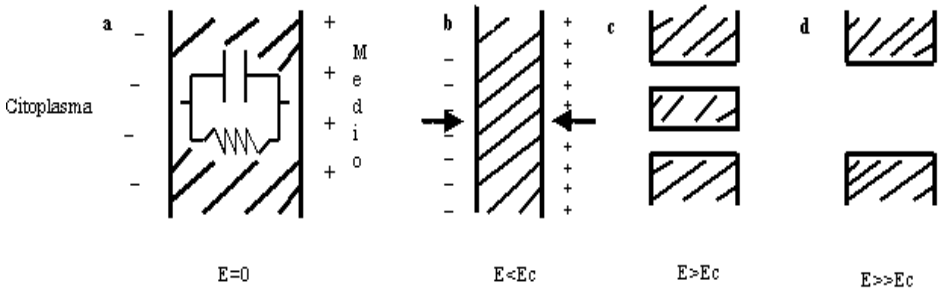

Figure 2.5.11: Effect of PEF treatment on cell membrane (Barbosa et al., 1999).

Within microorganism factors related to PEF it can be remarked:

2.5.4.1 Type of microorganism

Food prevailing natural flora (Gram+, Gram- , yeasts, molds or esporulated microorganisms) could give an idea of cell characteristics such as membrane size and structure being useful for an appropriate PEF treatment design.

Several studies have observed that Gram+ have greater PEF resistance than Gram-, mainly due to membrane composition (Hülsheger et al., 1981). Yeasts are more sensitive to PEF treatment because its higher size and pulses affect directly into their structures (Qin et al., 1998) (Figure 2.5.12). On the other hand, PEF treatment has been demonstrated to be unable to inactivate spores. Pagán et al., (1998) and Cserhalmi et al., (2002) did not observe significant differences in the inactivation of *B. subtilis* and *B. cereus* spores after the combined PEF and thermal treatment (60ºC). The combination of PEF treatment with other barrier technologies (natural antimicrobials or germinants) would lead an extent of inactivation degree.

2.5.4.2 Microorganism inoculum size

Different studies have tried to asses the effect of the inoculum size on PEF microbial inactivation and results have raised different conclusions. Several studies observed no significant effect on PEF inactivation after increasing the microorganism initial concentration (Álvarez et al., 2000). On the contrary, Zhang et al., (1994) and Damar et al., (2002) observed that by increasing *S. cerevisiae* and *E. coli* O157:H7 initial concentration (10^3-10^8 CFU/mL) PEF inactivation diminished possibly due to a protecting mechanism by the formation of aggregates. In conclusion, it seems that the influence of this parameter on pulses effectiveness is affected by the microorganism and treatment conditions.

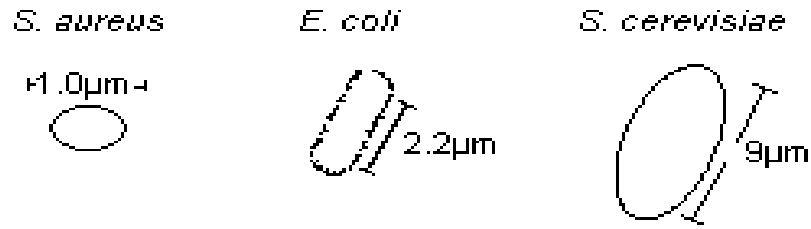

Figure 2.5.12: Cellular size comparison (Qin et al., 1998).

2.5.4.3 Growth phase

Membrane cell characteristics are different in each microorganism development stage. In logarithmic phase cell membrane is more sensitive to an external factor because its continuous division (Pothakamury et al., 1996; Wouters et al., 1999; Álvarez et al., 2000; Rodrigo et al., 2003 a). Wouters et al., (2001) did not observe significant differences on membrane permeability after PEF treatment between cells recovered in stationary and logarithmic phase. The authors admitted that other factors could also affect PEF inactivation. Stationary phase cells adapt better to stress conditions to assure their survival in the environment, causing physiological changes that could induce to a greater resistance to PEF treatment.

2.5.5 PEF enzyme inactivation

Enzymes structure is formed by noncovalent bonds (hydrogen bonds, electrostatic interactions, van der Waals forces and hydrophobic bonds) and in some cases bisulphite bonds that provide great stability. Nevertheless, any change might cause their denaturation (Ho et al., 1997). PEF treatment could impact such interactions affecting the three-dimensional structure (secondary, tertiary and quaternary) or the globular protein conformation. The difference in the enzyme inactivation degree could be mainly related to the secondary and tertiary structure.

Different studies based on PEF enzyme inactivation have observed conformational changes and alteration of protein helix alignment due to the movement of charges produced by the PEF treatment. Zhang et al., (2005)

observed loss in α-helix conformation content in a POD after combined PEF and thermal treatment. Bendicho et al., (2003) observed perturbations in the charged groups due to changes in the electric field value. This fact caused activity loss due to the difficulty of active site-substrate union. Enzyme PEF resistance varies depending on the number of hydrogen bonds and the perturbation around metal site (Ca^{2+}) necessary to maintain its activity.

Yang et al., (2004) observed the inactivation mechanism of several enzymes by PEF concluding that increasing the size and complexity of the enzyme structure was an indicator of a higher thermoresistance but not pulse-resistance. Higher inactivation at higher electrical conductivity was also observed due to a higher temperature increase for the same treatment conditions and higher ions flux affecting to the enzyme electrostatic interactions. Despite of the results obtained, the information about PEF inactivation of enzymes is very limited.

2.5.6 PEF advantages and disadvantages

2.5.6.1 Advantages

- Effective to inactivate pathogen and spoilage microorganisms.
- Synergy with moderate temperature, natural antimicrobials and pH.
- Similar shelf-life that conventional process but preserving better vitamins and flavor.
- It allows continuous food processing.
- Similar energy consumption that thermal treatment in a counterflow process.

2.5.6.2 Disadvantages

- High equipment and operation cost.
- Only valid for non-particulate and low electrical conductivity liquid foods.

- Unable for a sterilization process purpose.

2.6 ORANGE JUICE ENZYME ACTIVITY

2.6.1 Pectins

One of the most important physical characteristics of the orange juice is the turbidity. This parameter is also called juice "cloud" and its loss causes an important juice destabilization leading to a clarified juice with no commercial value.

Juice contained in the orange fruit vacuoles does not present turbidity. Turbidity comes from cell fragments colloidally dispersed in the juice corresponding to the cell disruption at the extraction moment. Colloidal suspension is composed by approximately 30% of proteins, 20% of hesperidins, 15% of cellulose and hemicellulose, 5% of pectins and 30% unknown (Klavons et al., 1987).

One of the most important components of the juice "cloud" are pectins. Pectins are the juice natural colloid that contributes to the formation of turbidity, keeping pulp particles in suspension. The total proportion of pectins in the juice depends on the pressure used in the extraction and pulp residual content.

2.6.2 Pectolytic enzymes: pectin methyl esterase (PME)

The orange juice enzyme activity is mainly due to a pectolytic enzyme, pectin methyl esterase (PME). PME is mainly found in the solid parts of the fruit (pulp and juice cells) and subsequently in decreasing order in flavedo, albedo and seeds.

PME is a high specific enzyme that catalyzes the hydrolysis of pectin methylester groups (demethylation) releasing alcohol and pectinic acid. Due to this reaction the pH diminishes increasing the quantity of free carboxyl groups and producing methanol. This reaction has a great technological relevance in juice industry processing producing colloidal unbalance destabilizing the suspension

formed by pectin mycelia. The generated carboxylic groups by the demetolixation process react with the Ca^{2+} and Mg^{2+} ions of the juice having as a result aggregates that are precipitated and deposited on the bottom of packaging (Figure 2.6.1).

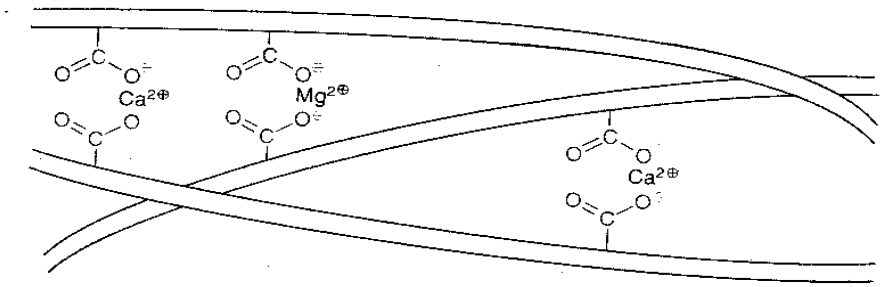

Figure 2.6.1: Three-dimensional structure by ions interaction (Plinik and Voragen, 1991).

As commented before, orange PME activity produces a suspension of pulp sediment, leading a whey with no commercial value. Orange PME also produces the gelification of concentrate juices. This is one of the major problems associated with the quality loss of these type of juices (Versteeg et al., 1980). Orange juice physical stability is traditionally obtained with thermal treatment but producing an overall food quality loss. Orange juice thermal preservation treatment (90 ºC, 1 min or 95 ºC, 30 s) is based on PME complete destruction (> 90%) (Cameron et al., 1994). Such conditions are higher than the microorganism thermoresistance found in the juice.

2.7 ORANGE JUICE MICROBIAL CHARACTERIZATION

2.7.1 *Salmonella typhimurium*

Salmonella enterica subgroup *enterica* serotype *Typhimurium* (also known as *Salmonella typhimurium* as simplification) belongs to the *Enterobacteriaceae*

family. *Salmonella* is a bacillus (1-2 μm), Gram-, facultative anaerobic, non spore-forming and motive bacteria. Is a glucose fermentative microorganism releasing acid and gas. Its optimum growth temperature is approximately 38ºC being able to growth in a range of temperatures between 5 and 46ºC. It is a relatively thermolabile microorganism and temperatures between 65-70ºC are enough to inactivate it. Recently, it has been known that the most common *Salmonella* food infection is caused by *S. typhimurium* (Forsythe et al., 2003).

It is estimated that 31% of USA annual food outbreaks belongs to *Salmonella* (35.000 outbreaks cases per year) (IFT, 2004). *S. typhimurium* infection does not cause a severe illness and normally is non-fatal. The illness is characterized for causing diarrhoeas, abdominal aches, vomits, nauseas and mild fever. The incubation period varies between 16-72 h and the sickness lasts between 2-7 days (Mossel, 2002).

The infective dose varies depending on the age and person health status, food and *Salmonella* strain but in general varies between 20 and 10^6 CFU/mL (Mossel, 2002) Unfortunately in immunedeficient people (elder people, children or hospitalised people) *Ssalmonella* infection ends up with death if appropriate treatment is not prescribed on time. The majority of *Salmonella* infections are taking place in summer.

This microorganism is frequently found in food such as: 1) beef, poultry and seafood; 2) nonpasteurized products such as egg products, milk and dairy products; 3) other *Salmonella*-free food but contaminated with some of those mentioned above (1 and 2). Lately, different cases of *Salmonella* contamination have been found in orange juice (Cook et al., 1998; Castillo et al., 2006 and Khan et al., 2007). The acid-tolerance of different *Salmonella* strains has been studied by several researchers (Parish et al., 1997 and Ray, 2004 in orange juice; Pao et al., 1998 on orange surface; Yuk and Schneider, 2006 in various fruit juices and simulated gastric juice).

Figure 2.7.1: Electron microscopy of *Salmonella typhimurium* (Mossel, 2002).

2.7.2 *Lactobacillus plantarum*

Lactobacillus plantarum is a non-starter homofermentative lactic bacteria (NSLAB) belonging to *streptobacterium* family. *L. plantarum* is Gram+, non spore-forming with no respiratory metabolism, microaerobic and catalase-producing a viscosity increase during its growth. *Lactobacillus* genus is often associated with diary products. It is often responsible of the final stages in lactic fermentation due to its acid-tolerance growing at pH values less than 5. *Lactobacillus* genus is rarely pathogen.

Within the spoilage microorganisms *L. plantarum* is frequently found in fruit juice based products. It is characterized to convert additives found in fruit juice such as malic and citric acid into lactic acid producing a sour flavor and taste (Bay, 2004). Many authors have observed a high acid-tolerance (Pao and Davis, 2001; McDonald et al., 1990).

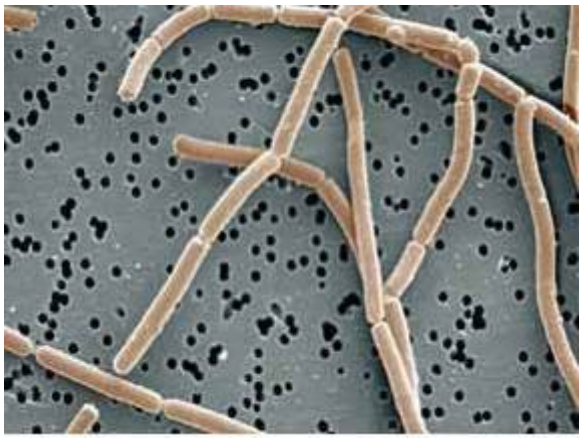

Figure 2.7.2: Electron microscopy of *Lactobacillus plantarum* (Mossel, 2002).

2.7.3 Orange juice microbial safety

During last decades, natural squeezed juice has acquired great relevance. It is prepared at the moment of purchasing or is storaged in a container in continuous agitation without having any preservation treatment. In occasions, the hygienic conditions are not appropriate (inadequate cleaning surfaces and equipments and inappropriate orange storage) leading to a pathogen inoculation through cross-contamination having as a result a public health problem.

In the latest years the United States have suffered pathogen microorganism's outbreaks in non-pasteurized orange juice. *Salmonella Hartford*, *Bacillus cereus*, *Clostridium botulinum* and *Escherichia coli* 0157:H57 were detected in non-pasteurized orange juice, causing 66 infection cases and the death of a child (Morris, 1998). The survival in acid environments is considered unlikely probable. However recent studies have showed that some *E. coli* strains, including three pathogens of 0157:H7 and various *Salmonella* strains (including *S. typhimurium*) have become acid-tolerant and thermoresistant after a previous adaptation at pH=5, as it possible occurs in orange juice processing (Morris, 1998).

Due to these facts, the FDA (USA Food and Drug Administration) proposed the implantation of a HACCP system in the entire non-pasteurized fruit juice processing. It should be also included an information label indicating that the

juice had not been processed to guarantee pathogen destruction. It was also recommended an educational programme for industries and consumers in order to minimize the risk associated to fresh juice consumption. After that, FDA made public a regulation indicating that the process that could obtain an equivalent reduction of five log cycles of *E. coli*, could guarantee microbiological safety of those products. The above came up at the end of 1998 and with the available information thermal pasteurization was the unique procedure to obtain such inactivation level (Morris, 1998). However, the regulation was only applied at industrial level leaving aside bars, restaurants and other groups where non-pasteurized juice is sold.

As stated earlier, the application of a mild thermal pasteurization process produces overall quality loss avoiding using by companies the term "fresh" to satisfy consumer tendencies. A possible solution to this crisis might come by the application of a nonthermal food process, such as High Hydrostatic Pressure (HHP) or Pulsed Electric Field (PEF)

2.8 INACTIVATION KINETIC MODELS

Generally the use of survival/inactivation curves has been extended since years to study microbial resistance, enzyme inactivation and quality factor degradation kinetics to different lethal agents. These survival/inactivation curves represent the survivors/enzyme activity/compound concentration number against the treatment. These graphs are often described through several mathematic models from which different parameters are obtained allowing the quantification and prediction of bacterial resistance/enzyme inactivation.

The acquisition of these kinetic parameters is basic in the development of a food preservation process. The industrial practical application of microbial modelling comes from optimising the process conditions, improving food safety and allowing starting up a HACCP plan. Lately, food safety policies have been designed by different European governments and Food safety Agencies (European and Spanish) based on microbiological risk analysis studies. Within these studies,

predictive microbiology and inactivation kinetic models are playing a fundamental role.

2.8.1 Microorganism inactivation kinetic models

2.8.1.1 Bigelow

Classic kinetic models suppose a linear relation between the survivors fraction and treatment time. The model developed by Bigelow, (1921) has been mainly used to explain survival curves by thermal treatment:

$$Log\,(S) = -\frac{t}{D}$$ **Equation 2.8.1**

where S is the survival fraction calculated as the relation between the survivors after the treatment (N) and the initial number of microorganisms (N_0), t is the treatment time and D is the kinetic parameter, showing the time required to achieve one decimal reduction and it can be calculated as the negative inverse of the inactivation curve slope.

The evidence of deviations from the traditional models with the appearance of shoulders and tailing phenomena in the survival curves have been observed in PEF treatment (Figure 2.8.1). The validity of Bigelow model for interpreting PEF kinetic inactivation data should be evaluated.

2.8.1.2 Hülsheger

The pioneers on microbial mathematical modelling were Hülsheger and Niemann (1980). The model was based on the survivors fraction dependency (S), treatment time (t) and critical treatment time (tc) for which S=1, according to the following relationship:

$$Ln(S) = -b_t * Ln\left(\frac{t}{t_c}\right)$$ **Equation 2.8.2**

where *bt* is the regression coefficient, *t* is the treatment time and *t*c is the critical time expressed (the maximum treatment time in which the survival fraction equals 1).

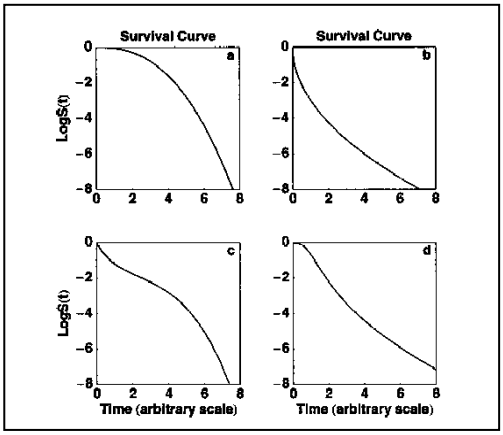

Figure 2.8.1: Illustration of different nonlinear survival curves.

2.8.1.3 Weibull

This model consists in a distribution function that considers the microorganism as a population where every individual has a different resistance to the treatment. The inactivation curves represent the resistance distribution of the population to the treatment. Under this perspective, it is easier to explain the non-existing linear relation between treatment time and microorganism death. Besides its simplicity (two parameters) is very versatile, allowing to describe curves with shoulders and tailing phenomena and straight lines. This distribution has served for describing heat and PEF inactivation curves in several studies (Peleg et al., 1995; Rodrigo et al., 2003 a) and it is defined as:

$$Ln(S) = -\left(\frac{t}{a}\right)^{b}$$ **Equation 2.8.3**

where t is the treatment time, a and b are scale and shape parameters, respectively. The b value gives an idea of the form of the curve, if $b>1$ the curve is concave upwards (it forms shoulders) indicating some activation phenomenon is produced or higher treatment resistance at the initial stage. If $b<1$ the curve is concave downwards (it forms tails) indicating that survival fraction is the most resistant to the treatment conditions. If $b=1$ the curve is a straight line and can be described by linear models indicating that every cell has the same resistance to the treatment

Other kinetic parameter used from the Weibull equation is the mean critical time ($\overline{tcw}$) that can be defined by the time when microorganism death is high in response to the PEF treatment, expressed by the following equation:

$$t\overline{cw} = a * \Gamma\left(1 + b^{-1}\right)$$ **Equation 2.8.4**

where a and b are the parameters of the Weibull equation and Γ is the gamma function.

2.8.2 Enzyme inactivation kinetic models

2.8.2.1 Primary models

Traditionally linear models have been applied to describe enzyme inactivation kinetics by heat, obtaining parameters such as D and z values (described later). However, in the case of different enzymes thermal inactivation curves have been observed to behave differently due to the presence of different fractions exhibiting different stabilities (Hou et al., 1997; Lee et al., 2003;

Tajchakavit and Ramaswamy, 1997; Van den Broeck et al., 1999 a; Wicker and Temelli, 1988; Cameron et al., 1998; Han et al., 2000; Versteeg et al., 1980). Nevertheless, in many cases only part of the curve (activity related to the most abundant fraction) has been modelled to obtain the previously described parameters. Lately some microbial inactivation kinetic models (Weibull distribution) have been applied to describe enzyme inactivation kinetics (Soliva et al., 2006; Élez et al., 2006 a). However the most common equations used to describe thermal and high pressure inactivation kinetics of different enzymes are the following:

For a thermal and high pressure treatment, inactivation kinetics of different enzymes founded in fruit juices were properly described using the biphasic model (primary model) which is usually applied when different fractions are present with different processing stabilities inactivating according to first-order kinetics defined by (Van den Broeck et al., 2000):

$$A = A_L \exp(-k_L \cdot t) + A_S \exp(-k_S \cdot t) \qquad \textbf{Equation 2.8.5}$$

where A is the dependent variable and can be expressed as enzyme activity (U/mL), A_L and A_S refer, respectively, to the activity of the labile and stable fraction (U/mL), k_L and k_S the inactivation rate constant of the labile and stable fraction (min^{-1}) and t the independent variable expressed as the treatment time (min).

A special case of first-order kinetics is the fractional conversion model (primary model), which is usually applied when a fraction is inactivated and another fraction remains constant and a nonzero residual activity after prolonged treatment is observed (Van den Broeck et al., 1999 a and b):

$$A = A\infty + (A_0 - A_\infty) \exp(-k\, t) \qquad \textbf{Equation 2.8.6}$$

where A is the dependent variable and can be expressed as enzyme activity (U/mL), A_0 and A_∞ refer, respectively, to the initial enzyme activity and to the residual activity after prolonged treatment time (U/mL), k the inactivation rate constant (min^{-1}) and t the independent variable expressed as the treatment time (min).

2.8.2.2 Secondary models

The temperature dependence of inactivation rate constants of the different fractions can be estimated using the Arrhenius model (secondary model) (Arrhenius, 1889):

$$\ln(k_L) = \ln(k_{Lo}) + \left[\frac{E_{aL}}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right] \qquad \textbf{Equation 2.8.7}$$

$$\ln(k_s) = \ln(k_{So}) + \left[\frac{E_{aS}}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right] \qquad \textbf{Equation 2.8.8}$$

where $\ln(k_L)$ and $\ln(k_S)$ are the dependent variables and are expressed as the inactivation rate constant of the labile and stable fraction, respectively (min^{-1}), E_{aL} and E_{aS} are the activation energy of the labile and stable fraction respectively (kJ/mol), k_{L0} and k_{S0} the inactivation rate constant at a reference temperature of the labile and stable fraction, respectively (min^{-1}), T is the independent variable expressed as the absolute temperature (K), T_0 the reference temperature (K) and R (8.314 J/mol K) is the universal gas constant.

The pressure dependence of the enzyme inactivation rate constants at a constant temperature of different fractions can be calculated using the linearized Eyring equation (secondary model) (Eyring, Johnson, & Gensler, 1946):

$$\ln(k_L) = \ln(k_{Lo}) + \left[\frac{V_{aL}}{RT}\left(P - P_{ref}\right)\right] \qquad \textbf{Equation 2.8.9}$$

$$\ln(k_s) = \ln(k_{So}) + \left[\frac{E_{aS}}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right] \qquad \textbf{Equation 2.8.10}$$

where k_L and k_S are the dependent variables and are expressed as the inactivation rate constant of the labile and stable fraction respectively (min^{-1}), V_{aL} and V_{aS} are the activation volume of the labile and stable fraction, respectively (cm^3/mol), k_{L0} and k_{S0} are the inactivation rate constant at a reference pressure of the labile and stable fraction, respectively (min^{-1}), T is the absolute temperature (K), P is the independent variable expressed as the pressure (MPa), *Pref* is the reference pressure (MPa) and R (8.314 J/mol K) is the universal gas constant.

2.8.3 *z* parameter

This parameter has been frequently used in microorganism and enzyme thermal inactivation. It describes the primary parameter variation (secondary parameter). Generally it has been used to describe the variation of D parameter toward an external factor such as temperature, pH, aw, among others. It is obtained from the logarithm of D values at different temperatures as the negative inverse of curve slope. It can be also calculated by the following equation (secondary model):

$$\frac{Log(D_1) - Log(D_2)}{T_1 - T_2} = \frac{1}{z}$$

Equation 2.8.11

where D is the decimal reduction time defined as the time required to achieve one decimal reduction and T the temperature (ºC).

z parameter could also be used to describe the variation of other kinetic parameters such as a and $\overline{tcw}$ of Weibull model or tc of Hülsheger model among others. Further discussion is presented in this doctoral thesis.

2.8.4 Model fit validity

A least square procedure was used to fit the models to the experimental data. This method consists basically on searching the parameters values that minimize the sum of squares of residuals between experimental and predicted

values. Least-squares fitting was applied checking the assumptions of normal data distribution, constancy of variance and independent distribution of residues.

2.8.5 Goodness of fit

To estimate the model fitting to the experimental data the Accuracy factor (*Af*) and Mean square error (*MSE*) parameters were used (Ross, 1996) and can be defined as follows:

$$Af = 10^{\frac{\sum \left| Log(fitted / observed) \right|}{n}}$$ **Equation 2.8.12**

where *n* is the number of observations and the predicted and observed values are referred to the survival fraction. The meaning of this statistic is the closer to 1 the *Af* values, the better the model fit the data.

Mean square error (*MSE*) is also calculated as follows:

$$MSE = \frac{\sum (fitted - observed)^2}{n - p}$$ **Equation 2.8.13**

where *n* is the number of observations, the predicted and observed values are referred to the survival fraction and *p* is the number of parameters to be estimated by the model. The meaning of this statistic is the smaller the *MSE* values, the better the model fit the data.

2.-OBJECTIVES

The through knowledge of the state of art of the different nonthermal technologies and juice processing industry leaded to the definition of the main objective of the present doctoral thesis based on the application of a combined PEF, HHP and thermal processing in a new beverage based on the mixture of orange juice and milk. To reach such objective, the following partial objectives were proposed:

- Preparation and physicochemical and sensorial characterization of a new product based on orange juice and milk.
- Study the PME inactivation and volatile compounds content loss by HHP, PEF and thermal treatment in the orange juice-milk based product.
- Obtain the combined HHP and thermal inactivation kinetics of pectin methyl esterase (PME) in the orange juice-milk based product.
- Study the influence of PEF processing variables on the inactivation of *L. plantarum* in the orange juice-milk based product.
- Obtain the combined PEF and thermal inactivation kinetics of a spoilage microorganism (*Lactobacillus plantarum*) in the orange juice-milk based product.
- Obtain PEF inactivation kinetics of a pathogen microorganism (*Salmonella typhimurium*) based on the influence of food characteristics such as pH and stabilizer concentration in the orange juice-milk based product.
- Study the shelf-life of the orange juice-milk based product after PEF and thermal treatment at refrigeration conditions.

3.-RESULTS

Development of a new product based on orange juice and milk ideal for PEF processing

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4.1.1 ABSTRACT

A new product based on the mixture of orange juice and milk suitable for Pulsed Electric Field (PEF) processing was developed. New methodology was established and four formulations were chosen based on orange juice concentration (15, 25, 50 and 70%). The election of the final formulation was based on the physicochemical characteristics, PEF inactivation of *E. coli* and sensorial evaluation. *E. coli* inactivation degree was lesser by increasing orange juice concentration due to a conductivity and viscosity sample increase but without significant differences between 15, 25 and 50% samples ($p>0.05$). There were no significant differences among the 50 and 70% samples on aspect, viscosity and global appreciation sensory parameters. The 50% sample was chosen as the most appropriate for PEF treatment due to its adequate inactivation degree, appropriate physicochemical characteristics and good sensorial acceptance.

Keywords: Orange juice; Milk; Formulation; Sensory analysis; *E. coli*; PEF

4.1.2 INTRODUCTION

Acidified milk drinks comprise a large range of products, from those usually prepared from fermented milk with stabilizers and sugar to those prepared by direct acidification with fruit juices and/or acids. The pH of these products ranges from 3.4 to 4.6 and because of the instability of caseins in this pH range, a stabilizer needs to be added to prevent protein aggregation and achieve optimal mouth feel (Nakamura et al., 2006).

Caseins at neutral pH in milk are present in the form of micelles, and these protein particles are stabilized by steric repulsive interactions. During acidification, at a pH around the isoelectric point (pH 4.6) the micelles aggregate, mainly because of the collapse of the extended layer formed by κ-casein on the surface of the micelle (De Kruif, 1998). At a pH below their isoelectric points, milk proteins are positively charged; negatively charged pectins are employed as stabilizers in products such as acidified milk drinks and yoghurt (Pereyra et al., 1997).

Pectin, an anionic polysaccharide generally extracted from citrus fruits or apple pomace, is mainly composed of a backbone of galacturonic acid, partly methyl esterified, and by hairy regions containing rhamnose, galactose, arabinose, xylose, and glucose. It has been demonstrated that, below pH 5.0, the polysaccharide chains of high methoxyl pectin (HMP) adsorb on the surface of the casein micelles, via the charged blocks of the pectin chains and the uncharged blocks of HMP extend into solution contributing to stabilization via steric repulsion (Pereyra et al., 1997).

The initial characteristics of an orange juice-milk based product to be suitable for PEF processing should be the following: i) juice concentration that provide an important Vit-C supply ii) simplest product as possible iii) good sensorial acceptance; iv) adequate physicochemical characteristics (low electrical conductivity and viscosity that allows an adequate flow rate) and with no suspension particles.

Recent foodborne *E. coli* outbreaks involving apple cider, fresh apple juice and orange juice have highlighted the acid-tolerance and probable low infective

dose of this pathogen (Zhao et al., 1993; Parish et al., 1997; Cook et al., 1998; Sivapalasingam et al., 2004). These findings strengthen the need to carry out extensive studies in order to check the nature of death of *E. coli* in an acid food pasteurized by using PEF technology. FDA made public a new regulation based on the requirement of an equivalent five log cycles reduction of *E. coli* in these products to guarantee their microbiological safety (Morris, 1998).

The objective of the present study was to develop a new beverage based on the mixture of orange juice and milk suitable for a PEF treatment.

4.1.3 MATERIALS AND METHODS

4.1.3.1 Composition and product physicochemical characterization

The product was composed by pasteurized orange juice from squeezed oranges (García-Carrión, Spain) kept frozen until used (the pulp was removed), commercial UHT skimmed milk, high methoxyl citrus pectin such as stabilizer (Unipectine AYD 250, Cargill, USA), commercial citric acid, sugar, and distilled water .

The physicochemical characterization of the product was based on the following parameters: electrical conductivity (Crison 525 conductimeter, Crison Instruments, Spain), pH (Crison 2001 pHmeter, Crison Instruments, Spain), Brix degrees (Atago RX-1000 digital refractometer, Atago Company, Japan) and viscosity (Haake VT5 Viscotester, Thermo Electron Corporation, UK).

4.1.3.2 PEF treatment

An OSU-4D bench-scale continuous processing unit was used to treat the food sample. Six co-field chambers with a diameter of 0.23 cm and a gap distance of 0.293 cm between electrodes were connected in series. One cooling coil was connected before and after each pair of chambers and submerged in a circulating bath (Polystat, Cole Parmer, USA) to maintain the selected initial temperature at 35ºC (60ºC maximum temperature of the treatment). The temperature was recorded

by thermocouples (T type) at the entrance and exit of each pair of chambers. The first treatment chamber can be considered as the initial temperature and the exit of the last treatment chamber as the final temperature. The values were recorded with a data logger (Control Company, USA). Pulse waveform, voltage and current in the treatment chambers were monitored with a digital oscilloscope (Tektronix TDS 210, Tektronix, USA). The flow rate was set at 60 mL/min with a peristaltic pump (XX 80002 30, 6-600 r.p.m., Millipore, USA). A bipolar square-wave of 2.5 μs was selected. Treatment time ranged from 0 to 110 μs and the electric field was set at 40 kV/cm. Samples were collected after each treatment time. They were serially diluted in sterile 0.1% peptone water, plated in Nutrient Broth Agar, and incubated for 24 h at 37ºC. The beverage was inoculated with the contents of a thawed *E. coli* at a final concentration of approximately 10^6 CFU/mL.

4.1.3.3 *Escherichia coli*

Escherichia coli CECT 516 (ATCC 8739) was obtained from the Spanish Type Culture Collection (Valencia, Spain). This strain has been used in previous PEF studies, in apple and orange-carrot juices (Evrendilek et al., 1999; Rodrigo, et al., 2003 a; Selma et al., 2004), and its ability to grow under acidic conditions has been proved (Evrendilek, et al., 1999). Cells were obtained according to Rivas et al., (2006). For that, the culture was inoculated in nutrient broth (NB) (Scharlab Chemie, Spain) and incubated at 37ºC with continuous agitation at 200 rpm for 4 h to obtain the cells in the exponential growth stage. The cells were centrifuged twice at 3220 g at 4ºC for 15 min and then resuspended in NB. After the second centrifugation the cells were resuspended in NB with 20% glycerol and then dispensed in 2-mL vials. The 2-mL vials were immediately frozen and stored at -80ºC until needed.

4.1.3.4 Sensory analysis

A panel of 53 untrained assessors evaluated the differences in aspect, flavor, viscosity and global preference among samples by ranking test (ISO, 1988

a). The evaluation was carried out the same day of sample preparation in a standardised test room (ISO, 1988 b) with separate booths. Samples (30 mL) were served at refrigerated temperature (8±1)ºC in transparent glasses coded with three random digit numbers. Panellists tasted approximately the same volume of each sample and mineral water was provided to the assessors to rinse their mouth. Samples were arranged from higher to lesser preference. Data acquisition and analysis were performed using Compusense® *five* release 4.6 (Compusense, Canada) applying a Friedman analysis. The significance differences between samples were detected by a Tukey test ($p \leq 0.05$).

4.1.4 RESULTS AND DISCUSSION

4.1.4.1 Elaboration methodology and physicochemical product characterization

A previous study on fruit juice and milk based products commercialized in 2004 provided the basic information to characterize the type and proportion of the main ingredients.

Based on that information, four formulations were prepared varying orange juice content (15, 25, 50 and 70%) keeping milk concentration constant (20%) and completing up to 100% with water. A physicochemical characterization of the different formulations was performed.

The elaboration methodology was based on the addition of a milled sugar and pectin mixture to water pre-heated to 80ºC, keeping it in continuous agitation for 10 min (in order to dissolve completely the pectin). Once at room temperature, milk was added keeping it in continuous agitation for 5 min. This step is important since the pectin-milk casein union is formed avoiding its precipitation. Then, orange juice was added (previously filtered through a 0.29 mm sieve) keeping it in continuous agitation for 5 min to uniform the mixture. Finally the product was kept at cooling temperatures until use (Figure 4.1.1).

Results of physicochemical characterization are shown in Table 4.1.1. An increase in the orange juice content produced a decrease in the pH value while increasing the electrical conductivity and Brix degrees. Viscosity increased above 50% formulation. The most important physicochemical parameter on PEF treatment is the electrical conductivity because high values (>3.0 mS/cm) make difficult the application of the treatment. A viscosity increase also make difficult to obtain an adequate working flow-rate (120 mL/min, laminar-turbulent). As a result of the physicochemical characterization all formulations would be adequate for PEF treatment except the 70% sample due to its high viscosity and electrical conductivity.

4.1.4.2 Effect of food composition on PEF inactivation of *E.coli*

Figure 4.1.2 shows PEF inactivation degree of *E. coli* in the different formulations. It was observed an increase in the inactivation by decreasing the juice content, possibly due to a lower electrical conductivity, Brix degrees and viscosity. However, no significant differences among the 15, 25 and 50% samples were found (Table 8.2.1). In conclusion, based on microbial inactivation the three previously described formulations would be adequate for PEF treatment.

4.1.4.3 Sensory analysis

Table 4.1.2 shows the samples ranking based on the sensorial evaluation in relation to the aspect, flavor, viscosity and global appreciation. A lesser punctuation is an indicator of a greater acceptance. The greater acceptance sample in all the parameters studied was the 50% juice content, showing no significant differences with the 70% sample in aspect, viscosity and global appreciation ($p>0.05$). There were no significant differences between 70% and 25% samples ($p>0.05$) in global appreciation. In relation to the sensorial evaluation the 50% juice sample is the more suitable for PEF treatment.

Facing the results, it is concluded that the product with 50% juice content is the one that provide all suitable conditions for PEF treatment due to the

inactivation level obtained, physicochemical characteristics and sensorial evaluation. This formulation will be used in the present doctoral thesis for the inactivation kinetic studies.

Figure 4.1.1: Orange juice-milk based product.

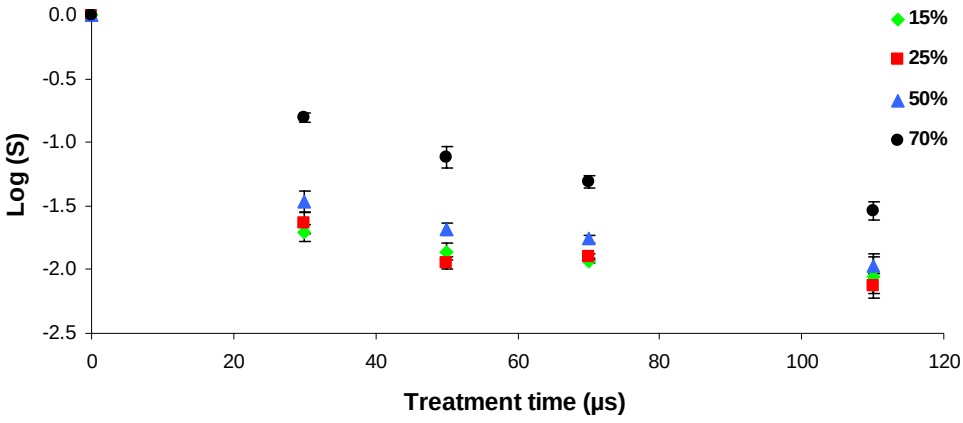

Figure 4.1.2: Effect of food composition on PEF inactivation of *E. coli* at 40 kV/cm. The standard deviation was expressed by error bars.

Table 4.1.1: Physicochemical characterization of different formulations.

Product	Juice (% v/v)	Water (% v/v)	Milk (% v/v)	Sugar (% w/v)	Pectin (% w/v)	pH	Electrical conductivity (mS/cm)	ºBrix	Viscosity (mPa·s)
1	15	65	20	7.5	0.3	4.37[a]±0.00[b]	2.13±0.00	11.87±0.02	20.00±1.00
2	25	55	20	7.5	0.3	4.29±0.01	2.27±0.01	12.15±0.07	20.00±0.00
3	50	30	20	7.5	0.3	4.09±0.00	2.86±0.01	14.30±0.01	22.00±1.00
4	70	10	20	7.5	0.3	4.04±0.01	3.13±0.01	15.80±0.00	24.00±1.00

[a] Value based on mean of three replicates.

[b] Standard deviation.

Table 4.1.2: Sample ranking based on aspect, viscosity, flavor and global appreciation.

SAMPLE	RANK SUMS[1]			
	ASPECT	FLAVOR	VISCOSITY	GLOBAL
50 %	86 [a2]	78[a]	91[a]	78[a]
70 %	89[a]	116[b]	106[a]	111[ab]
25 %	150[b]	146[b]	146[b]	143[b]
15 %	205[c]	190[c]	187[c]	198[c]

[1]Rank sums: Assessors order sum (1, 2, 3, 4).

[2] Different letters indicate significant differences among samples (95%, Tukey's test).

Effect of PEF, HHP and thermal treatment on PME inactivation and volatile compounds of an orange juice-milk based beverage

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4.2.1 ABSTRACT

The effects of thermal, pulsed electric field (PEF) and high hydrostatic pressure (HHP) processing on pectin methyl esterase (PME) activity and volatile compounds concentration in an orange juice-milk beverage (OJMB) were studied. Thermal treatment (85 ºC for 1 min) and combined thermal and PEF treatment (25 kV/cm at 65 ºC initial temperature) or thermal and HHP treatment (650 MPa at 50 ºC initial temperature) were needed to inactivate 90% of PME revealing the resistance of orange PME to the different preservation methods. At 25ºC PME activity was enhanced after PEF treatment. Twelve volatile compounds were extracted by SPME and selected for quantification by GC-MS following the application of the different treatments. The average loss in concentration of volatile compounds was between 16.0 and 43.0% after thermal treatment depending on the temperature. After PEF treatment the average loss was between -13.7 and 8.3% at an initial temperature of 25 ºC depending on electric field strengths, 5.8 to 21.0% at 45 ºC and 11.6 to 30.5% at 65 ºC. After HHP treatment the average loss was between -14.2 to 7.5% at an initial temperature of 30 ºC depending on the pressure levels and 22.9 to 42.3% at 50 ºC. The results showed the potential of the nonthermal technologies in providing food with a higher standard of quality compared to thermal processing.

Keywords: PEF; HHP; PME; Orange juice; Milk; Volatile compounds

4.2.2 INTRODUCTION

The use of High Hydrostatic Pressure (HHP) as a food preservation technology is gaining relevance in recent years, especially in Japan where the use of HHP has been adopted for the preservation of fruit juices, jams, sauces, rice, cakes and desserts (Norton and Sun, 2007). Nowadays in Europe and the USA, several companies in the meat, dairy and beverage industry have commercialized HHP treated products and it is thought that this trend will increase (Trujillo et al., 2002). Regarding the use of Pulsed Electric Fields (PEF), different research groups are working on the use of PEF for preservation of fruit and vegetable juices, milk and liquid egg (Sampedro et al., 2005; Rodrigo et al., 2005; Sampedro et al., 2006). The use of PEF by the juice processing industry is now becoming a reality in the USA where several types of fruit juices treated by PEF have been commercialized (Clark, 2006).

A large quantity of minimally processed foods have appeared on the market with characteristics similar to the original fresh food in response to a growing demand for natural foods that are perceived by consumers as healthy. Among them are beverages based on a mix of fruit juices and milk fortified with vitamins, minerals, and fiber as the most widely consumed functional foods (Pszczola, 2005); however, there are limited data related to quality and safety of these products.

Pectin Methyl Esterase (PME) is an important enzyme in orange juice based products and PME spoilage effects in orange juice are well known as cloud loss or gelification of juice concentrates (Tribess and Tadini, 2006). Thermal preservation treatments (88-95 ºC, 15-30 s) are based on the PME inactivation level achieved (>90%) due to its higher thermotolerance than those of majority of microorganisms found naturally in these products (Irwe and Olson, 1994). Due to the nature of the product (juice-milk mixture) a residual PME activity could instabilize the system.

The analysis of the volatile compounds has been performed by several authors to study the effect of PEF, thermal and HHP treatment on orange juice

aroma (Jia et al., 1999; Yeom et al., 2000; Ayhan et al., 2002; Min et al., 2003; Baxter et al., 2005) but there is no study on the effect of PEF and HHP treatment on the volatile compounds in a mixture of orange juice and milk.

The aim of this work was to study the effects of PEF, HHP and thermal processes on PME activity and volatile compounds in an orange juice-milk based beverage.

4.2.3 MATERIAL AND METHODS

4.2.3.1 Beverage preparation

Fresh Valencia var. oranges were purchased at a local supermarket. The oranges were squeezed with a juice extractor (Zumex 38, Zumex, S.A., Spain) and the juice was filtered with cheese cloth and stored at -40 ºC. The OJMB contained the following ingredients: fresh orange juice (500 mL/L), commercial UHT skimmed milk (200 mL/L), high methoxyl citrus pectin (Unipectine AYD 250, Cargill, USA) (3 g/L), sucrose (75 g/L), and deionized water (300 mL/L). Prior to mixing, solid ingredients were dissolved in water in the weight proportions indicated. The beverage was prepared just before use. The OJMB preparation and physicochemical characteristics were described in Chapter 1.

4.2.3.2 Thermal treatment

Isothermal inactivation experiments were carried out in a water bath with temperature control, in a range from 60-90 ºC for 1 min. One mL of sample was enclosed in a 1 mL thermal death time (TDT) disk (Jin et al., 2007). The samples were preheated to 40 ºC (results indicated that no PME inactivation was produced at this temperature, data not shown) in order to shorten and standardize the come-up time. The time needed to reach the final temperatures from the preheating temperature of 40 ºC was about 1 min. After treatment at the preset temperatures, the samples were withdrawn from the water bath and immediately cooled and kept in ice-water. The residual PME activity was measured within 2 h.

4.2.3.3 PEF treatment

An OSU-4F bench-scale continuous unit (Ohio State University, USA) was used to provide PEF treatment. Six co-field chambers with a diameter of 0.23 cm and a gap distance of 0.29 cm between electrodes were connected in series. One cooling coil was connected before and after each pair of chambers and submerged in a circulating bath (model 1016S, Fisher Scientific, PA, USA) to maintain the selected temperature (25, 45 and 65 ºC). The temperature was recorded by thermocouples (K type) at the entrance and exit of each pair of chambers. The entrance of the first treatment chamber can be considered as the initial temperature and the exit of the last treatment chamber as the final temperature. The values were recorded with a data logger (Model 800024, Sper Scientific, Taiwan). Pulse waveform, voltage, and current in the treatment chambers were monitored with a digital oscilloscope (Tektronix TDS 210, USA). The flow rate (120 mL/min) was controlled with a digital gear pump (Model 75211-30, Cole Parmer, USA). A bipolar square-wave of 2.5 μs was selected. Treatment time was set at 50 μs and the electric field at 15, 20, 25 and 30 kV/cm. One sample was collected after each treatment time and immediately cooled in ice-water.

4.2.3.4 HHP treatment

All pressure experiments were performed in a laboratory-scale vessel high-pressure processor (model 2L, Autoclave Systems Inc., USA). Combined thermal and high-pressure treatments were applied in the range of 450-650 MPa at initial temperatures of 30 and 50 ºC. The pressure medium was deionized water. A thermostated mantel, which surrounded the vessel, was connected to a cryostat keeping the vessel wall temperature constant during the experiment. Temperature was recorded by a thermocouple placed inside the vessel. The samples were filled in 2 mL eppendorf tubes and were enclosed in the pressure vessel already equilibrated at an initial temperature. The vessel was then pressurized and after a preset hold-time (15 min), decompressed. After pressure release the samples were immediately cooled in ice-water.

4.2.3.5 Analysis of headspace volatile compounds

Volatile compounds were extracted with a modification of the method described by (Fan and Gates, 2001) using a solid-phase microextraction (SPME) method. A 2 mL aliquot beverage was transferred into 6 mL serum vial. The vial, sealed by a teflon-lined septum and a screw cap, was pre-heated at 60 ºC for 2 min before a SPME fiber, coated with 100 μm of poly(dimethylsiloxane), was inserted into the headspace of the vial. After 30 min incubation, the SPME fiber with adsorbed volatile compounds was removed from the vial and inserted into the GC injection port at 250 ºC and held there for 5 min to desorb volatile compounds. Volatile compounds were separated by a Hewlett-Packard 6890N/5973 GC-MSD (Agilent Technologies, USA) equipped with a DB-Wax trace analysis column (30 m × 0.32 mm i.d., 0.5 μm film thickness). The temperature of the GC was programmed from 60 to 96 ºC at 8 ºC·min^{-1}, increased to 120 ºC at 12 ºC·min^{-1}, then increased to 220 ºC at 10 ºC·min^{-1} and held for 3 min at the final temperature. Helium was the carrier gas at a linear flow speed of 39 cm·sec^{-1}. Compounds were identified by comparing spectra of the sample with those contained in the National Institute of Standards and Technology Library (NIST02). The relative amount of each compound was expressed as peak area.

4.2.3.6 PME activity measurement

PME activity was determined by measuring the release of acid over time at pH 7 and 22 ºC following the procedure described by (Van den Broeck et al., 1999). The reaction mixture consisted of 1 mL of sample and 30 mL of 0.35 % citrus pectin solution (Sigma, USA) containing 125 mM NaCl. During hydrolysis at 22ºC, pH was maintained at 7.0 by adding 0.0001 N NaOH using an automatic pH-stat titrator (Titralab, Radiometer Analytical, SAS). After the first 1 min the consumption of NaOH was recorded every 1 s for a 3 min reaction period. PME activity was expressed in units (U/mL), defined as micromoles of acid produced per minute at pH 7 and 22 ºC. The detection limit was established at 0.019 U/ml. Residual activity was expressed as the relation between the PME activity after the

treatment (A) expressed in U/mL and the initial activity (A_0) expressed also in U/mL.

4.2.3.7 Experimental Design and Statistical Analysis

A hierarchical experimental design was used to study the influence of the different technologies studied: in thermal treatment, seven temperatures in PEF treatment, four electric field strengths and three temperatures and in HHP treatment, five pressures and two temperatures. The statistical analysis was performed using the Statgraphics software (Statistical Graphics Corp., USA), applying a univariant ANOVA test with a significance level of 95.0%.

4.2.4 RESULTS AND DISCUSSION

4.2.4.1 Effect of treatment on PME activity in the orange juice-milk based beverage

Figure 4.2.1 shows the thermal inactivation of PME in the OJMB in the range of 60-90 ºC for 1 min. Approximately 4-5 % of the PME remained after the 90 °C treatment. Several authors (Veersteg et al., 1980; Wicker and Temelli, 1988; Snir et al., 1996; Cameron et al., 1996; Tajchakavit and Ramaswami, 1997; Cameron et al., 1998; Lee et al., 2003; Do Amaral et al., 2005) have also observed a residual activity after thermal treatment, considered the stable fraction, between 4-8.3% in Navel and Valencia var. oranges reflecting the thermotolerance of the orange PME. To achieve a level of 90 % of PME inactivation, 85 ºC for 1 min or 90 ºC for 30 s was needed. No inactivation was found below 65 ºC for 1 min. At 60 ºC the PME activity was increased as a result of the treatment. This phenomenon could be explained by some activation effect produced by the heat. (Körner et al., 1980) found an optimum temperature of 60 ºC for the activity of two PME fractions purified from Valencia flavedo and Shamouti juice. The relationship between the temperature and the residual activity of PME seemed to be linear from

60-80 ºC and after that a tailing phenomenon was observed, suggesting the presence of a thermostable fraction in the OJMB.

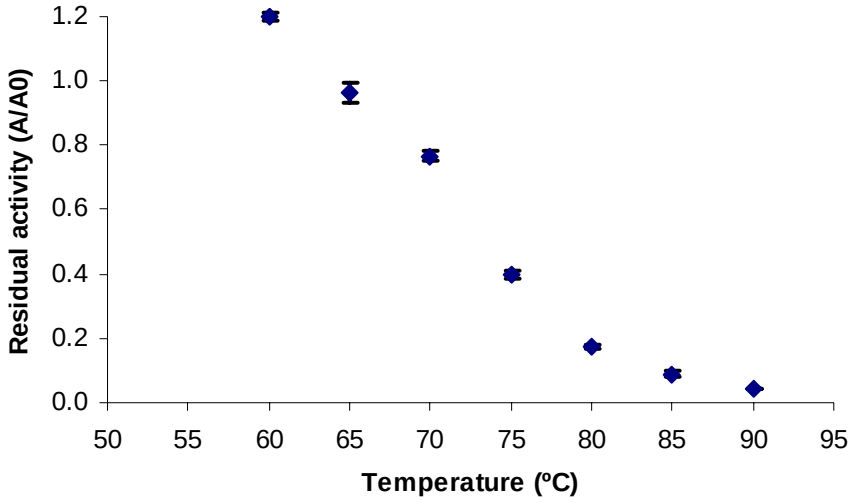

Figure 4.2.1: Isothermal inactivation of PME in the orange juice-milk based beverage. The standard deviation was expressed by error bars.

Table 4.2.1 shows the combined thermal and PEF inactivation of PME in the OJMB in the range of 15-30 kV/cm and initial temperatures of 25-65 ºC. At low treatment temperature (25 ºC) some activation effect was found as indicated by an increase in PME activity (between 11-60%) after the PEF treatment. PEF treatment has also been applied in different studies in recent years to improve the extraction of different components by increasing the permeability of plant cells from diverse foodstuff (Ade-Omowaye et al., 2001) and by improving the juice yield and quality parameters (Guderjan et al., 2007; Schilling et al., 2007). Therefore application of mild PEF treatments could increase the permeability of the orange pulp by facilitating the release of the bound PME. In those studies, after the PEF treatment, PME activity measured as "free" enzyme, increased. Van Loey et al., (2002) noticed that after PEF treatment, PPO in apple juice and PME in orange juice were activated due to increases in cell permeabilization and release of

the enzyme from plant cells. In addition, some authors have also observed an enhancing effect of diverse enzymes after PEF treatment, such as alkaline phosphatase and peroxidase in milk (Grahl and Märkl, 1996), lysozyme and pepsin in buffer solutions (Ho et al., 1997), PME in orange juice (Yeom et al., 2002), pepsin in an aqueous solution (Yang et al., 2004) and protease in milk (Bendicho et al., 2005). They argued that PEF treatment could create more active sites or increase the size of the existing sites converting the enzyme into a more active form.

Table 4.2.1: PME residual activity after combined PEF and thermal treatment in the orange juice-milk based beverage

E (kV/cm)	Residual Activity (A/A_0) T (ºC)		
	25	45	65
15	1.594[a]±0.150[b]	0.947±0.010	0.541±0.012
20	1.484±0.001	0.634±0.032	0.482±0.057
25	1.303±0.001	0.642±0.045	0.213±0.048
30	1.118±0.060	0.465±0.021	0.089±0.010

[a] Value based on mean of three replicates.

[b] Standard deviation.

By increasing the temperature, the inactivation reached a maximum of 91% inactivation after 30 kV/cm, 65 ºC (final temperature 80 ºC) and 50 µs. The residence time at this temperature was a few seconds. At these conditions the temperature could affect the enzyme to some extent. To check the thermal effect of the temperature in the PEF treatment, low electric field intensities, high frequency and low pulse duration were applied (3-5 kV/cm, 3000-3500 Hz, 1 µs) obtaining the same final treatment temperature (80 ºC). The results showed that slight PME inactivation was achieved after low intensity PEF treatment alone (<10%) demonstrating the synergetic effect between the temperature and PEF treatment.

Figure 4.2.2 represents the combined thermal and HHP inactivation of PME in the OJMB at 30 and 50 ºC for 15 min. At 25 ºC and low pressures (450 MPa) no inactivation effect was observed, revealing the pressure tolerance of the orange PME. By increasing the temperature to 50 ºC (final temperature 65 ºC) and pressure to 650 MPa the inactivation increased and reached a maximum of 90.5 %. Several authors have observed this resistance to pressure treatment. (Nienaber and Shellhammer, 2001; Truong et al., 2002) found that PME was stable under pressure below 400 MPa and combination of high temperature and high pressure (40-50 ºC and 600-700 MPa) were necessary to inactivate it.

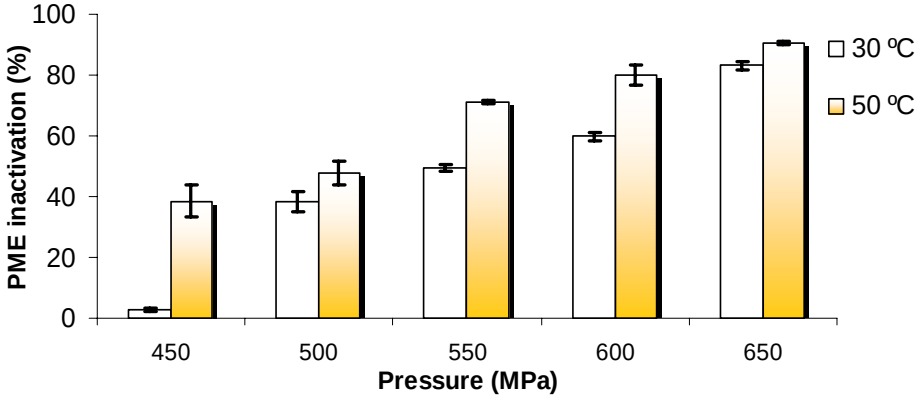

Figure 4.2.2: Combined HHP and thermal inactivation of PME in the orange juice-milk based beverage. The standard deviation was expressed by error bars.

4.2.4.2 Effect of treatment on the volatile compounds concentration in the orange juice-milk based beverage

Twelve volatile compounds could be identified accurately from the OJMB. The majority of the compounds were hydrocarbons among which limonene and valencene were the most abundant constituting more than 90% of the total compounds.

Figure 4.2.3 shows the effect of thermal treatment on the relative concentration of volatile compounds in the OJMB. The average loss of volatile compounds after thermal treatment at the different temperature range for 1 min was between 16.0-43.0%. There were no significant differences in the concentration of volatile compounds due to the increase in temperature up to 80ºC (Table 8.2.2). The compounds could be divided in two groups. One group considered as relatively low-molecular-weight (MW 136) including (β-pinene, α-pinene, β-myrcene, limonene, α-phellandrene, 3-carene and 4-carene) and a second group considered as relatively high-molecular-weight (MW 142-204) including nonanal, decanal, caryophyllene, dodecanal and valencene. The higher average loss was observed in β-pinene (48%), α-pinene (42%) and 3-carene (41%) which belong to the first group, and counted for an average loss of 35%. On the other hand the high-molecular-weight compounds seemed to be less sensitive to the thermal treatment with a slight increase in nonanal content at temperatures below 70 ºC. At higher temperatures, nonanal and caryophyllene (12%) which have higher boiling points had an average loss of 21%, lower than the loss of lower boiling point compounds. Boff et al., (2003) came to the same conclusions observing that in the thermal processing of an orange juice, low-molecular-weight compounds (β-myrcene, limonene and α-pinene) were more sensitive to the thermal treatment than relatively higher-molecular-weight compounds (decanal, caryophyllene and valencene).

Figure 4.2.4 shows the effect of the combined thermal and PEF treatment (at initial temperatures 25, 45 and 65 ºC) on the concentration of volatile compounds in the OJMB. The average loss was between -13.7 to 8.3% at 25 ºC, 5.8 to 21.0% at 45 ºC and 11.6 to 30.5% at 65 ºC after PEF treatment. An increase in the electric field strength did not produce a significant decrease in the average loss of volatile compounds content except at high electric fields (25 and 30 kV/cm at 25 and 65ºC) (Table 8.2.3). An increase in the temperature only decreased significantly the average loss of volatile compounds content at 65ºC (Table 8.2.4).

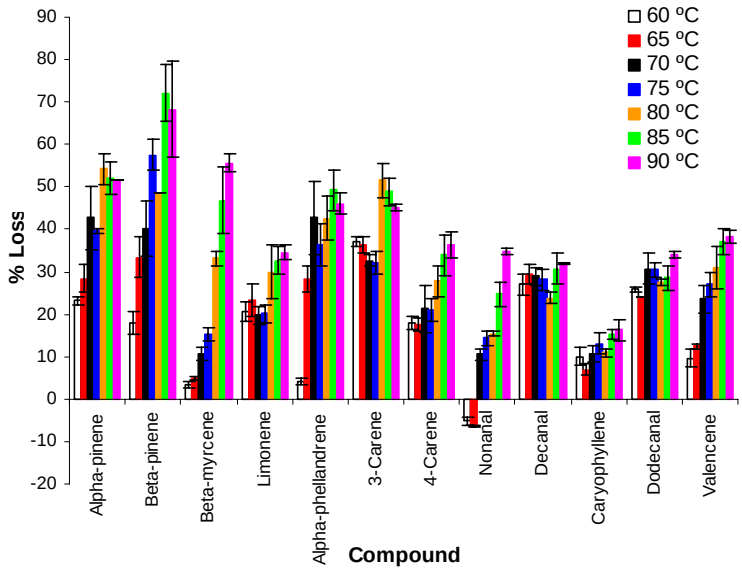

Figure 4.2.3: Effect of thermal treatment for 1 min on the volatile compounds content in the orange juice-milk based beverage. The standard deviation was expressed by error bars.

At 25 and 45 ºC and electric field strength below 25 kV/cm and 65 ºC at 15 kV/cm, several compounds increased its content after PEF treatment (β-pinene, limonene, 3-carene, 4-carene, nonanal, decanal and dodecanal). Ayhan et al., (2002) obtained similar results observing that several compounds (myrcene, limonene and α-pinene) increased its content after PEF. Steffen and Pawliszyn, (1996) found that in a complex matrix such as orange juice, with the presence of suspended solids, a portion of analytes could be trapped in the pulp. PEF technology has been used to improve the extraction of different compounds by increasing the membrane permeabilization. This fact could explain why PEF treatment could facilitate the release of several compounds from the suspended solids to the liquid phase facilitating its extraction into the headspace.

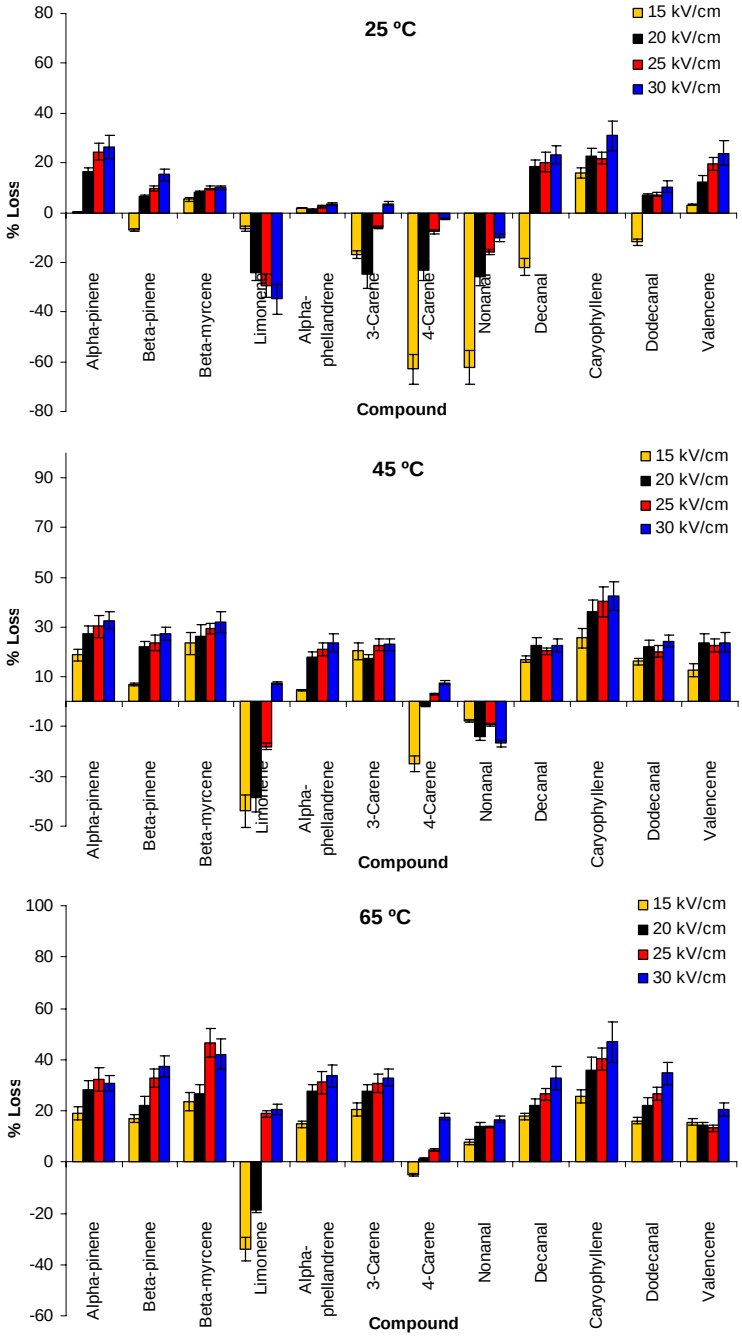

Figure 4.2.4: Effect of combined PEF and thermal treatment (25, 45 and 65ºC) on volatile compounds content in the orange juice-milk based beverage. The standard deviation was expressed by error bars.

To check the hypothesis of higher concentration of certain compounds in the pulp, the orange juice was centrifuged in order to separate into serum and pulp. The concentration of different compounds was studied in both fractions. Nonanal, 3-carene, 4-carene, limonene and α-phellandrene were found in higher quantity in the pulp (data not shown). This fact could explain that the compounds could be released to the liquid phase by the PEF treatment and be extracted in the headspace more readily. Compounds more sensitive to the PEF treatment were β-myrcene, α-pinene and caryophyllene whereas the less sensitive compounds were limonene, 4-carene and nonanal. Opposite to the thermal treatment, the compounds that were present in the pulp at higher proportions were also higher after PEF treatment.

Figure 4.2.5 shows the loss of volatile compounds concentration in the OJMB after the combined thermal and HHP (at 30 and 50 ºC initial temperatures) treatment. The average loss at 30 ºC was between -14.2 and 7.5%. An increase in the pressure value did not produce a significant decrease in the average loss of volatile compounds content (Table 8.2.5). However an increase in the initial treatment temperature produced a significant decrease in the loss of volatile compounds content (Table 8.2.6). Only β-myrcene content was lost at every pressure value (~50%) and limonene, α-pynene, β-pynene, 3-carene and α-phellandrene were not affected at 650 MPa. On the other hand, the rest of components in the OJMB increased in their contents after treatment with lower pressures. Similar to the PEF treatment, it seems that the HHP treatment released several compounds that are found in the solid phase of the orange juice. At 50 ºC (final temperature 65 ºC) the average loss was increased in all compounds (32.80%). At 650 MPa and 50 ºC the loss of volatile compounds was the same as the maximum which was reached after the thermal treatment (85-90 ºC). Valencene, limonene and caryophyllene were the compounds less sensitive to the HHP treatment whereas β-myrcene, α-phellandrene and 3-carene at 50 ºC were more sensitive to the HHP treatment.

The combination of different nonthermal technologies and thermal treatment (PEF at 65 ºC initial temperature and HHP at 50 ºC initial temperature) inactivated 90% of PME whereas high temperature treatments (85-90 ºC) were needed for thermal treatment. At this temperature the loss of different volatile compounds were significantly higher than PEF and similar to HHP at 50 ºC. Concentrations of several compounds were enhanced after PEF and HHP treatment at low temperatures. The sensitivity of volatile compounds differed with the different treatments applied. The high-molecular-weight compounds were more resistant to the thermal treatment and the pulp-related compounds were more resistant to the PEF and HHP treatments. Based on these results it is possible that PEF treatment can achieve a similar PME inactivation than thermal processing with a better orange juice-milk beverage fresh aroma.

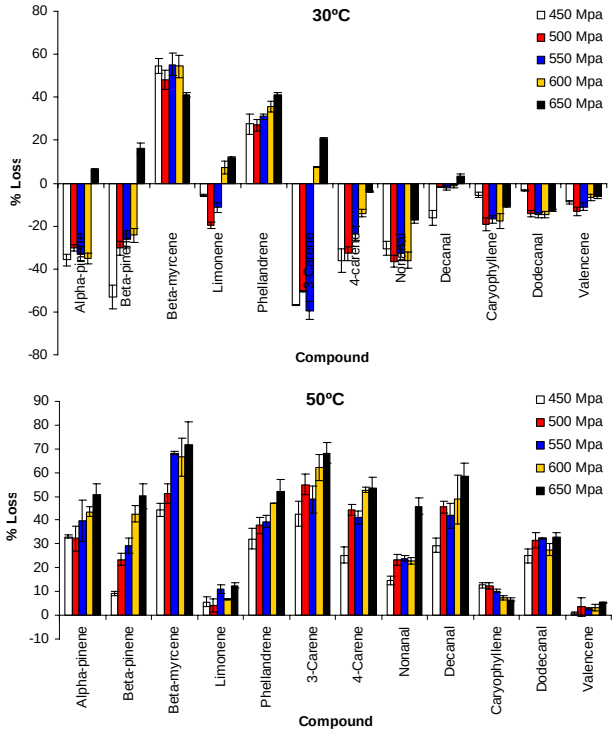

Figure 4.2.5: Effect of combined HHP and thermal treatment (30 and 50ºC) on volatile compounds content in the orange juice-milk based beverage. The standard deviation was expressed by error bars

Inactivation kinetics of pectin methyl esterase under combined thermal-high pressure treatment in an orange juice-milk beverage

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4.3.1 ABSTRACT

The inactivation kinetics of PME in an orange juice-milk beverage system treated by thermal (65-80ºC) and combined thermal (25-65ºC)-high pressure (0.1-700 MPa) processes were fitted using a biphasic model. About 6-8 % of the initial activity corresponding to the heat and pressure stable fraction was observed. For complete inactivation a treatment at 90ºC, 1 min or 700 MPa at 55ºC for 2 min was necessary showing the protective effect of the orange-milk media. The extent of inactivation was different in the orange matrices showing that PME was more thermostable in the orange juice-milk based beverage system as compared to the purified enzyme in a buffer system. On the other hand, the purified enzyme in a buffer system showed the highest pressure stability. Parameters such as pH (from acid in the orange juice matrices to basic in the buffer), matrix composition (from less to more complex) and purification level of the enzyme (purified in the buffer or nature in the orange juice) play an important role in the stability of the PME against the different processing technologies studied.

Keywords: Orange juice, Milk, Pectin methyl esterase, HHP, inactivation

4.3.2 INTRODUCTION

In recent years a large quantity of new pasteurised liquid foods requiring chilled storage and distribution have appeared on the European market. Among these, fruit-juice mixes, vegetable soups and different sauces are predominant; they are produced from food ingredients of very different natures, leading to complex compositions. The fruit juice mixes include beverages based on a mixture of fruit juices and milk, fortified with vitamins, minerals, fiber and these products represent the most widely consumed functional foods (Pszczola, 2005).

For these systems only limited data on quality and safety aspects are available in open literature and it is essential to evaluate the potential use of alternative technologies, such as High Hydrostatic Pressure (HHP), in order to preserve their freshness, as well as their nutritional and functional properties. Studies on the application of HHP to different juice matrices have recently become available but studies on more complex fruit juice based foods (including fat, proteins and additives such as pectin and sugar in an acid environment) are still lacking.

Pectin methyl esterase (PME) is an important enzyme in orange juice products and its effects on quality aspects such as cloud stability are well known (Cameron et al., 1998; Do Amaral et al., 2005). Orange PME is a thermal and high pressure stable enzyme composed by several fractions with different processing stabilities (stable and labile fraction). The orange PME stable fraction is mainly responsible for the destabilization of orange juice (cloud loss) at 5ºC and also to a lesser extent at 30ºC (Cameron et al., 1998; Versteeg et al., 1980). It has been shown that the enzyme remains active (between 90-95 % retention at pH 3.5 for 60 h and 80 % retention at 4ºC for 42 days) in a single strength orange juice (Hou et al., 1997). This fraction is thermo tolerant and severe conditions are necessary to inactivate it (90ºC, 1 min or 95ºC, 30 s) (Cameron et al., 1994; Do Amaral et al., 2005). Normally industry adopts these conditions to pasteurize orange juice (88-90ºC, 15-30 s) (Irwe and Olson, 1994). PME is also pressure tolerant (is stable at P<400 MPa); therefore, a combination of high T and high P (40-50ºC and 600-700

MPa) is necessary to inactivate it (Nienaber and Shellhammer, 2001; Truong et al., 2002).

There are a number of studies available on the heat inactivation of PME in orange juice (Versteeg et al., 1980; Tajchakavit and Ramaswamy, 1997; Lee et al., 2003), in orange pulp (Wicker and Temelli, 1988) as well as on the purified enzyme isolated from oranges (Cameron et al., 1994; Cameron and Grohmann, 1996; Van den Broeck et al., 1999 a; Han et al., 2000). HHP inactivation of purified PME has been studied in buffer systems (Van den Broeck et al., 1999 b; Van den Broeck et al., 2000) and in orange juice (Nienaber et al., 2001; Seyderhelm et al., 2004) but there are no studies on the behavior of PME during thermal and/or HHP treatment of beverages based on fruit juices and milk.

Traditionally linear models have been applied to describe enzyme inactivation kinetics by heat, obtaining parameters such as D and z values. However, in the case of orange PME the thermal inactivation curves have been observed to behave differently due to the presence of different fractions exhibiting different stabilities. A number of authors have demonstrated the appearance of such fractions; two fractions with different thermal stabilities were observed in orange Valencia var. juice or pulp (Wicker and Temelli, 1988; Hou, et al., 1997; Tajchakavit and Ramaswamy, 1997; Van den Broeck, et al., 1999 a; Lee, et al., 2003), four fractions with different thermostability (Cameron et al., 1998) and seven fractions in the peel (Han, et al., 2000). In the case of orange juice Navel var. three fractions with different thermostability were found (Versteeg, et al., 1980). Nevertheless, in many cases only part of the curve (activity related to the most abundant fraction) has been modelled to obtain the previously described parameters. In this study a biphasic model is proposed to characterize the behavior of the different fractions against the treatment.

The aim of the present work was to study the inactivation kinetics of PME in an orange juice-milk based beverage system as well as different orange matrices under combined conditions of HHP and heat.

4.3.3 MATERIAL AND METHODS

4.3.3.1 Orange juice and beverage preparation

Fresh Valencia Navel var. oranges were purchased at a local supermarket. The oranges were peeled and squeezed with the aid of a juice centrifuge and the juice was kept at -40ºC until use. The beverage contained the following ingredients: fresh orange juice (500 mL/L), commercial UHT skimmed milk (200 mL/L), high methoxyl citrus pectin (Unipectine AYD 250, Cargill, USA) (3 g/L), sugar (75 g/L), and distilled water (300 mL/L). The beverage preparation and physicochemical characteristics were described in Chapter 1. The beverage was prepared just before use.

4.3.3.2 PME activity measurement

PME activity was determined by measuring the release of acid over time at pH 7 and 22 ºC following the procedure described by (Van den Broeck et al., 1999). The reaction mixture consisted of 2.5 mL of sample and 30 mL of 0.35% apple pectin solution (70-75 % esterification, Fluka, Belgium) containing 125 mM NaCl. Before injection, the pectin solution was adjusted to pH 7.0. During hydrolysis at 22ºC, pH was maintained at 7.0 by adding 0.005 N NaOH using an automatic pH-stat titrator (Metrohm, Switzerland). After the first 300-400 s the consumption of NaOH was recorded every 10 s for a 10-15 min reaction period. PME activity expressed in units (U) is defined as micromoles of acid produced per minute at pH 7 and 22ºC. The detection limit was established at 0.057 U. Residual activity was expressed as the relation between the PME activity after the treatment (A) expressed in U/mL and the initial activity (A_0) expressed also in U/mL.

4.3.3.3 Experimental design

A hierarchical experimental design was used to study the influence of the different treatments on PME activity: six temperatures and six treatment times were used for the thermal treatment. Four pressure levels, four temperatures and

five treatment times were used for the combined thermal-high pressure treatment. The statistical analysis was performed using the SAS® software, applying a nonlinear regression. To study the influence of the orange matrices, between eighteen and fourteen temperatures and one treatment time were used for the thermal treatment and ten pressure levels and one treatment time for the high pressure treatment.

4.3.3.4 Thermal inactivation at atmospheric pressure

Isothermal inactivation experiments were carried out in a water bath with temperature control, in a temperature range from 45-90ºC for 1-90 min. Beverage sample (8 mL) was enclosed in 10 mL stainless steel tubes. Prior to each treatment the samples were preheated at 40ºC (measurements indicated that no inactivation was observed at this temperature, data not shown) in order to shorten and standardize the come-up time. Under these conditions a come-up time of 2.5 min was observed using a tube with an inner thermocouple connected to a data logger. After the preset time intervals including the come-up time, the samples were withdrawn from the water bath and immediately cooled in ice-water. The residual PME activity was measured within 2 h storage in an ice-water bath.

4.3.3.5 Combined thermal and high-pressure inactivation

All pressure experiments were performed in laboratory scale multi-vessel high-pressure equipment (HPIU-10000, Resato, The Netherlands). Combined thermal and high-pressure treatments were applied in the ranges of 25-65ºC and 0.1-700 MPa for 2-75 min. The pressure medium was a glycol-oil mixture (TR15, Resato, The Netherlands). A thermostated mantel, which surrounds each vessel, was connected to a cryostat keeping the temperature constant during the experiment. The samples were filled in 15 mL plastic tubes with flexible stoppers and were enclosed in the pressure vessel already equilibrated at a preset temperature. Pressure was built up slowly (100 MPa/min) to minimize the adiabatic heating. After pressure build-up, an equilibrium period of 2 min was taken into

account in order to allow temperature to evolve its desire value. After the equilibration period, one vessel was decompressed and the sample was immediately cooled representing the blank. Then the rest of vessels were decompressed after preset time intervals. After pressure release the samples were immediately cooled in ice-water and the residual PME activity was measured within 2 h storage in an ice-water bath.

4.3.3.6 Orange PME purification

Orange PME was extracted using a 0.2 M Tris buffer with 1M NaCl at pH 8. Afterwards, the extract was partially purified by ammonium sulphate precipitation. The fraction precipitating between 30% and 80% ammonium sulphate saturation was collected and dissolved in 20 mM Tris buffer, pH 7.5 and kept at -80ºC until use. This crude extract of orange PME was further purified following the procedure described by (Ly Nguyen et al., 2002) based on affinity chromatography using a NH-Sepharose 4B-PME-inhibitor column, pooling and desalting the different fractions of PME.

4.3.3.7 Data analysis and parameter estimation

For the thermal and high pressure ranges under study, inactivation kinetics of orange PME in the orange juice-milk based beverage was properly described using the biphasic model which is usually applied when two fractions are present with different processing stabilities, one stable and one labile fraction and both inactivating according to first-order kinetics and can be defined by (Van den Broeck et al., 2000):

$$A=A_L \exp(-k_L \cdot t)+As \exp(-k_S t) \qquad \textbf{Equation 4.3.1}$$

where A is the dependent variable and can be expressed as enzyme activity (U/mL), A_L and A_S refer, respectively, to the activity of the labile and stable fraction (U/mL), k_L and k_S the inactivation rate constant of the labile and stable fraction

(min^{-1}) and t the independent variable expressed as the treatment time (s). The different parameters (A_L, A_S, k_L, k_S) were estimated through a nonlinear regression using the SAS® software.

A special case of first order kinetics is the fractional conversion model, which is usually applied when a fraction is inactivated and another fraction remains constant and a nonzero residual activity after prolonged thermal/pressure treatment is observed (Van den Broeck et al., 1999 a and b):

$$A = A\infty + (A_0 - A_\infty) \exp(-k\,t) \qquad \textbf{Equation 4.3.2}$$

where A is the dependent variable and can be expressed as enzyme activity (U/mL), A_0 and A_∞ refer, respectively, to the initial enzyme activity and to the residual activity after prolonged treatment time (U/mL), k the inactivation rate constant (min^{-1}) and t the independent variable expressed as the treatment time (s). The different parameters (k and A_∞) were estimated through a nonlinear regression using the SAS® software.

The temperature dependence of inactivation rate constants of the labile and stable fraction can be estimated using the Arrhenius model (Arrhenius, 1889):

$$\ln(k_L) = \ln(k_{Lo}) + \left[\frac{E_{aL}}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right] \qquad \textbf{Equation 4.3.3}$$

$$\ln(k_s) = \ln(k_{So}) + \left[\frac{E_{aS}}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right] \qquad \textbf{Equation 4.3.4}$$

where $\ln(k_L)$ and $\ln(k_S)$ are the dependent variables and are expressed as the inactivation rate constant of the labile and stable fraction respectively (min^{-1}), Ea_L and Ea_S are the activation energy of the labile and stable fraction respectively (kJ/mol), k_{L0} and k_{S0} the inactivation rate constant at a reference temperature of the

labile and stable fraction respectively (min^{-1}), T is the independent variable expressed as the absolute temperature (K), T_0 the reference temperature (K) and R (8.314 J/mol K) is the universal gas constant. The different parameters (Ea_L, Ea_S, k_{L0} and k_{S0}) were estimated through a linear regression using the SAS® software.

The pressure dependence of the enzyme inactivation rate constants at a constant temperature of the labile and stable fraction can be calculated using the linearized Eyring equation (Eyring, et al., 1946):

$$\ln(k_L) = \ln(k_{Lo}) + \left[\frac{V_{aL}}{RT}\left(P - P_{ref}\right)\right] \qquad \textbf{Equation 4.3.5}$$

$$\ln(k_S) = \ln(k_{So}) + \left[\frac{V_{aS}}{RT}\left(P - P_{ref}\right)\right] \qquad \textbf{Equation 4.3.6}$$

where $\ln(k_L)$ and $\ln(k_S)$ are the dependent variables and are expressed as the inactivation rate constant of the labile and stable fraction respectively (min^{-1}), Va_L and Va_S are the activation volume of the labile and stable fraction respectively (cm^3/mol), k_{L0} and k_{S0} are the inactivation rate constant at a reference pressure of the labile and stable fraction respectively (min^{-1}), T is the absolute temperature (K), P is the independent variable expressed as the pressure (MPa), P_{ref} is the reference pressure (MPa) and R (8.314 J/mol K) is the universal gas constant. The different parameters (Va_L, Va_S, k_{L0} and k_{S0}) were estimated through a linear regression using the SAS® software.

The corrected R^2 was calculated to check how well the model fits to the experimental enzyme inactivation curve and can be defined as follows:

$$\text{Corrected R} = \left[1 - \frac{(m-1)\left(1 - \frac{SSQ_{regression}}{SSQ_{total}}\right)}{(m-j)}\right] \qquad \textbf{Equation 4.3.7}$$

where *m* is the number of observations, *j* is the number of model parameters, *SSQ* is the sum of squares, and *SD* is the standard deviation.

4.3.3.8 Effect of food matrix

The effect of different orange matrices on the thermal PME inactivation was carried out in a water bath with temperature control. Treatments of 5 min in a temperature range from 45-90ºC were carried out. Samples were enclosed in 10 mL metal tubes. The effect of the different matrices on high-pressure PME inactivation was performed applying pressures in the range of 350-800 MPa for 15 min at 25ºC. Samples were filled in 15 mL plastic tubes with flexible stoppers.

4.3.4 RESULTS AND DISCUSSION

4.3.4.1 Thermal inactivation of the PME in the orange juice-milk based beverage

Data for isothermal inactivation of PME in the orange juice-milk based beverage obtained in the range from 65-80ºC could be accurately modelled applying a special case of first-order kinetics, the biphasic model assuming the occurrence of two fractions with different stability levels and obtaining the rate constants (*k*) of both fractions and the percentage of stable fraction (Eq.4.3.1) (Table 4.3.1). The percentage (6-8%) of stable fraction estimated was in line with data obtained by several authors (4-8.3%) in different varieties of oranges (Navel and Valencia var.) (Cameron and Grohmann, 1996; Cameron et al., 1998; Do Amaral et al., 2005; Lee et al., 2003; Snir et al., 1996; Tajchakavit and Ramaswamy, 1997; Versteeg, et al., 1980; Wicker and Temelli, 1988). It has been stated that the amount of orange PME stable fraction is variable and depends to a large extent on the orange variety used and the time of harvest (Snir et al., 1996). Irwe and Olson (1994) and Van den Broeck et al., (1999 a) found strong differences in the thermal and pressure stability of PME from different orange varieties: Valencia and Navel var.

It was necessary to increase the temperature to 90ºC for 1 min to inactivate the stable fraction (data not shown). Snir et al., (1996) found that the stable fraction is the one that remains after treatment, at 70ºC, 5 min, but in our study slightly different conditions (applying 80ºC, 5 min or 90°C, 1 min) were found to distinguish the stable fraction. These results are in line with those obtained by Han et al., (2000), who also found similar conditions to distinguish the thermolabile and thermostable fractions.

From 75-80ºC only the rate constants of the heat stable fraction were estimated because the inactivation of the labile fraction was too fast to be determined accurately. Based on the inactivation rate constants, the activation energy of the labile and stable fraction (Ea_L and Ea_S) were estimated (Eq. 4.3.3 and Eq. 4.3.4) (Table 4.3.1). The inactivation rate constants of the stable fraction were found to be less temperature sensitive than the rate constants of the labile fraction. From 65-72.5ºC only the labile fraction was inactivated whereas the stable fraction did not inactivate. In this temperature domain a fractional conversion model was also used to estimate the rate constant of the heat labile fraction and the percentage of stable fraction (Eq. 4.3.2) (Table 4.3.1). It could be seen that the inactivation parameters and percentage of stable fraction obtained by both models were similar. The corrected R^2 values ranged from 0.995 to 0.999 indicating the biphasic model could accurately describe the thermal inactivation curves of PME in the orange juice-milk based beverage (Figure 4.3.1).

The *k* values obtained in the thermal inactivation study were compared to those obtained by other authors in less complex media. Van den Broeck et al., (1999 a) studied the thermal inactivation of PME purified from Navel oranges obtaining *k* values at 65ºC of 0.889, 1.536, 0.288 and 0.234 min^{-1} in deionized water and citric buffer at pH of 3.2, 3.7 and 4.2 respectively. The *Ea* values ranged from 404.9 to 292.6 kJ/mol. Comparing these data with those obtained in the orange juice-milk based beverage at the same conditions, it can be observed that the k values are much lower while the value of the activation energy is higher

(528.23 kJ/mol). This indicates that the PME in the milk-juice beverage is more thermally resistant and the k values less sensitive to heat.

Table 4.3.1: Biphasic and fractional conversion kinetic parameters estimate ± standard error describing isothermal inactivation of PME in the orange juice-milk based beverage at atmospheric pressure.

T (ºC)	Biphasic Model		Fractional Conversion
	k_L[a] (min^{-1})	k_S[b] (min^{-1})	k (min^{-1})
65	0.173[c]±0.057[d]	-	0.085±0.019
70	0.215±0.115	-	0.166±0.024
72.5	1.789±0.194	-	1.538±0.130
75	-	1.747±0.241	-
77.5	-	1.740±0.084	-
80	-	6.617±0.839	-
% heat stable fraction	-	7.90±0.250	6.29±0.639
Ea_S[e] (kJ/mol)	-	605955±89312	-
Ea_L[f] (kJ/mol)	532603±32043	-	528233±32810.3

[a]: Kinetic constant of the labile fraction

[b]: Kinetic constant of the stable fraction

[c]: Value based on mean of three replicates

[d]: Standard error

[e] Activation energy of the heat stable fraction

[f] Activation energy of the heat labile fraction

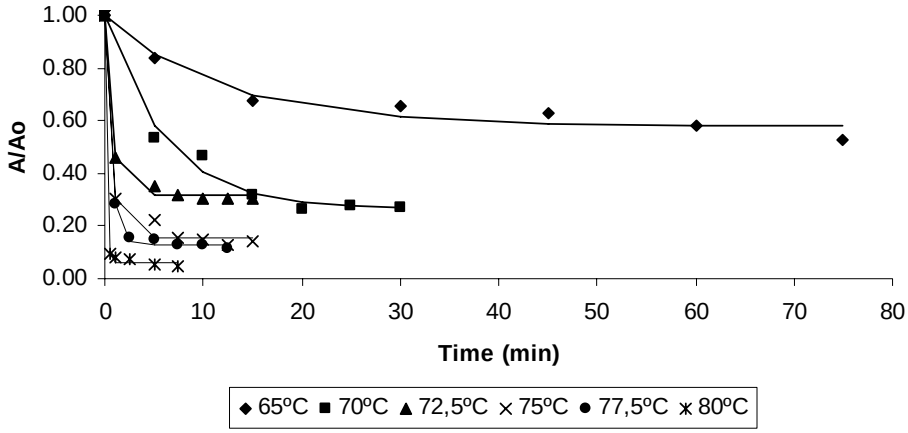

Figure 4.3.1: Isothermal inactivation of PME in the orange juice-milk beverage fitted by a biphasic model.

4.3.4.2 Combined thermal and high-pressure inactivation of PME in the orange juice-milk based beverage

Inactivation kinetics of PME in the orange juice-milk based beverage were studied by combining pressures and temperatures and the occurrence of two fractions with different high pressure stability was also observed. Taking this fact into account, the biphasic model was used to estimate the inactivation rate constants (k) and the percentage of stable fraction (Eq. 4.3.1) (Table 4.3.2). Goodner et al., (1998) and Van den Broeck et al., (2000) also observed several fractions with different pressure stabilities in an orange juice from Valencia var. and in a PME fraction purified from Navel var. oranges respectively.

A 7 % of the initial activity was estimated as the pressure stable fraction. At pressure level until 550 MPa and temperature until 55ºC only the labile fraction was inactivated. Based on the inactivation rate constants, the activation volume of the labile and stable fraction (Va_L and Va_S) were estimated (Eq. 4.3.5 and Eq. 4.3.6) (Table 4.3.2).

Based on the activation volume, the inactivation rate constant of the labile fraction was more pressure dependent than the inactivation rate constant of the

stable fraction. The biphasic model could also describe the combined thermal and high pressure treatment with corrected R^2 values ranging from 0.961 to 0.998 (Figure 4.3.2). Van den Broeck et al., (2000) obtained the k values after the combined thermal and high pressure treatments of the PME purified from Navel oranges in citric buffer at pH of 3.7. The k values ranged from 3.95 to 5.41 min^{-1} after the combined 25ºC and 600 to 700 MPa treatment and from 2.92 to 20.80 min^{-1} for the combined 45ºC and 550 to 700 MPa treatment. As for thermal treatments, the k values were much higher than those obtained in our study meaning that the enzyme is more pressure resistant in the orange juice-milk based beverage.

4.3.4.3 Effect of orange matrices

Based on the results obtained comparing the orange juice-milk based beverage with other matrices in the literature was found interesting to check the effect of different orange matrices on the stability of orange PME against thermal and high pressure treatment. In the Figure 4.3.3 is represented the thermal inactivation of orange PME in different matrices in the range of 45-90ºC. As for PME inactivation in the orange juice-milk based beverage, no inactivation was found below 63ºC for 5 min and around 3.5% remaining activity was found at 91°C. In the case of orange juice no inactivation was achieved below 50ºC and around 8% remaining activity was found at 83°C. As for inactivation of purified PME around 6% remaining activity was found at 72.5°C. Analyzing the results it could be stated that the PME inactivation in the orange juice-milk based beverage was slower than in the other media, meaning that PME was more thermostable in the orange juice-milk based beverage environment. In addition, PME was found to be more thermostable in orange juice compared to the purified enzyme in a buffer system at pH 7. This fact could mean that the pH or level of PME purification affects the thermal inactivation mechanism of PME.

Table 4.3.2: Biphasic kinetic parameters estimate ± standard error describing the combined thermal and high pressure inactivation of PME in the orange juice-milk beverage.

P (MPa)	25ºC		45ºC		55ºC		65ºC	
	k_L[a] (min^{-1})	k_S[b] (min^{-1})	k_L (min^{-1})	k_S (min^{-1})	k_L (min^{-1})	k_S (min^{-1})	k_L (min^{-1})	k_S (min^{-1})
500	-	-	-	-	-	-	0.353±0.064	-
550	0.264[c]±0.071[d]	-	0.245±0.031	-	0.365±0.046	-	0.415±0.023	0.011±0.001
600	0.293±0.033	0.014±0.001	0.320±0.024	0.014±0.001	0.794±0.051	0.015±0.001	0.775±0.048	0.031±0.002
650	0.399±0.056	0.020±0.002	0.704±0.045	0.026±0.001	0.935±0.124	0.027±0.001	0.927±0.067	0.037±0.001
700	0.709±0.077	0.023±0.001	1.130±0.097	0.025±0.002	1.672±0.101	0.032±0.002	-	-
Va_L[e] (cm^3/mol)	-66.608±4.80		-74.615±7.63		-59.081±7.49		-44.227±3.86	
Va_S[f] (cm^3/mol)	-49.987±4.45		-54.243±6.87		-57.653±6.03		-52.786±4.23	

[a]: Kinetic constant of the labile fraction [b:] Kinetic constant of the stable fraction [c]: Value based on mean of three replicates

[d]: Standard error [e:] Activation volume of the heat stable fraction [f:] Activation volume of the heat labile fraction

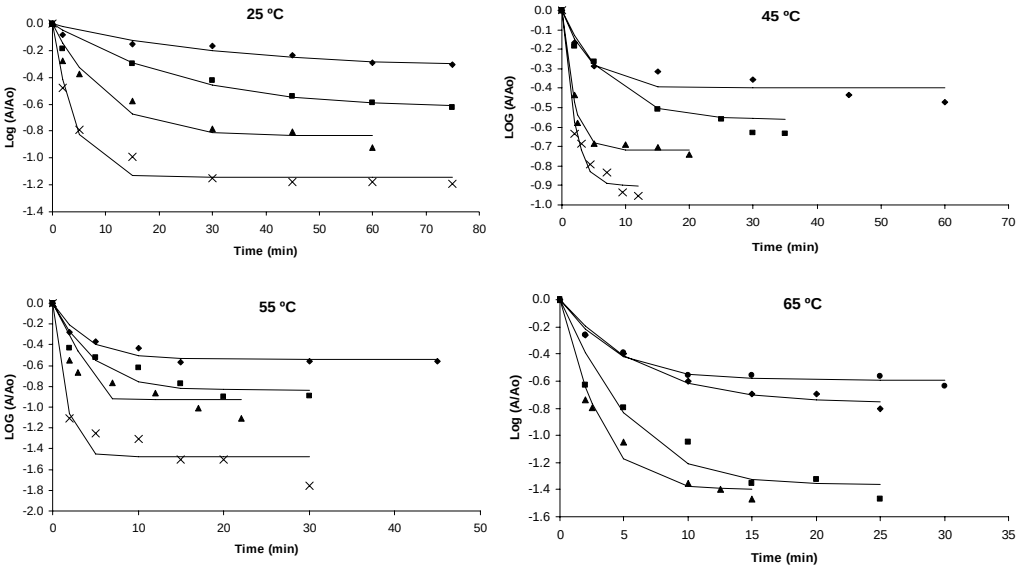

Figure 4.3.2: Combined thermal and high-pressure inactivation of PME in the orange juice-milk beverage fitted by a biphasic model.

The pressure inactivation of orange PME in different matrices in the range of 350-800 MPa is presented in Figure 4.3.4. No pressure inactivation was observed below 400 MPa in the orange juice-milk based beverage and orange juice environment and below 550 MPa for the purified enzyme in a buffer system. Truong, (2002) also found a pressure threshold of 400 MPa below which no PME inactivation was observed in a single-strength orange juice. A remaining activity of 17% and 6% was observed respectively after a treatment at 750 MPa in the orange juice-milk based beverage system and a treatment of 700 MPa in orange juice. A remaining activity of 20% was observed after a treatment at 800 MPa for the purified enzyme. Opposite to thermal treatment, the high pressure inactivation of purified PME was less pronounced than in all other matrices tested meaning that the purified enzyme was more pressure-stable. As observed in the thermal treatment, the level of purification of the enzyme could affect the stability of the enzyme, increasing in this case its pressure resistance. On the other hand, the pH of the buffer was close to 7 so the enzyme pressure resistance is increased at neutral

pH values showing that the inactivation mechanism differs between both technologies.

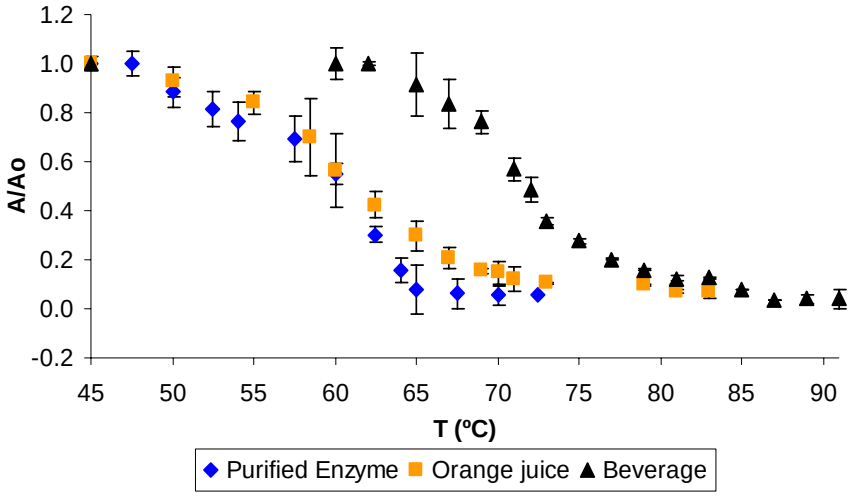

Figure 4.3.3: Isothermal inactivation of purified PME, PME in the orange juice-milk based beverage and PME in orange juice. The standard deviation was expressed by error bars.

The higher stability of PME in the orange juice-milk based beverage can be explained by the fact that factors, such as pH, ºBrix, Ca^{2+}ion content, orange variety, time of harvesting and purification level of the enzyme affect the sensitivity of the orange PME to the different preservation treatments. There is also a theory that when the composition of the orange juice-milk based beverage is more complex it protects PME against thermal treatment. This could be due to the milk environment, in which proteins can bind with the enzyme, thus becoming more resistant (Kumura et al., 1993). The presence of sucrose can also protect the enzyme against thermal or pressure treatment. Seyderhelm et al., (2004) and Van den Broeck et al., (1999 a) found that at higher sucrose content the pressure inactivation of pectinarase was lower arguing that sucrose decreased the water

activity and since denaturation of enzymes requires water it protects the PME against the thermal treatment.

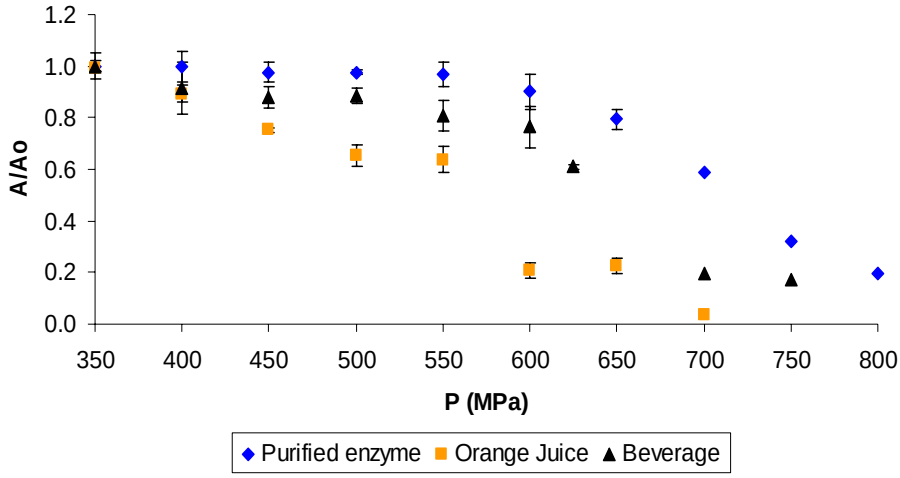

Figure 4.3.4: Pressure inactivation of purified PME, PME in an orange juice-milk based beverage and PME in orange juice. The standard deviation was expressed by error bars.

A biphasic model was fitted to the thermal and combined thermal and high pressure inactivation data of PME in juice and milk-based beverage assuming that two fractions with different stabilities occur. After thermal and combined thermal and high pressure treatments between 6-8% of stable fraction was found. Severe conditions based on combining high temperatures and high pressures were necessary to completely inactivate the enzyme. Similar treatments performed by other authors in simpler media yielded higher PME inactivation rate, which implies that the complex composition of the matrix play an important role in enzyme stability. Different PME thermal and high pressure stabilities were found depending on the media studied. PME was found to be more thermostable in the orange juice-milk beverage media but more pressure-stable in the purified enzyme in a buffer system, meaning that the composition, pH or enzyme purification level affect the PME stability against the different technologies studied. The inactivation mechanism of PME against thermal and high pressure differs and more studies are needed to understand it.

Pulsed electric fields inactivation of *Lactobacillus plantarum* in an orange juice–milk based beverage: Effect of process parameters

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4.4.1 ABSTRACT

This work studies the effect of electric field strength, treatment time, process temperature, and pulse width on the inactivation of *Lactobacillus plantarum* inoculated in an orange juice-milk based beverage. For any given quantity of energy applied, the highest degree of inactivation was achieved with high field intensities and short treatment times. The inactivation curve had different slopes, one up to application of 200-285 J/mL, a second stage up to application of 813-891 J/mL in which the inactivation did not increase significantly, and a third stage up to application of 1069-1170 J/mL. When the process temperature was raised to 55ºC the inactivation increased by 0.5 log, achieving an energy saving of up to 60%. No increase in inactivation was achieved when the pulse width was increased from 2.5 to 4 μs. The inactivation achieved with *L. plantarum* in this beverage is less than that reported by other authors in foods with a simpler composition.

Keywords: PEF; Inactivation; Beverage; *Lactobacillus plantarum*; Orange juice; Milk

4.4.2 INTRODUCTION

Mixed fruit juices and milk beverages fortified with vitamins, minerals and fiber are among the most widely consumed functional foods (Pszczola, 2005) and consumption of these products has increased by 30% in Europe in the last 10 years, but only 10% belong to the short shelf-life group that needs a chilled chain for their storage and distribution (MAPA, 2003). Nevertheless, it is thought that the trend will increase in the next few years, partly owing to the application of emerging nonthermal technologies such as High Hydrostatic Pressure (HHP) and Pulsed Electric Fields (PEF), which find an interesting field for application in these kinds of products because they allow cold pasteurization while at the same time preserving their nutritional components. Studies on the possibility of applying PEF in fruit juices have proliferated in recent years, concentrating mainly on apple and orange juice and other substrates of a different nature such as soymilk (Li and Zhang, 2004), apple cider (Evrendilek et al., 2000), chocolate milk (Evrendilek et al., 2001), mixed juices (Rodrigo et al., 2001; Rodrigo et al., 2003 a), model beer (Ulmer et al., 2002), horchata (Spanish beverage) (Selma et al., 2003) and beer with gas (Evrendilek et al., 2004). However, there are no studies on the application of PEF in the inactivation of microorganisms in juice-milk mixtures such as the beverage under study.

In studies on inactivation of microorganisms by PEF, some works were focused on analyzing the influence of process variables on the inactivation, using buffers or foods with a simple composition as a suspension medium such as skim milk (Martín et al., 1997), phosphate buffer (Wouters et al., 1999), NaCl and peptone solution (Aronsson et al., 2001; Aronsson and Rönner, 2001), sodium phosphate buffer (Abram et al., 2003), citrate phosphate McIlvaine buffer (Álvarez et al., 2003) and apple juice from concentrate (Heinz et al., 2003). Some of these studies (Wouters et al., 1999; Abram et al., 2003; Álvarez et al., 2003; Heinz et al., 2003) are accompanied by a calculation of the energy applied, which makes it possible to assess their possible applicability in the industry by comparison with the energy cost of traditional heat treatments, and it can also be useful for

comparing PEF inactivation treatments of microorganisms in various conditions. To establish the optimum PEF treatment conditions in a new product requires not only studies on the influence of the various process parameters on the death of the microorganism and quality factors, but also the choice of a reference microorganism on which to carry out the studies.

Lactobacillus plantarum is capable of growing over a wide pH range and spoiling minimally processed or fresh fruit juices owing to its aciduric nature, producing a ‘‘butter’’ off-flavor and swelling of packages. Parish and Higgins (1988) isolated four strains of *L. plantarum* in a commercial fresh orange juice and then, in a later study, Parish et al., (1990) observed that the population did not decrease in a reconstituted orange juice of pH 3.9 after 14 days at 4 ºC. Some authors have studied the inactivation of *L. plantarum* by PEF in different substrates, orange-carrot juice (Rodrigo et al., 2001), HEPES buffer (Wouters et al., 2001), model beer (Ulmer et al., 2002), sodium phosphate buffer (Abram et al., 2003), buffered peptone water (Rodrigo et al., 2003 b), buffered peptone water and orange-carrot juice (Selma et al., 2004). The growth of the microorganism in this beverage was studied in a previous work demonstrating its capability to spoilage these kinds of products (Sampedro et al., 2006).

The aim of this work was to study the influence of the process variables (electric field intensity (E), treatment time (t), pulse width (W), and temperature (T)) on the inactivation by PEF of *L. plantarum* inoculated in an orange juice–milk based beverage, and to make an analysis of the energy applied in each case to determine the efficiency of the process from the viewpoint of the possible use of PEF in the industry.

4.4.3 MATERIALS AND METHODS

4.4.3.1 Food sample

The beverage contained the following ingredients: pasteurized orange juice from squeezed oranges (García-Carrión, Spain) kept frozen until used (the pulp

was removed), commercial UHT skimmed milk, high methoxyl citrus pectin such as stabilizer (Unipectine AYD 250, Cargill, USA), commercial citric acid, sugar, and distilled water. The beverage preparation and physicochemical characteristics were described in Chapter 1. The beverage was prepared just before use.

4.4.3.2 *Lactobacillus plantarum*

The culture of *L. plantarum* CECT 220 was provided by the Spanish Type Culture Collection. Cells were obtained according to Sampedro et al., (2006). For that, the freeze-dried microorganism was dissolved in 15 mL of MRS broth (Scharlau Chemie S.A., Barcelona, Spain) during 30 min at 37ºC. Then, the content was placed in 500 mL of MRS broth with continuous agitation at 37ºC. The exponential phase was reached after 12-16 h and the stationary growth stage after 16-20 h. 500 mL of the culture was centrifuged twice (Beckman J-25) at 3220g, 5 min and 4ºC and dissolved in 100 and 50 mL of MRS broth, respectively. The content was transferred to 2 mL vials with 1 mL of suspension and 1 mL of glycerol 20% diluted with MRS broth and kept at 80ºC. No cell viability decrease was observed during frozen storage (data not shown). Just before the PEF treatment (5 min) the beverage was inoculated with the thawed microorganism, reaching a final concentration of 3-4 10^7 CFU/mL.

4.4.3.3 PEF treatment

An OSU-4D bench-scale continuous unit (Ohio State University, USA) was used to treat the food sample. Six co-field chambers with a diameter of 0.23 cm and a gap distance of 0.293 cm between electrodes were connected in series. One cooling coil was connected before and after each pair of chambers and submerged in a circulating bath (Polystat, Cole Parmer, USA) to maintain the selected initial temperature (35 or 55ºC, final maximum temperature 55 and 72ºC). The temperature was recorded by thermocouples (T type) at the entrance of the first treatment chamber (initial temperature) and at the exit of the last treatment chamber (final temperature). The values were recorded with a data logger (Control

Company, USA). Pulse waveform, voltage, and current in the treatment chambers were monitored with a digital oscilloscope (Tektronix TDS 210, Tektronix, USA). The flow rate was set at 60 mL/min with a peristaltic pump (XX 80002 30, 6–600 rpm, Millipore, USA). A bipolar square-wave of 2.5 μs was selected. Treatment time ranged from 0 to 180 μs, and the electric field was set at 35 and 40 kV/cm. A negative control was carried out immediately after the microorganism was added to the beverage (700 mL) and after the treatment to ensure no inactivation took place because of the acid environment. One sample was collected after each treatment time. They were serially diluted in sterile 0.1% peptone water, plated in MRS agar, and incubated for 48 h at 37ºC. The experiments were performed to obtain three valid repetitions.

The influence of the temperature was studied by applying electric field strengths of 35 and 40 kV/cm treatment times between 0 and 180 μs, pulse width of 2.5 μs (110-356 Hz) and initial temperature of 55ºC. The influence of the pulse width was also studied by applying 4 μs width pulses (68-123 Hz), electric field strength of 35 and 40 kV/cm and treatment times ranging from 0 to 180 μs combined with temperature (35 and 55ºC). The energy input (Q) was calculated by the following equation used by Abram et al., (2003), Min et al., (2003) and Pérez and Pilosof, (2004):

$$Q\ (J\ L^{-1}) = E^2 \sigma t \qquad \textbf{Equation 4.4.1}$$

where E is the electric field strength (kV/cm), σ is the electrical conductivity of the product (S/m) calculated for each process temperature (Figure 8.2.1) and t is the treatment time (s).

4.4.3.4 Statistical analysis

A hierarchical experimental design was used to study the influence of the different parameters: two electric field strengths, seven treatment times, two pulse

widths, and two temperatures. The statistical analysis was performed using the SPSS 12.0 software, applying a univariant ANOVA test with a significance level of 95.0%.

4.4.4 RESULTS

4.4.4.1 Effect of electric field strength

Figure 4.4.1 showed the survival curves against treatment time (Figure 4.4.1a) and energy applied (Figure 4.4.1b) for *L. plantarum* with the application of E=35 and 40 kV/cm. The curves had different slopes. The cell population of the microorganism decreased rapidly with short treatments (40 μs or 200-285 J/mL) with an inactivation of 1.5 log but there was a more resistant fraction remaining (40-110 μs with 40 kV/cm or 130 μs with 35 kV/cm with the application of 813-891 J/mL). This residue was inactivated slowly when the treatment times were increased, without producing significant differences in the survival fraction (Table 8.2.7). A final application of 110-130 μs (40 kV/cm) or 180 μs (35 kV/cm) with 1069-1170 J/mL increased the inactivation to 1.9-2.1 log. A maximum inactivation of 2.12 log was achieved using E = 40 kV/cm, t = 130 μs, and T<55ºC.

Comparison of the two field intensities studied showed that greater inactivation was achieved by applying 40 kV/cm and 130 μs (2.12 log), using 1069 J/mL of energy, than by 35 kV/cm, 180 μs and 1170 J/mL (1.9 log). When short treatment times (40-60 μs) were applied, there were non-significant differences between the 35 and 40 kV/cm fields (Table 8.2.8). When treatment time was increased (80 and 130 μs), the differences were higher and significant differences among the electric field strengths were found (Table 8.2.8 and Table 4.4.1). Taking these differences into account, during the first phase of inactivation treatment time was the variable with the greatest influence, and as longer treatment times were applied the inactivation was increasingly due to the electric field intensity.

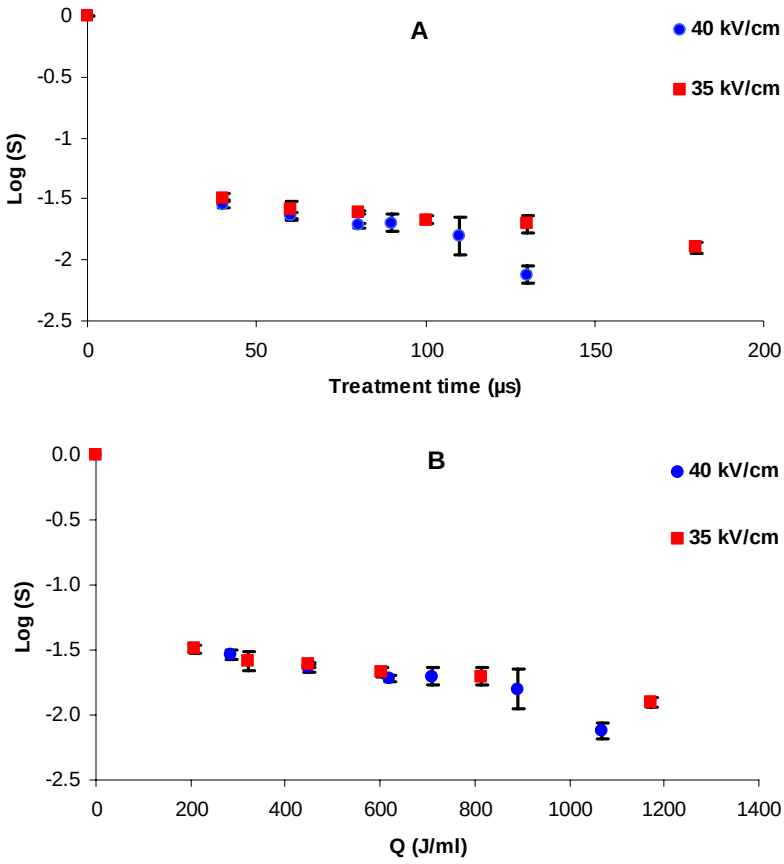


Figure 4.4.1: Inactivation curves as a function of the treatment time (A) and energy applied (B) for *L. plantarum* with T=35 ºC and pulse width of 2.5 µs. The standard deviation was expressed by error bars.

4.4.4.2 Effect of temperature

The influence of process temperature was studied by setting the bath temperature at 35 or 55ºC. The results showed that when temperature increased there was an increase on inactivation for all the treatment times. The analysis of variance showed significant differences in the logarithm of the survivor fraction between the two values of temperature at 35 kV/cm (Figure 4.4.2a) and 40 kV/cm (Figure 4.4.2b) (Table 8.2.9). A maximum inactivation of 2.46 log was achieved with E=40 kV/cm, 130 µs, 2.5 µs, and 1358 J/mL, which was an increase of almost

0.5 log with respect to the temperature of 35ºC. The inactivation at 55ºC followed the same trend as at 35ºC, with a rapid inactivation being produced with 40 μs and not increasing significantly for intermediate times (Table 8.2.10).

Table 4.4.1: Effect of electric field strength on the inactivation of *L. plantarum* in the juice-milk beverage.

Treatment time (μs)	E (kV/cm)	S (CFU/mL)	Differences (%)
40	40	0.029[a]±0.002[b]	8.6
	35	0.032±0.002	
60	40	0.023±0.002	11.90
	35	0.026±0.005	
80	40	0.019±0.001	21.15[*]
	35	0.024±0.001	
130	40	0.008±0.001	52.87[*]
	35	0.016±0.003	

[a] The value is the average of three replicates

[b] Standard deviation

[*] Showing significant differences

From the point of view of energy, was observed that for a given quantity of energy applied (890 J/mL) the inactivation increased with temperature, attaining 1.8 log at 35ºC and 2.0 log at 55ºC at 40 kV/cm (Figure 4.4.2 c). When the temperature was increased to 55ºC the same degree of inactivation was achieved with a reduction in the energy consumed. To achieve 1.8 log reductions with 35 ºC it was necessary to apply 890 J/mL whereas with 55ºC only 352 J/mL was needed.

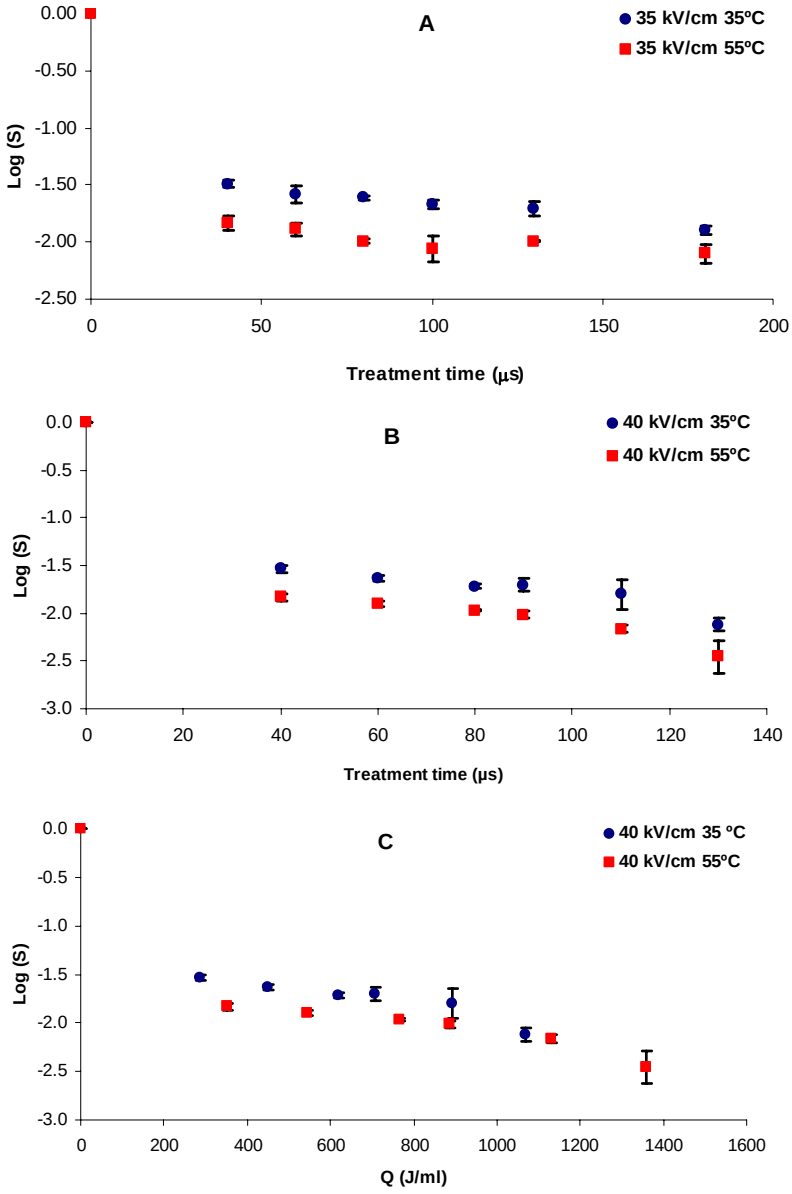
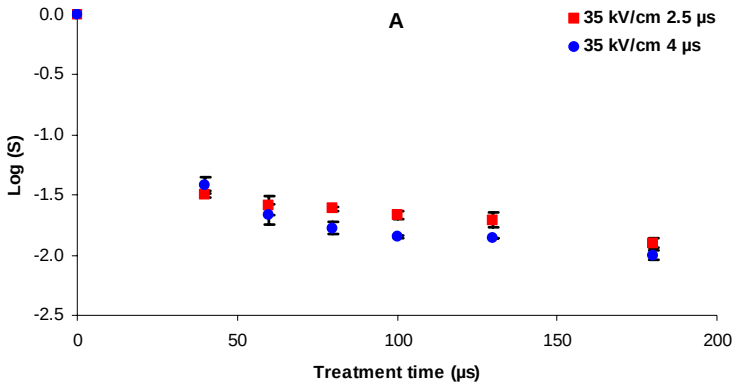


Figure 4.4.2: Effect of treatment temperature on the inactivation of *L. plantarum* as a function of treatment time with E=35 kV/cm (A) and E=40 kV/cm (B) and as a function of energy applied with E=40 kV/cm (C) and pulse width of 2.5 µs. The standard deviation was expressed by error bars.

4.4.4.3 Effect of pulse width

The influence of pulse width was studied by applying pulse widths of 2.5 and 4 µs. When 40 kV/cm was applied, varying the pulse width did not produce a significant increase in the inactivation (Table 8.2.11), and in fact a greater inactivation was achieved with 2.5 µs (Figure 4.4.3b). With 35 kV/cm there were also no significant differences when the pulse width was increased (Table 8.2.11), but a slightly greater inactivation was achieved with 4 µs (Figure 4.4.3a).

Analysis of the energy applied showed that for a given quantity of energy an increase in pulse width did not produce an increase in the degree of inactivation at 35 kV/cm (Figure 4.4.3c) and 40 kV/cm (Figure 4.4.3d). When the temperature was increased to 55ºC results were similar to those obtained for 35ºC. There were no significant differences on inactivation between using pulse widths of 2.5 or 4 µs with the same quantity of energy applied for 40 kV/cm (Table 8.2.12) (Figure 4.4.4a). Applying 35 kV/cm was also confirmed that there was a slightly greater inactivation with a pulse width of 4 µs but without significant differences (Table 8.2.12) (Figure 4.4.4b). With 4 µs there was also a greater inactivation when the process temperature was increased from 35 to 55ºC, which also implied a saving in the energy consumed to attain the same degree of inactivation (Figure 4.4.5 a and b).



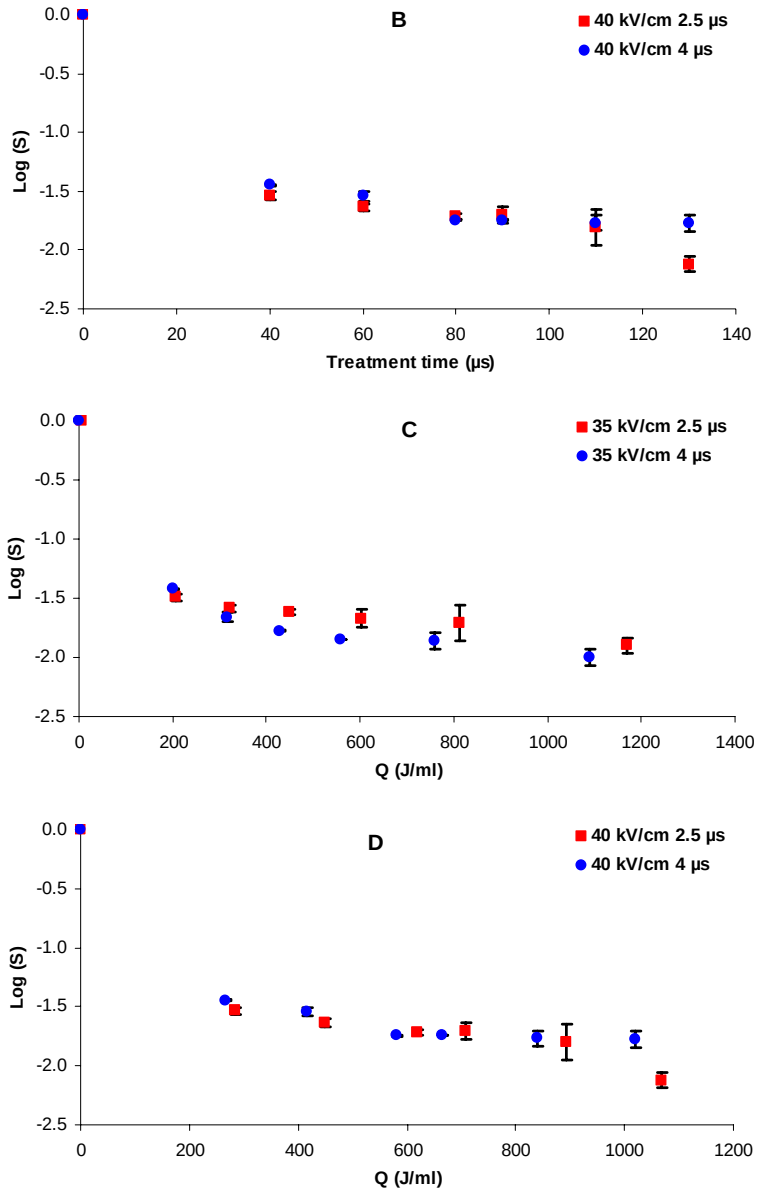


Figure 4.4.3: Influence of pulse width in the inactivation of *L. plantarum* at 35 ºC as a function of treatment time with E=35 kV/m (A) and E=40 kV/cm (B) and energy applied with E=35 kV/cm (C) and E=40 kV/cm (D). The standard deviation was expressed by error bars.

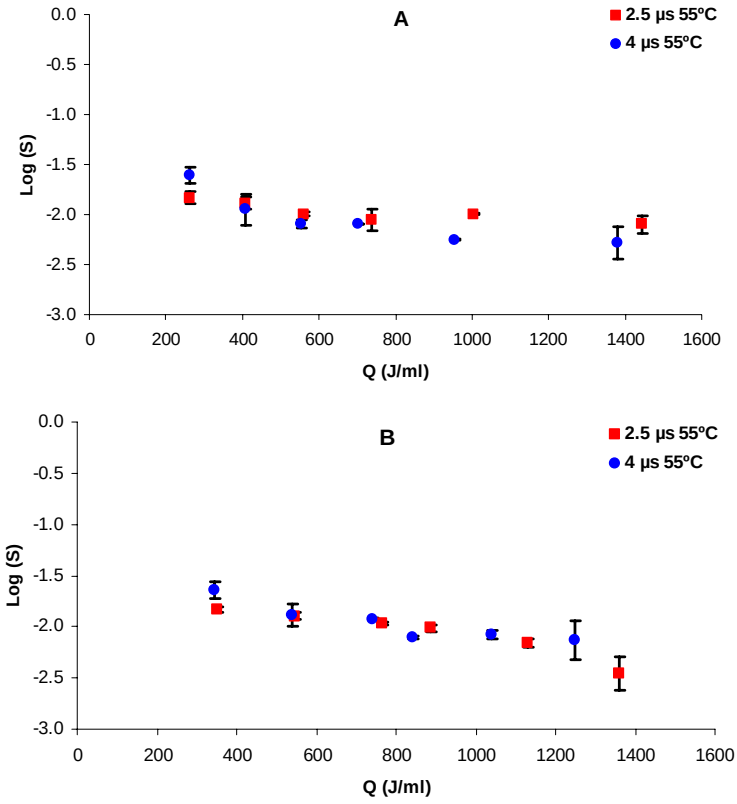
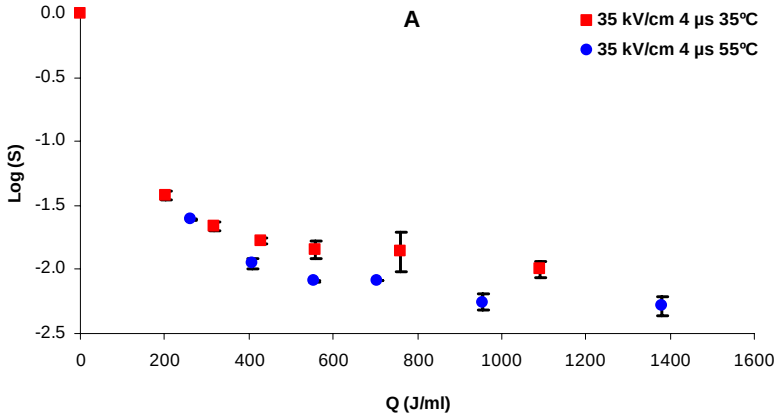


Figure 4.4.4: Influence of pulse width in the inactivation of *L. plantarum* at 55 ºC as a function of energy applied with E=35 kV/cm (A) and E=40 kV/cm (B). The standard deviation was expressed by error bars.



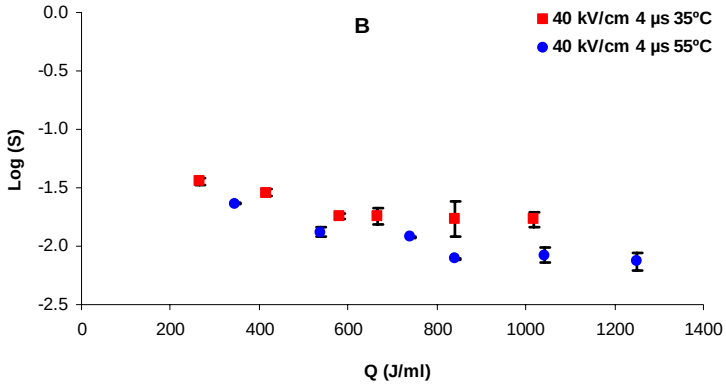


Figure 4.4.5: Effect of treatment temperature on the inactivation of *L. plantarum* as a function of energy applied with E=35 kV/cm (A) and E=40 kV/cm (B) and pulse width of 4 µs. The standard deviation was expressed by error bars.

4.4.5 DISCUSSION

Various factors influence the effectiveness with which the application of PEF inactivates microorganisms. In general, when the electric field or the treatment time increases there is an increase in the inactivation, but in this study there was a very rapid decrease in the number of microorganisms at the start of the treatment and as the treatment continued the microorganism became more pulse-resistant. The same conclusions were reached by Raso et al., (2000) and Aronsson et al., (2001), who observed that at any electric field strength investigated the inactivation was very fast in the first moments of the treatment and then the number of survivors decreased slowly. The reduction in the viability was caused mostly by the first pulses, and increasing the number of pulses did not have the same effect as the first ones.

If the effect of electric field strength is expressed in terms of energy, it would succeed in inactivating a substantial fraction of microorganisms with a small amount of energy at the start of the treatment and it would be necessary to go beyond a minimum level to increase the inactivation for a given electric field

intensity. Some authors showed that there were minimum field intensities or treatment times at which pore formation took place in the membrane of the microorganism, depending among other things on the geometry and size of the cell (Li and Zhang, 2004; Picart et al., 2002; Ulmer et al., 2002; Wouters et al., 1999). In the smallest cells it is usually necessary to apply higher field intensities to cause electroporation (Heinz et al., 2002). In our case, this was applicable to the quantity of energy, and therefore, once electroporation took place, it was necessary to go beyond a certain energy level to increase the inactivation.

When the variable temperature is taken into account, an increase in process temperature produced an increase in the degree of inactivation achieved. The increase in inactivation with temperature is attributed to a decrease in the electrical breakdown potential of the membrane (Reina et al., 1998), which increases the fluidity of the cell membrane, affecting its physical stability and decreasing the critical field intensity so that electroporation takes place (Aronsson et al., 2001). Some authors have studied the heat inactivation of *L. plantarum* in different substrates. Tait et al., (1991) studied the heat inactivation of *L. plantarum* in a buffer at pH 4 obtaining a D value of 1.81 min at 50ºC and at pH 7 obtaining a D value of 3.64 min at 55ºC. In a further study, De Angelis et al., (2004) obtained a D value of 32.9, 14.7 and 7.14 s at 60, 72 and 75ºC of *L. plantarum* in sterile milk. It could be deduced that the treatment time used in this study is not enough to produce heat inactivation of the microorganism. Moreover, increasing the initial treatment temperature achieved equal or greater levels of inactivation with the application of much small quantities of energy, which in our case meant a saving of up to 60%. Wouters et al., (1999) observed that when they applied 40 J/mL a greater inactivation was achieved with a process temperature of 40ºC than with 20 or 30ºC for *Listeria innocua* in sodium-phosphate buffer. In order to obtain a reduction of 5 log, Heinz et al., (2003) observed that with an initial temperature of 45ºC it was necessary to apply 100 kJ/kg, whereas if the temperature was increased to 65ºC less than 10 kJ/kg was needed.

It could be also verified that an increase in pulse width did not produce an increase in inactivation independently from temperature and electric field strength. Raso et al., (2000) and Mañas et al., (2001) also found that for electric fields of 22 and 33 kV/cm and same treatment time the inactivation was independent of the pulse width when it ranged between 2-15 μs and 1.2-1.9 μs, respectively. However, some authors have demonstrated that at a higher pulse width the inactivation is also higher, but by applying a higher treatment time and in consequence more energy (Martín et al., 1997; Martín-Belloso et al., 1997). But in this work the treatment time and energy were the same for the different pulse widths. Nevertheless, some authors concluded that applying the same quantity of energy, the inactivation achieved was greater at higher pulse width. Aronsson et al., (2001) increased the pulse duration from 2 to 4 μs for the same treatment time, they found that the inactivation was greater with 4 μs but the differences were greater at high electric field intensities and Abram et al., (2003) also found that when they increased the pulse width over 1 μs, with E=25 kV/cm and the same energy input, the inactivation increased with the pulse width. The effect of pulse width is not clear and seems to vary depending on the conditions applied such as electric field.

According to this, in our study it could be seen that the field intensity was related with the effectiveness of the pulses, because at smaller intensities there was greater inactivation with the larger pulse width. Wouters et al., (1999) came to the same conclusion, observing that varying the pulsed width (2, 3 and 3.9 μs) the inactivation achieved was greater applying the largest pulse width at an intensity of 28 kV/cm but at 36 kV/cm there were not significant differences in the inactivation when the pulse width was varied. Further experiments are needed to clarify the effect of pulse width on the inactivation of microorganisms by PEF.

In connection with what was stated earlier, *L. plantarum* is not a particularly small cell and therefore, a priori, it should not be a particularly pulse-resistant microorganism. In this respect, other authors have achieved greater levels of inactivation for other strains of the same microorganism in simpler substrates.

Reductions of 3.6 log was reached by Wouters et al., (2001) after E=25 kV/cm and 120 J/mL in a phosphate buffer (pH 4.5 and 0.15 S/m), and Ulmer et al., (2002) reached 4 log after E=34.8 kV/cm and 50 kJ/kg in a model beer (pH 3.6 and electrical conductivity of 0.120-0.178 S/m). Abram et al., (2003) reached 3.66 log reductions after E=33.4 kV/cm, t = 90 μs, pulse width of 5 μs, and energy applied of 100 kJ/L in a Na phosphate buffer (pH 4.5 and 0.1 S/m).

If the treatment intensity expressed as quantity of energy applied is compared, in this work was applied 100 kJ/L and achieved 2.1 log or 135 kJ/L and achieved 2.5 log. The authors cited earlier achieved greater levels of inactivation by applying energy quantities 10 times smaller in other substrates and with different PEF equipment. One of the causes could be the electric conductivity, which in many cases was considerably less than that of our beverage (0.286 S/m) and could affect the effectiveness of the treatment. Nevertheless, in similar conditions with the application of exponential wave pulses, Rodrigo et al., (2001) achieved 2.5 log after E=35.8 kV/cm and 46.3 μs in orange-carrot juice with an electric conductivity of 0.455 S/m. The composition also seemed to affect the effectiveness of the treatment. Some works show that fat and other components could protect the microorganism (Martín et al., 1997; Min et al., 2002) although there are other authors who did not observe this phenomenon (Dutreux et al., 2000; Mañas et al., 2001; Reina et al., 1998).

In a study to optimize process conditions, the treatments and the design of the equipment for products of this kind should be aimed at the application of high field intensities and short treatment times, which, on the lines of heat treatment based on HTST, could be called high electric field short time (HFEST). It would be necessary to preheat the sample to 30-50ºC and apply pulses of short duration. This combination of factors would involve a smaller energy cost to obtain a given degree of inactivation, so that in future it could be possible to equate pulses with heat treatment and perform an industrial scale-up. Moreover, optimization of these factors could increase the inactivation and thus obtain degrees of inactivation close to what is recommended by the authorities concerned with food safety.

Nevertheless, there is a need for systematic studies on the influence of pulses in foods with a more complex composition to compare their effectiveness and understand the mechanisms by which they act.

Effect of temperature and substrate on PEF inactivation of *Lactobacillus plantarum* in an orange juice–milk beverage

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European Food Research and Technology (2006), 223: 30–34.

4.5.1 ABSTRACT

The inactivation kinetics of *Lactobacillus plantarum* in an orange juice-milk beverage treated by Pulsed Electric Fields (PEF) were studied. Experimental data were fitted to Bigelow and Hülsheger kinetic models and Weibull frequency distribution function. Results indicate that both Hülsheger model and Weibull function fit well the experimental data being Accuracy factor values (*Af*) closer to 1 and Mean Square Error (*MSE*) closer to 0. The $\overline{tcw}$ parameter can be considered as a kinetic indicator as it expresses the microorganism's resistance to treatment by electric pulses. An increase in temperature favored the inactivation of *L. plantarum* by PEF as reflected by a decreased in $\overline{tcw}$ value. Under the same conditions to those studied by other authors we reached less inactivation of *L. plantarum* in the beverage used in this study than in substrates with a simpler composition.

Keywords: PEF; Orange juice; Milk ; *Lactobacillus plantarum ;* Inactivation ; Modelling

4.5.2 INTRODUCTION

The consumption of fruit juices and drinks based on juice and milk subjected to a low pasteurization and stored under refrigeration has experienced a spectacular increase in the last few years, particularly in developed countries. This is in response to a growing demand for minimally processed foods that are perceived by consumers as healthier. The potential attraction of these products is that they preserve the original characteristics of fresh food whilst offering innovative new flavor mixtures. Over the last few years, nonthermal technologies such as the Pulsed Electric Fields (PEF) have been studied on both, laboratory and industrial scale and can be used in the preservation of food as fruit juices, milk and liquid whole egg (Sampedro et al., 2005; Rodrigo et al., 2005). They have the potential to inactivate microorganisms while maintaining the products original characteristics. However, studies showing the capability of PEF as an alternative to heat in the inactivation of microorganism in foods with a complex composition are scarce (Vega-Mercado et al., 1996; Sharma et al., 1998; Evrendilek et al., 2000; Evrendilek et al., 2001; Ulmer et al., 2002; Rodrigo et al., 2001; Rodrigo et al., 2003 a; Selma et al., 2003; Selma et al., 2004; Li and Zhang, 2004; Evrendilek et al., 2004).

Lactobacillus plantarum is a spoilage microorganism frequently found in juice based products. Between 2.7 and 8.9% of *L. plantarum* on the orange's surface enters the juice during extraction and that this level remained constant overtime (Pao and Davis, 2001). When the juice is mixed with other substrates to produce a more sophisticated or nutritional foodstuff, contamination is transferred and there is the likelihood that *L. plantarum* grows and spoils the product. The inactivation of this microorganism by PEF has been studied in different substrates and foodstuffs (Rodrigo et al., 2001; Wouters et al., 2001; Ulmer et al., 2002; Rodrigo et al., 2003 a; Abram et al., 2003; Selma et al., 2003). However, the nature of the inactivation of the microorganism by pulse treatment has not been studied in foods made by a mixture of fruit juice and milk where different components and their interactions can affect the death of the microorganism.

Different authors have tried to model the microorganism inactivation curves through predictive mathematical models that fit approximately to the curve shape which relates the survivor fraction with treatment time at different field intensities. The majority of inactivation curves have been analyzed by predictive models which presume a linear behavior (Bigelow, 1921; Hülsheger et al., 1981) and several authors have applied it to PEF technology (Pothakamury et al., 1995; Martín-Belloso et al., 1997 a and b; Sensoy et al., 1997; Raso et al., 1998; Wouters et al., 1999). As in many emergent preserving technologies, the relationship between the log of the survival fraction and the treatment time does not follow in many cases a lineal behavior, rendering curves with tails or shoulders that make difficult the application of a lineal model. Trying to give an explanation for such behavior, several authors have assumed that each cell has a different resistance to pulse treatment and that the resulting inactivation curve is the sum of a series of lethal events expressed in the form of distribution. One frequency distribution model widely applied to interpret biological phenomena was developed (Weibull, 1951). This distribution has been applied by some authors to describe inactivation curves after PEF treatment (Rodrigo et al., 2001; Rodrigo et al., 2003 a and b, Álvarez et al., 2003 a and b; Lebovka and Vorobiev, 2004). The aim of this work is to characterize the inactivation by PEF of *L. plantarum* in an orange juice-milk beverage as an influence of temperature throughout the treatment and substrate as well as select the best model to describe the survival curves.

4.5.3 MATERIAL AND METHODS

4.5.3.1 Food sample

The beverage contained the following ingredients: pasteurized orange juice from squeezed oranges (García-Carrión, Spain) kept frozen until use (with the pulp removed) (500 mL/L), commercial UHT skimmed milk (200 mL/L), high methoxyl citrus pectin (3 g/L) (Unipectine AYD 250, Cargill, USA), commercial citric acid (1 g/L), sugar (75 g/L) and distilled water (300 mL/L). The beverage

preparation and physicochemical characteristics were described in Chapter 1. The beverage was prepared just before use.

4.5.3.2 *Lactobacillus plantarum*

The culture of *L. plantarum* 220 was provided by the Spanish type culture collection. Cells were obtained according to Sampedro et al., (2006). For that, the frozen microorganism was placed in 2 mL vials with MRS broth (Scharlab Chemie, Spain) and 20% sterile glycerol with an initial concentration of 5×10^9 CFU/mL in exponential growth stage. A vial was diluted and inoculated in 500 mL of MRS broth to achieve an initial concentration of 10^3-10^4 CFU/mL in continuous agitation at 37ºC. The exponential phase was reached between 12 and 16 h and the stationary growth stage between 16 and 20 h. The microorganism was kept for 13 h to reach the early exponential growth stage. A 500 ml of culture was centrifuged twice (Beckman J-25) at 3220 g, 5 min and 4ºC and dissolved in 100 and 50 mL of MRS broth, respectively. The content was transferred into 2 mL vials with 1 mL of suspension and 1 mL of glycerol 20% diluted with MRS broth and kept at -80ºC. Before the PEF treatment, the beverage was inoculated with the defrozen microorganism reaching a final concentration of $3\text{-}4\times10^7$ CFU/mL.

4.5.3.3 PEF treatment

An OSU-4D bench-scale continuous processing unit was used to treat the food sample. Six co-field chambers with a diameter of 0.23 cm and a gap distance of 0.293 cm between electrodes were connected in series. One cooling coil was connected before and after each pair of chambers and submerged in a circulating bath (Polystat, Cole Parmer, USA) to maintain the selected initial temperature (35 and 55ºC) (55 and 72ºC maximum temperature of the treatment). The temperature was recorded by thermocouples (T type) at the entrance of the first treatment chamber (initial temperature) and at the exit of the last treatment chamber (final temperature). The values were recorded with a data logger (Control Company, USA). Pulse waveform, voltage and current in the treatment chambers were

monitored with a digital oscilloscope (Tektronix TDS 210, Tektronix, USA). The flow rate was set at 60 mL/min with a peristaltic pump (XX 80002 30, 6-600 r.p.m., Millipore, USA). A bipolar square-wave of 2.5 μs was selected. Treatment time ranged from 0 to 700 μs and the electric field was set at 15, 25, 35, and 40 kV/cm. Samples were collected after each treatment time. They were serially diluted in sterile 0.1% peptone water, plated in MRS agar and incubated for 48 h at 37ºC. The experiments were performed to obtain three valid repetitions. The influence of the temperature was studied applying electric field strengths of 35 and 40 kV/cm, treatment times between 40 and 180 μs and reaching temperatures below 72ºC.

4.5.3.4 Mathematical models

The data analysis of two valid replicates used the following models:

Bigelow, (1921):

$$Log\,(S) = -\frac{t}{D} \qquad \textbf{Equation 4.5.1}$$

where *S* is the survival fraction after the treatment, *t* is the treatment time expressed in μs and *D* is the kinetic parameter, showing the time required to achieve one decimal reduction, is also expressed in μs and it can be calculated as the negative inverse of the inactivation curve slope.

Hülsheger et al., (1981):

$$Ln\,(S) = -b_t * Ln\left(\frac{t}{t_c}\right) \qquad \textbf{Equation 4.5.2}$$

where *bt* is the regression coefficient, *t* is the treatment time in μs and *t*c is the critical time expressed in μs (the maximum treatment time in which the survival fraction equals 1)

Weibull, (1951):

$$Ln(S) = -\left(\frac{t}{a}\right)^{b}$$ **Equation 4.5.3**

where *a* and *b* are scale and shape parameters, respectively. The *b* value gives an idea of the form of the curve, if $b>1$ the curve is concave downwards (it forms shoulders), if $b<1$ the curve is concave upwards (it forms tails) and if $b=1$ the curve is a straight line and can be described by linear models.

The critical mean time ($\overline{tcw}$), can be defined by the maximum death per time in response to the PEF treatment and can be expressed by the following equation:

$$\overline{tcw} = a * \Gamma\left(1 + b^{-1}\right)$$ **Equation 4.5.4**

where *a* and *b* are the parameters of the Weibull equation and Γ is the gamma function.

4.5.3.5 Statistical analysis

The models were externally validated with a third valid replicate by using the Accuracy factor parameter (*Af*) (Ross, 1996). This factor gives and idea of the model fitting to the survivor curve of the microorganism and can be defined as follows:

$$Af = 10^{\frac{\sum\left|Log(fitted/observed)\right|}{n}}$$ **Equation 4.5.5**

where *n* is the number of observations and the fitted and observed values are referred to the survival fraction. The meaning of this statistic is the closer to 1 the *Af* values, the better the model fit the data.

Mean square error (*MSE*) was also calculated as follows:

$$MSE = \frac{\sum (fitted - observed)^2}{n - p} \qquad \textbf{Equation 4.5.6}$$

where n is the number of observations, the fitted and observed values are referred to the survival fraction and p is the number of parameters to be estimated by the model. The meaning of this statistic is the smaller the *MSE* values, the better the model fit the data.

4.5.4 RESULTS AND DISCUSSION

The growth of *L. plantarum* in the juice-milk beverage has been studied in this work and results showed an increase of 3 log after 20 h (Figure 4.5.1). The growth ability of *L. plantarum* in acid products has been demonstrated before. McDonald et al., (1990) studied the microorganism growth in a buffer with a pH of around 3 Results of our study indicate that *L. plantarum* could be a spoilage problem for the juice processing industry if it is not adequately controlled. Even more, the problem could be more acute in this kind of beverages because milk and sugar can supply additional nutrients to the microorganism.

The survivor curves show the relationship between the survivor's fraction of *L. plantarum* in the beverage with treatment time at different electric field intensities and are characterized by a rapid decrease in the number of microorganisms followed by a tail. Different mathematical models were fitted to the data in order to select one which best describe this concave upwards behavior.

Table 4.5.1 shows the calculated kinetic parameters and the *Af* and *MSE* values of different models for each survivor curve. Results indicate that both Hülsheger model and Weibull function fit well the experimental data being *Af* values closer to 1 and *MSE* closer to 0 but only Bigelow (*D* value) and Weibull ($\overline{tcw}$) equations have parameters that decrease significantly as the field intensity increases (Table 8.2.13 and 8.2.14). Consequently they can be considered as behavior indexes for the death of *L. plantarum* by PEF in the beverage.

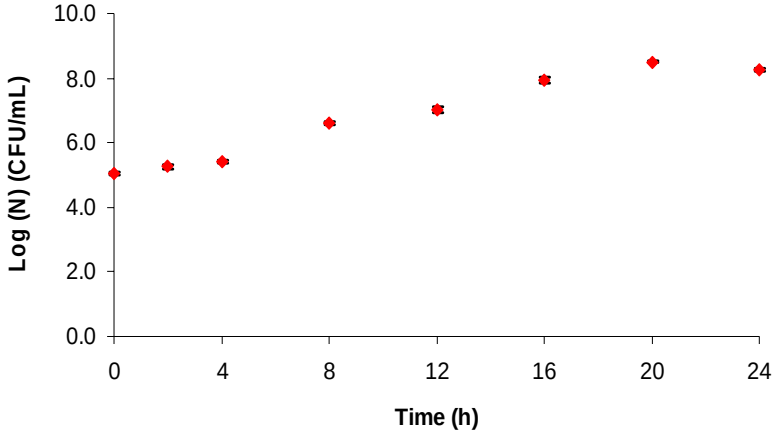

Figure 4.5.1: Growth curve of *L. plantarum* in the orange juice-milk beverage at 37ºC.

Applying the critical time parameter (*t*c) or the scale parameter (*a*) of the Hülsheger and Weibull models, respectively, are therefore unable to provide a logical interpretation of the behavior of the microorganism as they are not representative of the treatment intensity. *Af* values ranged from 1.4 to 2.0 and from 1.0 to 1.1 for the Bigelow and Weibull models, respectively. Accuracy factors closer to 1 indicate better predictions. Considering the *MSE* values, in general values are closer to 0 in the case of Weibull as well as in the Bigelow model. Consequently, in this study the Weibull model appears to be the more appropriate model to interpret the death of *L. plantarum* in the juice–milk beverage. Figure 4.5.2 shows the fitted curves using the Weibull model. It appears that the model fits better the experimental data obtained at higher field intensities, probably because at those high field intensities tails are less apparent.

Temperature can play an important role in pulsed electric field treatments. Figure 4.5.3 shows the evolution of the survivor's fraction with treatment time and temperature at 40 kV/cm. Increasing the initial process temperature (from 35 to 55ºC) increased the actual inactivation. The lower pulse-resistance was reflected by

a significant decrease in the $\overline{tcw}$ value, from 7.0 μs to 5.1 μs at 35 and 55ºC respectively (Table 8.2.15).

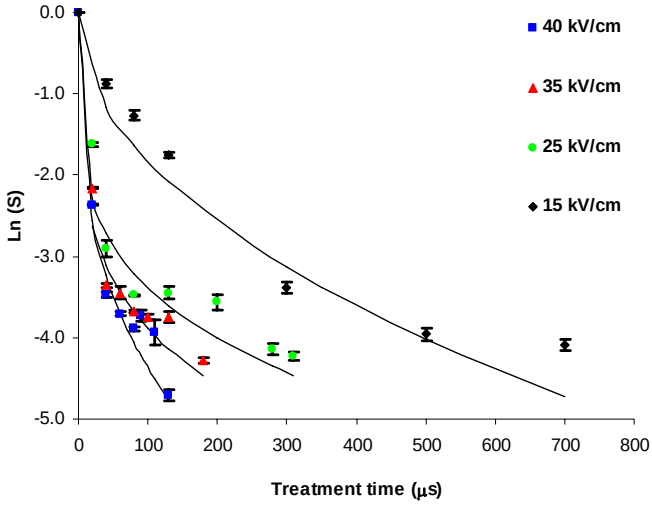

Figure 4.5.2: Survival curves of *L. plantarum* in juice-milk based beverage at different electric field strengths at initial *T* of 35◦C adjusted to the Weibull model. The standard deviation was expressed by error bars.

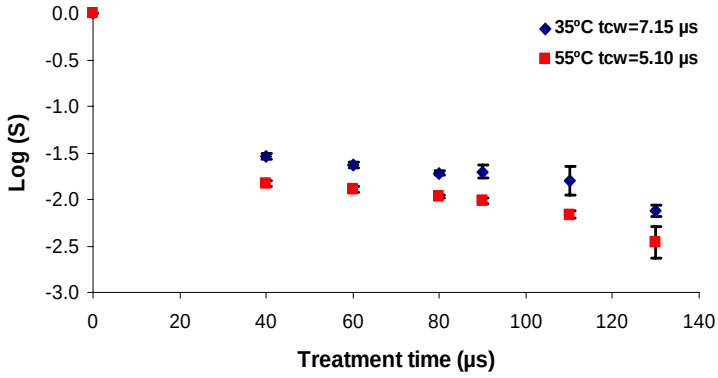

Figure 4.5.3: Effect of T in the survival fraction of *L. plantarum* and $\overline{tcw}$ of the Weibull model in the beverage with E=40 kV/cm. The standard deviation was expressed by error bars.

Other authors have observed a higher inactivation level than ours combining pulses and temperature in different microorganisms and substrates (Pothakamury et al., 1995; Aronsson and Rönner, 2001; Heinz et al., 2003). For this reason, it is necessary to study in greater depth how to optimize pulse treatment alone or combined with heat to achieve improved inactivation in complex foods.

In the last few years, some authors have observed that by decreasing electric conductivity of food the effectiveness of pulses increases (Raso et al., 1998; Wouters et al., 1999; Rodrigo et al., 2003 b). Some explanations have been proposed to this event. According to Sensoy et al., (1997) when the medium conductivity is decreased, it increases the conductivity difference between the microorganism's cytoplasm and the medium, causing an additional pressure in the microorganism membrane due to the osmotic forces and making it more sensitive to the pulse treatment.

Food composition (proteins, fat, etc.) can cause resistance to the treatment and protect the microorganism against pulses. Inactivation achieved in *E. coli* was greater in milk diluted two or three times compared to milk diluted only once, this may be because of lower protein content and greater resistance, enabling the absorption of free radicals and ions that actively participate in cell breaking (Martín-Belloso et al., 1997) and the same conclusions were obtained by (Min et al., 2002) with *E. cloacae* in chocolate liquor. Whereas Reina, et al., (1998), Dutreux et al., (2000) and Mañas et al., (2001) did not found differences in the inactivation of *L. monocytogenes*, *E. coli* and *L. innocua* and *E. coli* respectively in substrates with different concentration in fat.

To illustrate the effect of food composition, the results of this work were compared with the $\overline{tcw}$ values for the same strain of *L. plantarum* obtained in other products, orange-carrot juice (conductivity of 0.455 S/m) and peptone water (conductivity of 0.6 S/m) (Rodrigo et al., 2001; Rodrigo et al., 2003 a) in Table 4.5.2. For 25 kV/cm a $\overline{tcw}$ value of 32.0 μs in peptone water and 50.5 μs in the

juice-beverage was obtained. When applying 35 kV/cm a 6.7 μs $\overline{tcw}$ for the orange-carrot juice and 9.4 μs for the juice-milk beverage were achieved. This appears to indicate that the more complex the substrates, the more resistant the microorganism to pulses. Furthermore, because of distinct substrate composition the differences are more pronounced between the peptone water and the orange-milk beverage than between the orange-milk beverage and the orange-carrot juice. So, pulses are more effective in simpler foods despite of pulsed electric fields are more effective at low conductivity levels. Therefore, we can conclude despite that conductivity is the main cause affecting pulse effectiveness, there are other factors that can influence its effect. Among the reasons for the lower inactivation might be the incorporation of additives such as pectin, milk protein and fat which seem to protect the microorganisms from pulses.

To conclude, the Weibull model gives a good adjustment and the $\overline{tcw}$ parameter can describe the lethality of treatment it is therefore the model which best describes the inactivation curves of *L. plantarum* in the beverage. The combination of temperature and PEF produces an increase in inactivation as shown by a decreasing $\overline{tcw}$. Compared with other substrates studied in similar conditions by other authors, the complexity of the beverage produces a decrease in final inactivation.

Table 4.5.1: Kinetic parameters *Af* and *MSE* values of Bigelow, Hülsheger and Weibull models for *L. plantarum* survivor curves at initial treatment temperature of 35ºC.

E (kV/cm)	Bigelow			Hülsheger				Weibull				
	D (μs)	*Af*	*MSE*	tc (μs)	b_t	*Af*	*MSE*	$\overline{tcw}$ (μs)	a	b	*Af*	*MSE*
15	294.74[a] ± 6.79 [b]	1.63	0.16	22.44 ± 1.57	1.31 ± 0.01	1.14	0.16	61.11 ± 4.26	29.07 ± 3.66	0.49 ± 0.01	1.17	0.20
25	130.21 ± 3.87	2.01	0.48	0.18 ± 0.10	0.57 ± 0.03	1.09	0.22	50.55 ± 2.84	0.03 ± 0.03	0.16 ± 0.01	1.10	0.22
35	68.02 ± 0.65	1.65	0.41	1.38 ± 0.20	0.94 ± 0.06	1.09	0.14	9.40 ± 1.21	0.44 ± 0.10	0.26 ± 0.02	1.09	0.15
40	51.17 ± 1.29	1.41	0.24	2.55 ± 0.47	1.18 ± 0.09	1.05	0.07	7.15 ± 0.56	1.18 ± 0.24	0.33 ± 0.02	1.05	0.08

[a] :Value based on mean of three replicates

[b]: Standard deviation

Table 4.5.2: Kinetic parameters of Weibull model for *L. plantarum* in orange-carrot juice (Rodrigo et al., 2001), peptone water (Rodrigo et al., 2003) and the beverage studied.

	Peptone Water	Orange-Carrot Juice	Orange Juice-Milk Beverage
E (kV/cm)	Calculated $\overline{tcw}$ (μs)	$\overline{tcw}$ (μs)	$\overline{tcw}$ (μs)
25	32.06	-	50.55
35	-	6.77	9.40

Effect of pH and pectin concentration on the inactivation kinetics by PEF of *Salmonella typhimurium* in an orange juice-milk beverage

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4.6.1 ABSTRACT

The inactivation kinetics of *Salmonella typhimurium* in an orange juice-milk beverage treated by Pulsed Electric Fields (PEF) were studied. Experimental data were fitted to Bigelow and Hülsheger kinetic models and Weibull frequency distribution function. Results indicate that both Hülsheger model and Weibull function fit well the experimental data being Accuracy factor values (*Af*) closer to 1. The $\overline{tcw}$ parameter can be considered as a kinetic indicator as it expresses the microorganism's resistance to treatment by electric pulses. The effect of pH (3.4, 4 and 4.5) and pectin concentration (0.1, 0.3 and 0.6%) were also studied. A decrease in pH favored the inactivation of *S. typhimurium* by PEF as reflected by a decreased in $\overline{tcw}$ value. The z_{pH} parameter based on $\overline{tcw}$ indicated a value of 3.41 and 1.74 for 15 and 40 kV/cm respectively. Variation in the pectin concentration had no significant effect on the inactivation of *S. typhimurium* ($p>0.05$). The $z_{\%}$ was 8.11 and 4.85 for 15 and 40 kV/cm respectively.

Keywords: Orange juice; Milk; *Salmonella typhimurium*; Inactivation; PEF; Modelling; pH; Pectin concentration

4.6.2 INTRODUCTION

Consumption of juices and non-carbonated beverages based on mixes of citric juices and milk has increased by 30% in Europe in the last ten years, but only 10% belong to the short shelf-life group that needs a chilled chain for their storage and distribution (Artiach, 2005). Nevertheless, it is thought that the trend will increase in the next few years, partly owing to the application of emerging non-thermal technologies such as High Hydrostatic Pressure (HHP) and Pulsed Electric Fields (PEF), which find an interesting niche for application in those type of products because they allow cold pasteurization of the product preserving its nutritional components. Studies on the possibility of applying PEF in fruit juices have proliferated in recent years, concentrating mainly on apple and orange juice and other substrates of a different nature with complex composition such as soymilk (Li and Zhang, 2004), apple cider (Evrendilek et al., 2000), chocolate milk (Evrendilek et al., 2001), mixed juices (Rodrigo et al., 2001; Rodrigo et al., 2003 a), model beer (Ulmer et al., 2002), horchata (Spanish beverage) (Selma et al., 2003) and beer with gas (Evrendilek et al., 2004). However studies showing the capability of PEF as an alternative to heat in the inactivation of microorganisms in food with a complex composition such as the beverage under study are scarce.

Different outbreaks in orange juice products involving *Salmonella* genus have appeared in the last years around the world. Cook et al., (1998) and Khan et al., (2007) reported 2 *Salmonella* outbreaks in Florida and Arizona states (USA) in non-pasteurized orange juice. Five *Salmonella enterica* serotypes were found in the juice and packaging samples. Different samples recovered from the industry revealed cleaning installation deficiencies. These studies demonstrated the capability of *Salmonella* to contaminate and survive in non-pasteurized orange juice. Castillo et al., (2006) performed a study on the sanitary and cleaning conditions of different small street sellers in the city of Guadalajara (Mexico) during 2005. The study was based on samples from orange juice, orange surface and different utensils used for cleaning purposes. The authors observed a high *Salmonella* prevalence (14.3, 20.0 and 22.8%) in orange juice, orange surface and

cleaning utensils respectively. It were found 3 principal serotypes and among them was *S. typhimurium*. The contamination found in the orange juice samples reached in some cases 10^5 CFU/mL. The lack of properly hygienic conditions in utensils and equipments, cross-contamination or the non adequate storage conditions are the possible causes of the high *Salmonella* prevalence.

The acid-tolerance of different *Salmonella* strains has been previously studied by different authors. Parish et al., (1997) studied the growth of different *Salmonella* serovars in orange juice at different pH values (3.5-4.4) incubated at 0 and 4ºC. The juice was inoculated with 10^6 CFU/mL surviving 27 days at pH 3.5, 46 days at pH 3.8, 60 days at pH 4.1 and 73 days at pH 4.4. Later on, Pao et al., (1998) studied the growth of different pathogen microorganisms (among them *Salmonella*) on the orange surface. They obtained a survival period of 14 days at 24ºC and pH of 6-6.5 being inhibited at 4-8ºC. The authors concluded that the refrigeration of raw material could be a good practice to reduce *Salmonella* prevalence in the orange fruit. Yuk and Schneider (2006) showed that the acid pre-adaptation of several *Salmonella* strains in fruit juices produced higher resistance to the gastric juice. The resistance varied depending on the adaptation temperature, type of juice (organic acid) and serotype used. The study demonstrated that the acid pre-adaptation in diverse fruit juices could increase the probability of infection developing.

Parameters such as pH, initial contamination, sugars content or enzyme activity have a great variability during the orange harvest season. Due to this fact, is important to know the effect of the product physicochemical characterization on PEF microbial inactivation. Several authors have studied PEF inactivation of *Salmonella typhimurium* (Liang et al., 2002; Álvarez et al., 2003) and the effect of the pH in several *Salmonella* strains (Álvarez, et al., 2000) but there are no studies on a mixture of orange juice and milk. On the other hand, companies are continuously creating new food formulation and composition to make it more suggestive to the consumer. Food based on the mixture of different fruit juices and milk with the addition of an adequate stabilizer (pectin derivatives) is essential to

maintain the product stability. For this reason, is important to know the effect of pectin concentration on PEF *Salmonella* inactivation.

The aim of the present work is to study the influence of the pH and pectin concentration on the inactivation by PEF of *S. typhimurium* inoculated in an orange juice-milk based beverage.

4.6.3 MATERIALS AND METHODS

4.6.3.1 Food Sample

The beverage contained the following ingredients: 50% pasteurized orange juice from squeezed oranges provided by (García-Carrión, Spain) kept frozen until used (the pulp was removed), 20% commercial UHT skimmed milk, 0.3% high methoxyl citrus pectin such as stabilizer (Unipectine AYD 250, Cargill, USA), 7.5% sugar, and 30% distilled water. The beverage preparation and physicochemical characteristics were described in Chapter 1. The beverage was prepared just before use.

4.6.3.2 *Salmonella typhimurium*

The culture of *Salmonella typhimurium* CECT 443 was provided by the Spanish Type Culture Collection. Cells were obtained according to Sampedro et al., (2006). For that, the frozen microorganism was placed in 2-mL vials with Tryptic Soy Broth (TSB) (Scharlab CHEIME, Spain) and 20% sterile glycerol with an initial concentration of $6x10^8$ CFU/mL in stationary growth stage. A vial was diluted and inoculated in 500 mL of TSB to achieve an initial concentration of 10^3-10^4 CFU/mL at 37 ºC. The exponential phase was reached after 10 hours and the stationary growth stage after 14 hours. The 500 mL of the culture was centrifuged twice (J-25, Beckman, USA) at 3220 x g, 5 min and 4ºC, and was dissolved in 100 and 50 mL of TSB respectively. The content was transferred to 2-mL vials with 1 mL of suspension and 1 mL of glycerol 20% diluted with TSB and kept at -80ºC observing no cell viability decrease during the frozen storage (data not shown). Just

before the PEF treatment (5 min) the beverage (700 mL) was inoculated with the defrozen microorganism in stationary growth phase reaching a final concentration of $3.5x10^6$ CFU/mL.

4.6.3.3 PEF treatment

An OSU-4D bench-scale continuous unit was used to treat the food sample. Eight co-field chambers with a diameter of 0.23 cm and a gap distance of 0.293 cm between electrodes were connected in series. One cooling coil was connected before and after each pair of chambers and submerged in a circulating bath (Polystat, Cole Parmer, USA) to maintain the selected temperature at 5ºC. The temperature was recorded by thermocouples (K type) at the entrance of the first treatment chamber (initial temperature) and at the exit of the last treatment chamber (final temperature). The values were recorded with a data logger (Control Company, USA). Pulse waveform, voltage, and current in the treatment chambers were monitored with a digital oscilloscope (Tektronix TDS 210, Tektronix, USA). The flow rate was set at 30 mL/min with a peristaltic pump (XX 80002 30, 6-600 rpm, Millipore, USA). A bipolar square-wave of 2.5 μs was selected. Treatment time ranged from 0 to 2500 μs, and the electric field was set at 15, 25, 35 and 40 kV/cm for the product at pH=4 and 0.3% of pectin concentration. A negative control was carried out immediately after the microorganism was added to the beverage and after the treatment to ensure no inactivation took place because of the acid environment. Samples were collected after each treatment time. The different treatments were serially diluted in sterile 0.1% peptone water, plated in Tryptic soy agar (TSA) and incubated for 24 h at 37 ºC. The experiments were performed to obtain three valid repetitions. The effect of pH (3.5, 4 and 4.5) and pectin concentration (0.1, 0.3 and 0.6%) was studied at 15 and 40 kV/cm for treatment times ranged from 0 to 2500 μs.

4.6.3.4 Mathematical models

The data analysis of two valid replicates used the following models:

Bigelow, 1921:

$$Log\,(S) = -\frac{t}{D}$$ **Equation 4.6.1**

where S is the survival fraction after the treatment, t is the treatment time expressed in μs and D is the kinetic parameter, showing the time required to achieve one decimal reduction, is also expressed in μs and it can be calculated as the negative inverse of the inactivation curve slope.

Hülsheger et al., (1981):

$$Ln\,(S) = -b_t * Ln\left(\frac{t}{t_c}\right)$$ **°Equation 4.6.2**

where bt is the regression coefficient, t is the treatment time in μs and tc is the critical time expressed in μs (the maximum treatment time in which the survival fraction equals 1)

Weibull, 1951:

$$Ln(S) = -\left(\frac{t}{a}\right)^b$$ **Equation 4.6.3**

where a and b are scale and shape parameters, respectively. The b value gives an idea of the form of the curve, if $b>1$ the curve is concave downwards (it forms shoulders), if $b<1$ the curve is concave upwards (it forms tails) and if $b=1$ the curve is a straight line and can be described by linear models.

The critical mean time ($\overline{tcw}$) can be defined by the maximum death per time in response to the PEF treatment and can be expressed by the following equation:

$$\overline{tcw} = a * \Gamma\left(1 + b^{-1}\right) \qquad \textbf{Equation 4.6.4}$$

where a and b are the parameters of the Weibull equation and Γ is the gamma function.

4.6.3.5 Statistical analysis

The models were externally validated with a third valid replicate by using the Accuracy factor parameter (*Af*) (Ross, 1996). This factor gives and idea of the model fitting to the survivor curve of the microorganism and can be defined as follows:

$$Af = 10^{\frac{\sum\left|Log(fitted/observed)\right|}{n}} \qquad \textbf{Equation 4.6.5}$$

where n is the number of observations and the fitted and observed values are referred to the survival fraction. The meaning of this statistic is the closer to 1 the *Af* values, the better the model fit the data.

4.6.4 RESULTS

Figure 4.6.1 shows the *S. typhimurium* counts in the orange juice-milk based product. It could be observed a 12 h latent phase demonstrating the survival of the microorganism in these kind of products. An increase in the microorganism growth was observed reaching a concentration of 6.88×10^7 CFU/mL after 24 h.

Figure 4.6.2 shows the survival curves for *S. typhimurium* in the orange juice-milk product at pH=4 after PEF treatment. Different mathematical models were fitted to the data in order to select one which best describe the different behavior.

Table 4.6.1 shows the kinetic parameters and *Af* values of the Bigelow, Hülsheger and Weibull models in the different survival curves. Results indicate that Bigelow, Weibull and Hülsheger equations had parameters (*D*, *tc* and $\overline{tcw}$) that decreased significantly as the field intensity increased with no significant

differences between 35 and 40 kV/cm (Table 8.2.16, 8.2.17 and 8.2.18). Consequently they can be considered as behavior indexes for the death of *S. typhimurium* by PEF in the product.

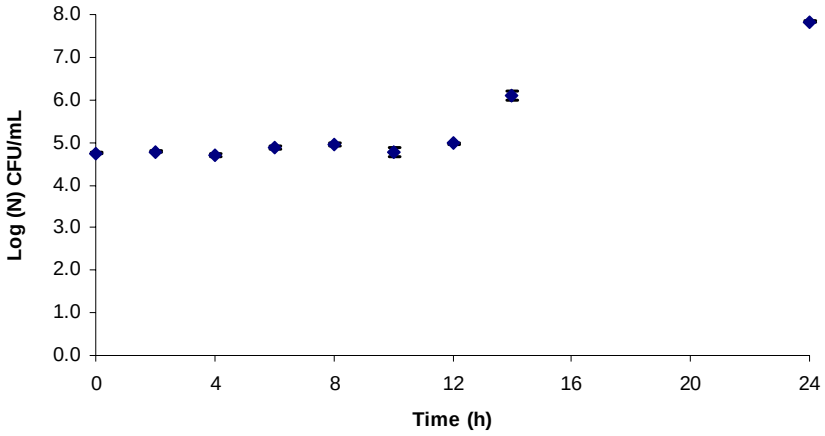

Figure 4.6.1: *Salmonella typhimurium* counts at 37 ºC in the orange juice-milk based product. The standard deviation was expressed by error bars.

The scale parameter (*a*) of the Weibull model was unable to provide a logical interpretation of the behavior of the microorganism as it was not representative of the treatment intensity. The $\overline{tcw}$ parameter could represent an index for the *S. typhimurium* resistance to the PEF treatment. The shape parameter (*n*) of the Weibull model was always below 1 indicating the tail phenomenon with a more pronounced behavior at high electric fields. *Af* values ranged from 1.56-1.22, 1.04-1.24 and 1.04-1.12 for the Bigelow, Hülsheger and Weibull models, respectively. *Af* values closer to 1 indicate better predictions. Consequently, in this study the Weibull model appeared to be the more appropriate model to describe the death of *S. typhimurium* after PEF treatment in the juice-milk product (Figure 4.6.2).

Sampedro et al., (2006) and Rivas et al., (2006) studied the PEF inactivation kinetics of *L. plantarum* (Gram+) and *E. coli* (Gram-) recovered in logarithmic phase in the orange juice-milk based product. Comparing the $\overline{tcw}$ values for both microorganisms it could be observed a higher pulse-resistance of *L. plantarum* reflected by an increase in $\overline{tcw}$ value (Table 4.6.2). Different studies have showed higher PEF treatment resistance in Gram+ bacteria due principally to differences in membrane composition (Hülsheger et al., 1981). Comparing $\overline{tcw}$ values of both microorganisms with the one of *S. typhimurium* (Gram-) recovered in stationary phase, it was observed that $\overline{tcw}$ value was significantly higher than the rest of microorganisms studied (Table 8.2.19) demonstrating a higher pulse-resistance. In consequence the growth phase plays an important role in the microorganism resistance to PEF treatment.

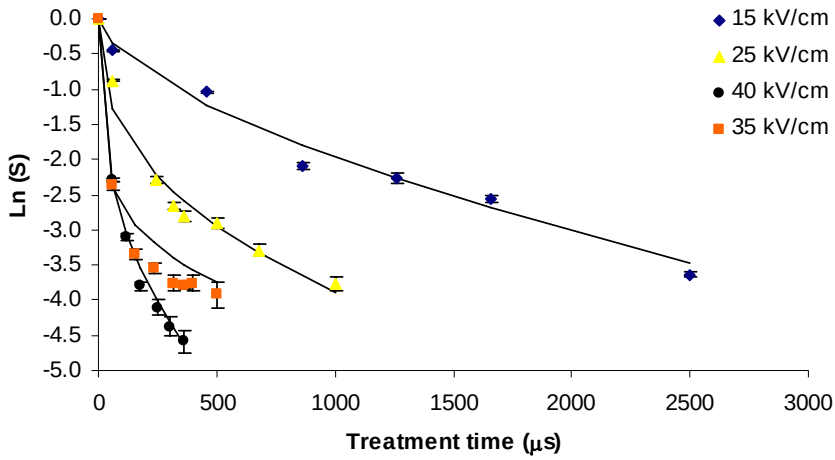

Figure 4.6.2-Survival curves of *S. typhimurium* in the juice-milk based beverage at (♦) 15 kV/cm, (▲) 25 kV/cm, (■) 35 kV/cm and (●) 40 kV/cm at pH=4 adjusted to the Weibull model (—).The standard deviation was expressed by error bars.

Table 4.6.1: Kinetic parameters and *Af* and *MSE* values of Bigelow, Hülsheger and Weibull models for *S. typhimurium* survivor curves.

E (kV/cm)	Bigelow		Hülsheger			Weibull			
	D (μs)	*Af*	tc (μs)	b_t	*Af*	$\overline{tcw}$ (μs)	a	b	*Af*
15	1454.65[a] ± 27.33[b]	1.22	53.82 ± 1.48	0.77 ± 0.00	1.24	478.51 ± 9.21	331.38 ± 2.24	0.62 ± 0.01	1.10
25	454.25 ± 15.31	1.47	23.33 ± 1.09	0.99 ± 0.01	1.10	111.45 ± 0.47	33.19 ± 1.29	0.50 ± 0.00	1.12
35	234.81 ± 7.32	1.56	4.60 ± 1.07	0.89 ± 0.09	1.04	38.38 ± 8.54	2.51 ± 0.81	0.27 ± 0.03	1.04
40	149.01 ± 2.17	1.44	1.36 ± 0.02	0.76 ± 0.02	1.12	24.61 ± 1.60	6.48 ± 1.03	0.38 ± 0.01	1.08

[a] :Value based on mean of three replicates

[b]: Standard deviation

Table 4.6.2: Comparison of $\overline{tcw}$ values of *Salmonella typhimurium* (Gram-, Stat. phase), *Lactobacillus plantarum* (Gram+, Log. phase) and *Escherichia coli* (Gram-, Log. phase) in the orange juice-milk based product.

E (kV/cm)	*Salmonella typhimurium*	*Lactobacillus plantarum*	*Escherichia coli*
	$\overline{tcw}$ (μs)	$\overline{tcw}$ (μs)	$\overline{tcw}$ (μs)
15	478.51[a] ± 9.21[b]	61.11 ± 4.26	5.37 ± 0.69
25	111.45 ± 0.47	50.55 ± 2.84	2.80 ± 0.06
35	38.38 ± 8.54	9.40 ± 1.21	1.98 ± 0.55
40	24.61 ± 1.60	7.15 ± 0.56	1.60 ± 0.99

[a] :Value based on mean of three replicates [b]: Standard deviation

4.6.1 Effect of pH

Figure 4.6.3 and 4.6.4 show the evolution of the survivor's fraction with treatment time and pH at 15 kV/cm and 40 kV/cm respectively fitted to the Weibull model. Decreasing the pH (from 4.5 to 3.5) and increasing the electric field strength increased the actual inactivation. The lower pulse-resistance was reflected by a decreasing in the $\overline{tcw}$ value but with no differences between pH value of 4 and 4.5 (Table 4.6.3 and Table 8.2.20). The $\overline{tcw}$ parameter could represent a resistance index of S. *typhimurium* to the PEF treatment varying the product pH. Other authors have observed higher inactivation level than the reached in this study after PEF treatment in simpler substrates at different pH values. Liang et al., (2002) obtained 5.9 log in *Salmonella typhimurium* after 50 pulses, 90 kV/cm at 55 ºC in a freshly squeezed orange juice at pH=3.8. Álvarez et al., (2003) reached a 7 log cycle inactivation in *Salmonella typhimurium* after 28 kV/cm, 1300 µs and 35ºC in citrate-phosphate McIlvaine buffer at pH 7.0. The higher complexity of the product composition could be a cause of this phenomenon. For this reason, it is necessary to study in greater depth how to optimize pulse treatment alone or combined with an acidic environment to achieve improved inactivation in complex foods.

Table 4.6.3: Effect of pH and electric field on $\overline{tcw}$ value.

E (kV/cm)	3.5 $\overline{tcw}$ (µs)	4 $\overline{tcw}$ (µs)	4.5 $\overline{tcw}$ (µs)
15	249.05[a]±25.83[b]	371.40±39.65	488.74±16.69
40	16.66±1.50	40.03±9.21	61.36±4.45

[a] :Value based on mean of three replicates

[b]: Standard deviation

Figure 4.6.5 shows the estimation of z_{pH} parameter at different pH values. z_{pH} value was 3.41 (R^2=0.989) and 1.74 (R^2=0.962) at 15 kV/cm and 40 kV/cm

respectively. It was observed that the z_{pH} value was lower at 40 kV/cm indicating that $\overline{tcw}$ sensitivity to pH variation was higher than at 15 kV/cm.

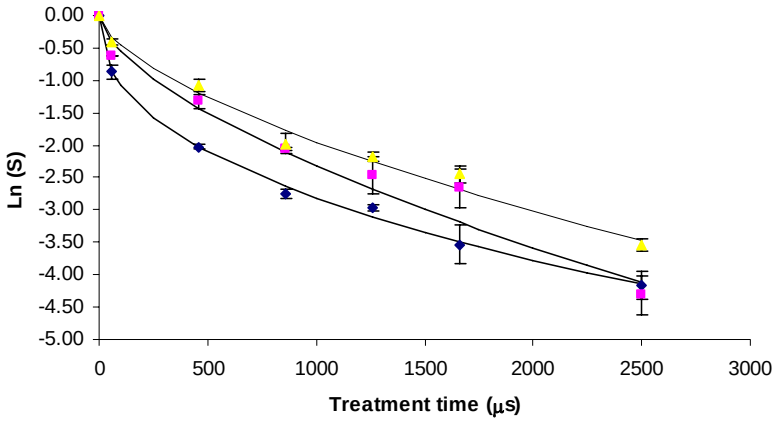

Figure 4.6.3-Survival curves of *S. typhimurium* in the juice-milk based beverage at 15 kV/cm at pH=3.5(♦), pH=4(■) y pH=4.5(▲) adjusted to the Weibull model (—).The standard deviation was expressed by error bars.

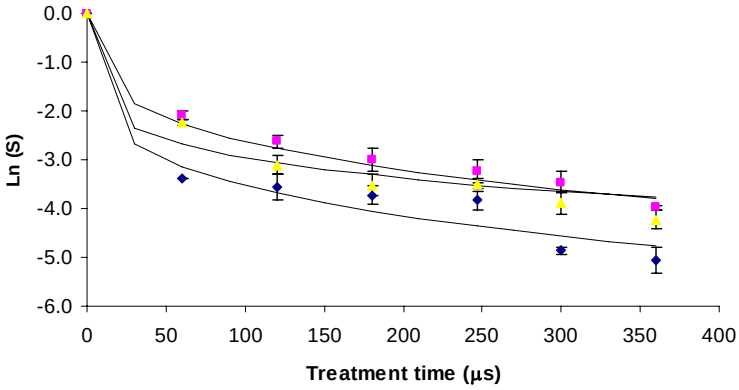

Figure 4.6.4-Survival curves of *S. typhimurium* in the juice-milk based beverage at 40 kV/cm at pH=3.5(♦), pH=4(■) y pH=4.5(▲) adjusted to the Weibull model (—).The standard deviation was expressed by error bars.

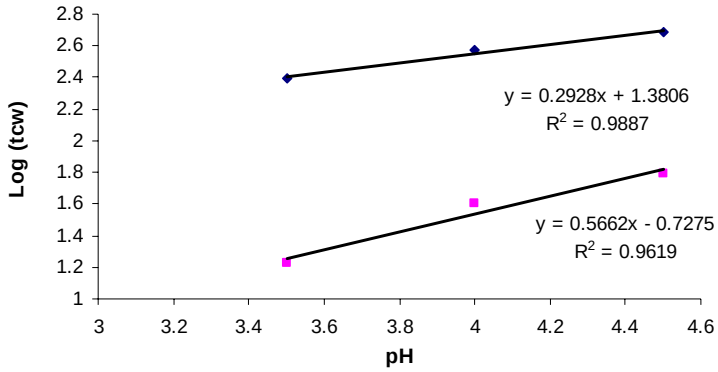

Figure 4.6.5: z_{pH} estimation at E = 15 kV/cm (♦) and E = 40 kV/cm (■).

4.6.2 Effect of pectin concentration

Figure 4.6.6 and 4.6.7 show the evolution of the survivor's fraction with treatment time and pectin concentration at 15 kV/cm and 40 kV/cm at pH 4 fitted to the Weibull model. Variation of the pectin concentration (from 0.1 to 0.6%) did not produce any significant effect in the *S. typhimurium* inactivation after PEF treatment (Table 8.2.21). However increasing the electric field an inactivation increase was produced (Table 8.2.22). Table 4.6.4 shows the $\overline{tcw}$ value at 15 kV/cm and 40 kV/cm and different pectin concentrations. There were no significant differences among the different pectin percentage samples (Table 8.2.21). This indicates a non protective effect of pectin concentration on PEF inactivation of *S. typhimurium*.

Table 4.6.4: Effect of pectin concentration and electric field strength on $\overline{tcw}$ value.

E (kV/cm)	0.10% $\overline{tcw}$ (μs)	0.30% $\overline{tcw}$ (μs)	0.60% $\overline{tcw}$ (μs)
15	402,67 ± 5.81	488,74 ± 16.69	516,93 ± 60.10
40	37.37 ± 9.76	61.36 ± 4.45	45.50 ± 0.58

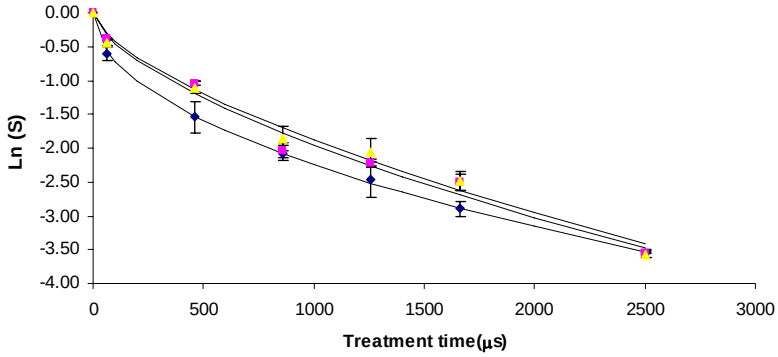

Figure 4.6.6-Survival curves of *S. typhimurium* in the juice-milk based beverage at 15 kV/cm at 0.1% (♦), 0.3% (■) and 0.4% (▲) adjusted to the Weibull model (—).The standard deviation was expressed by error bars.

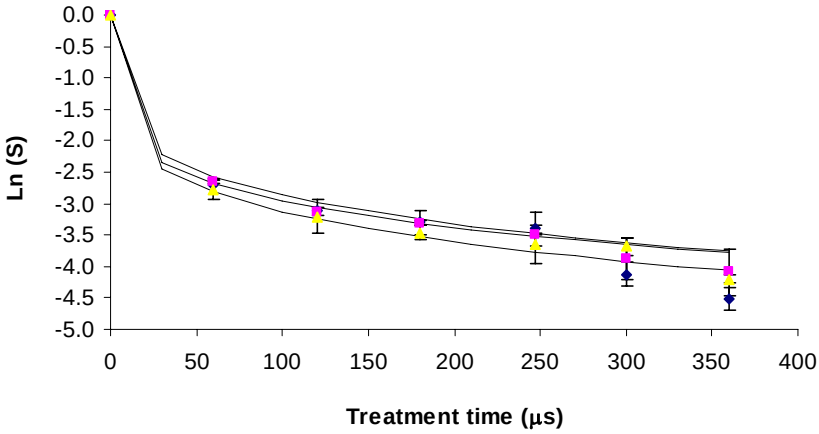

Figure 4.6.7-Survival curves of *S. typhimurium* in the juice-milk based beverage at 40 kV/cm at 0.1% (♦), 0.3% (■) and 0.4% (▲) adjusted to the Weibull model (—).The standard deviation was expressed by error bars.

The $z_{\%}$ value obtained was 4.85 (R^2=0.831) and 8.11 (R^2=0.819) at 15 and 40 kV/cm respectively (Figure 4.6.8).

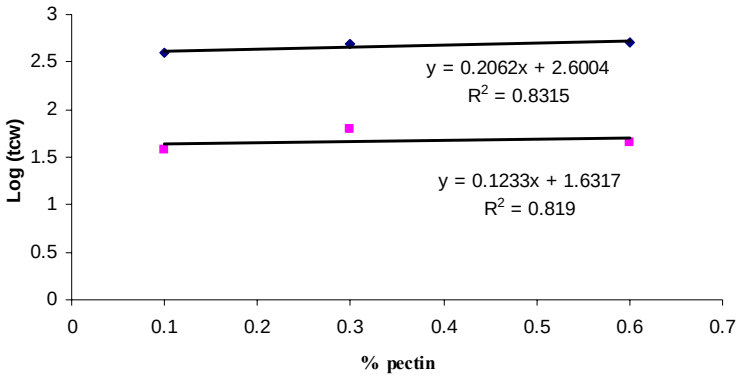

Figure 4.6.8: $z_{\%}$ estimation at E = 15 kV/cm (♦) and E = 40 kV/cm (■).

To conclude, Weibull model gives a good fitting and $\overline{tcw}$ parameter can describe the lethality of treatment, it is therefore the model which best describes the inactivation curves of *S. typhimurium* in the product. The combination of pH and PEF produced an increase in the inactivation by a decreasing in $\overline{tcw}$ value. Variation in the pectin concentration did not produce any added effect. Compared with other substrates studied, the complexity of the product produces a decrease in final inactivation. In our opinion development of new PEF equipment and PEF processing combined with pH and other technologies (heat and antimicrobials) will conduct to an overall effectiveness improvement.

Shelf-life study of an orange juice-milk based beverage after combined PEF and thermal processing

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4.7.1 ABSTRACT

The effect of thermal and pulsed electric field (PEF) processing, that produced similar PME inactivation, on shelf-life of an orange juice-milk beverage (OJMB) was studied. Physical properties (pH, ºBrix), microbial population, pectin methyl esterase (PME) activity, color measurement and volatile compounds concentration were investigated during the storage of the product at 8-10 ºC for 4 weeks. The pH was not affected by any treatment but decreased during the storage in the untreated sample. The Brix degrees were decreased by the two treatments. The thermal and PEF treatments initially inactivated the PME activity by 90%. During the storage the PME activity remained constant in the treated samples and decreased slightly in the untreated sample. The bacterial and mold and yeast counts reduction was similar after the two treatments (4.5 and 4.1 log CFU/mL -thermal- 4.5 and 5.0 log CFU/mL -PEF-). Based on the initial bacterial load of the control, it was estimated that the shelf-lives of the OJMB treated with thermal and PEF treatment stored at 8-10 °C were 2 and 2.5 weeks respectively. Differences were observed in the color parameters of the OJMB among the two treatments with a higher difference observed in the thermally processed sample respect to the control. The relative volatile compounds concentration was higher in the OJMB processed by PEF treatment than thermally processed sample. During storage the loss of volatile compounds was lower in the PEF sample while thermal and control samples had a similar rate of loss. For an OJMB, the combination of PEF and mild heat achieved the same degree of microbial and enzyme inactivation as thermal treatment, but preserving better the original product characteristics during the storage.

Keywords: Orange juice; Milk; Shelf-life; PEF; Thermal

4.7.2 INTRODUCTION

A high quantity of new minimally processed foods have appeared on the market in response to a growing demand for natural products that are perceived by consumers as healthier. Among them, are beverages based on a mixture of fruit juices and milk, fortified with vitamins, minerals, and fiber, which are the most widely consumed functional foods (Pszczola, 2005); however, there are little data related to quality and safety of these products.

These products need a chilled chain for their storage and distribution. Many refrigerators in Europe are set at a temperature around 8ºC. This situation points to conducting the research on the shelf-life of these products at more realistic conditions using temperatures higher than 4ºC. Many studies on shelf-life that compare different technologies (PEF, thermal and HHP) apply different intensity treatments (Jia et al., 1999, Yeom et al., 2000, Rivas et al., 2006, Élez, et al., 2006, Aguilar et al., 2007) being the results not comparable. When fairly comparing different technologies it is very important to choose the right conditions in order to apply the same intensity to the product. This comparison can be done using a microbiological index (inactivation of a selected microorganism or microbial flora) or enzymatic index (inactivation of a selected enzyme). In our study the conditions to obtain the same degree of PME inactivation by thermal and PEF treatment were selected from a previous work (Sampedro et al., 2008).

Pectin methyl esterase (PME) is an important enzyme in orange juice based products and thermal preservation treatments are based on the PME inactivation level of >90 % because its thermotolerance is higher than the majority of microorganisms found naturally in this type of product, causing the cloud loss or gelification of juice concentrates (Tribess et al., 2006). Severe conditions (90 ºC, 1 min or 95 ºC, 30 s) are necessary to inactivate orange PME (Cameron et al., 1994, Do Amaral et al., 2005). Normally industry adopts these conditions to pasteurize orange juice 88-95 ºC, 15-30 s (Irwe and Olson, 1994). Unfortunately, these treatments can modify the original aroma, color and other attributes of the fresh orange juice (Farnworth, et al., 2001, Lee and Coates, 2003).

Several authors have studied the shelf-life of different foodstuff after PEF and thermal treatments such as blended orange and carrot juice (Rivas et al., 2006), apple juice and cider (Evrendilek, et al., 2000), cranberry juice and chocolate milk (Evrendilek et al., 2001), tomato juice (Min and Zhang, 2003, Min et al., 2003) and orange juice (Yeom et al., 2000, Ayhan et al., 2002, Min et al., 2003 a, Élez, et al., 2006) but there are no studies on the effects of PEF and thermal treatment on the shelf-life of a mixture of orange juice and milk.

The aim of this work was to perform a shelf-life study of an orange juice-milk based beverage after thermal and PEF treatment.

4.7.3 MATERIAL AND METHODS

4.7.3.1 Beverage preparation

Fresh Valencia var. oranges were purchased at a local supermarket. The oranges were squeezed with the aid of a juice extractor (Zumex 38, Zumex, S.A., Spain) and the juice was filtered with cheese cloth and stored at -40 ºC until use. The OJMB contained the following ingredients: fresh orange juice (500 mL/L), commercial UHT skimmed milk (200 mL/L), high methoxyl citrus pectin (Unipectine AYD 250, Cargill, USA) (3 g/L), sucrose (75 g/L), and deionized water (300 mL/L). Prior to mixing, solid ingredients were dissolved in water in the weight proportions indicated. The OJMB was prepared just before use. The OJMB physicochemical characteristics were reported in a previous article (Sampedro et al., 2007).

4.7.3.2 Thermal treatment

Thermal treatment conditions were chosen based on the results obtained in a previous work in order to obtain a 90% of PME inactivation (Sampedro et al., 2008). The experiments were carried out in a plate and frame heat exchanger equipped with nominal 66 s hold time tube (FT74X/HTST/UHT, Armfield, Inc., UK). OJMB placed in a feeding tank was driven by a pump to the heat exchanger

at 17 ml/s where it was rapidly heated to 85 ºC. Then the product reached the holding tube where the treatment conditions (85 ºC, 66 s) were maintained. After the treatment, the OJMB was immediately cooled with cold water in a cooler (FT61, Armfield, Inc., UK), and it was packaged and stored until needed for analysis.

4.7.3.3 PEF treatment

PEF treatment conditions were chosen based on the results obtained in a previous work in order to obtain a 90% of PME inactivation (Sampedro et al., 2008). An OSU-4F bench-scale continuous unit (Ohio State University, USA) was used to treat the food sample (Sampedro et al., 2007). Six co-field chambers with a diameter of 0.23 cm and a gap distance of 0.29 cm between electrodes were connected in series. One cooling coil was connected before and after each pair of chambers and submerged in a circulating bath (model 1016S, Fisher Scientific, USA) to maintain the selected initial temperature (65ºC). The temperature was recorded by thermocouples (K type) at the entrance and exit of each pair of chambers. The entrance of the first treatment chamber can be considered as the initial temperature and the exit of the last treatment chamber as the final temperature. The values were recorded with a data logger (Sper Scientific, Taiwan). Pulse waveform, voltage and current in the treatment chambers were monitored with a digital oscilloscope (TDS 210, Tektronix, USA). The flow rate was set at 120 mL/min with a digital gear pump (Cole Parmer, USA). A bipolar square-wave of 2.5 μs was selected. Treatment time was set at 50 μs and the electric field at 30 kV/cm. The sample was immediately cooled in ice-water and it was packaged and stored until needed for analysis.

4.7.3.4 Packaging and storage

The treated product was packaged in clean, sterile twist-off jars inside a laminar flow hood. The closed jars were stored in a refrigerator at 8-10 ºC in

darkness. Quality analyses discussed in the following sections were carried out after 1, 2, 3 and 4 weeks.

4.7.3.5 Analysis of headspace volatile compounds

Volatile compounds were extracted with a modification of the method described by (Fan and Gates, 2001) using a solid-phase microextraction (SPME) method. A 2 mL aliquot sample was transferred into 6 mL serum vial. The vial, sealed by a teflon-lined septum and a screw cap, was pre-heated at 60 ºC for 2 min before a SPME fiber, coated with 100 µm of poly(dimethylsiloxane), was inserted into the headspace of the sample bottle. After 30 min incubation, the SPME fiber with adsorbed volatile compounds was inserted into the GC injection port at 250 ºC and held there for 5 min to desorb volatile compounds. Volatile compounds were separated by a Hewlett-Packard 6890N/5973 GC-MSD (Agilent Technologies, USA) equipped with a DB-Wax trace analysis column (30 m x 0.32 mm i.d., 0.5 µm film thickness). The temperature of the GC was programmed from 60 to 96 ºC at 8 ºC·min^{-1}, increased to 120 ºC at 12 ºC·min^{-1}, then increased to 220 ºC at 10 ºC·min^{-1} and held for 3 min at the final temperature. Helium was the carrier gas at a linear flow rate of 39 cm·sec^{-1}. Compounds were identified by comparing spectra of the sample compounds with those contained in the National Institute of Standards and Technology library (NIST02). The relative amount of each compound was expressed as peak area.

4.7.3.6 PME activity measurement

PME activity was determined by measuring the release of acid over time at pH 7 and 22 ºC. The reaction mixture consisted of 1 mL of sample and 30 mL of 0.35 % citrus pectin solution (Sigma, USA) containing 125 mM NaCl. During hydrolysis at 22ºC, pH was maintained at 7.0 by adding 10^{-4} N NaOH using an automatic pH-stat titrator (Titralab, Radiometer Analytical, SAS). After the first 1 min the consumption of NaOH was recorded every 1 s for a 3 min reaction period. PME activity was expressed in units (U), defined as micromoles of acid produced

per minute at pH 7 and 22 ºC. The detection limit was established at 0.019 U/ml. Residual activity was expressed as the relation between the PME activity after the treatment (A) and the initial activity (A_0) expressed in U/mL.

4.7.3.7 Physical property measurements

The physical properties such as pH, ºBrix and visual inspection (phases separation) were measured at room temperature. An pH meter (model 370, Fisher, USA) and hand refractometer (Fisher, USA) was used to determine the pH and ºBrix respectively.

4.7.3.8 Color measurement

Color was measured with a color meter (HunterLab MiniScan XE, Hunter Associates Laboratory, USA) using a 27 mm measuring aperture. The color meter was calibrated using the standard white and black plates. D65/0° was the illuminant/viewing geometry Triplicate measurements were recorded for each sample. 75 mL of product were placed into a 2.5 inch (diameter) glass sample cup. The cup was then placed onto 2-inch port for color measurement. A black cover over the sample cup was used. The parameter "L^*" is a measure of brightness/whiteness that ranges from 0 to 100 (white if L^*=100, black if L^*=0). The parameter "a" is an indicator of redness that varies from $-a^*$ to $+a^*$ ($-a^*$=green, a^*=red). The third parameter "b^*" is a measure of yellowness that varies from $-b^*$ to $+b^*$ ($-b^*$=blue, $+b^*$=yellow). Also the total color difference between the control and treated sample was calculated by the equation proposed by Cserhalmi et al., (2006):

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \qquad \textbf{Equation 4.7.1}$$

Depending on the value of ΔE the color difference could be estimated such as not noticeable (0-0.5) slightly noticeable (0.5-1.5), noticeable (1.5-3.0), well visible (3.0-6.0) and great (6.0-12.0).

4.7.3.9 Microbiological assay

The microbial inactivation and growth were examined by diluting the samples in 0.1 % (w/v) sterile peptone water and plating in Tryptic Soy Agar (TSA) for bacteria counts and in acidified Potato Dextrose Agar (PDA) for mold and yeast counts every week for one month. Plates were incubated at 37 ºC for 48 h for bacteria and yeast counts and at 30 ºC for 5 days for mold counts.

4.7.3.10 Statistical Analysis

The statistical analysis was performed using the software Statgraphics® Centurion XV (Statistical Graphics Corp.) applying an univariant ANOVA test with a significance level of 95.0 % ($p<0.05$). The means of three replicates were differentiated by Tukey's test.

4.7.4 RESULTS AND DISCUSSION

4.7.4.1 Effects of processing and storage on physical properties

The effect of thermal and combined PEF and thermal processing on the OJMB physical properties are shown in Table 4.7.1. There were significant differences in the pH values after the different treatments (Table 8.2.23), however the differences were less than 0.3. Many authors have observed no variation in the pH value after different thermal and PEF treatments in different fruit and vegetable juices (Min, et al., 2003 a and b, Cserhalmi, et al., 2006, Rivas et al., 2006, Élez, et al., 2006, Aguilar et al., 2007). During the storage, pH value decreased significantly in the untreated sample during the first and fourth week due to the growth of microorganisms that could use the carbohydrates producing lactic acid (Table 8.2.24). The pH of the thermal and PEF processed samples decreased

significantly during the storage due to the increase in the microbial counts (Table 8.2.24). Élez et al., (2007) found a decrease in pH in the unprocessed sample from 28 to day 56 at 22ºC in an orange juice. Yeom et al., (2000) and Min et al., (2003 a) found no differences in the pH value in the treated samples during the storage of an orange juice for 112 days at 4 and 22ºC. Rivas et al., (2006) found a decrease in the pH value in a PEF treated sample (25 kV/cm, 280 μs) at 12ºC after the 8.5th week owing to the beginning of microbiological spoilage of a blended orange-carrot juice.

The Brix degrees content decreased significantly after the PEF treatment with no differences between thermal and untreated sample (Table 8.2.25). However the differences between the mean values of the untreated and treated samples were less than 0.5 so practically were negligible. Cserhalmi et al., (2006) and Rivas et al., (2006) also observed a slight decrease in the Brix content after the PEF treatment in an orange and blended orange-carrot juice respectively. During the storage the Brix degrees decreased significantly in the untreated and thermal sample (Table 8.2.26). The growth of microorganisms could cause this phenomenon by fermentation of sugars. However there were no significant differences in the PEF treated samples (Table 8.2.26). Several authors also found no changes in Brix degrees during the storage of any treated samples at 2 and 12ºC for 10 weeks in a blended orange-carrot juice and orange juice for 112 days at 4 and 22ºC (Yeom et al ., 2000, Rivas et al., 2006, Min et al., 2003 a).

4.7.4.2 Effects of processing and storage on microbial flora

The effect of thermal processing and combined PEF and thermal treatment on the total plate count and mold and yeast count in the OJMB are also shown in Table 4.7.1. Microbial loads of the untreated sample were 5.99 and 5.43 log for bacteria and yeast and mold counts, respectively. The bacteria and mold and yeast counts were reduced by 4.5 and 4.1 log cycles by thermal treatment and 4.6 and 5.0 log cycles by the PEF treatment, respectively, with no significant differences in the microbial reduction among the two technologies (Table 8.2.27). Min et al., (2003 a

and b) reached 6 log cycles inactivation of endogenous bacteria in a tomato juice and orange juice after the thermal (90ºC, 90 s) and PEF treatment (40 kV/cm, 57 μs and 45ºC).

Table 4.7.1: Effect of thermal and PEF processing on different parameters of an orange juice-milk beverage

Parameters	Untreated	Thermal	PEF+Thermal
pH	4.31±0.02[1a]	4.39±0.01[b]	4.36±0.01[c]
Bacteria (log CFU/mL)	5.99±0.02[a]	1.42±0.08[b]	0.92±0.25[b]
Molds and Yeasts (log CFU/mL)	5.43±0.03[a]	0.92±0.11[b]	0.43±0.09[b]
ºBrix	15.07±0.06[a]	14.83±0.06[a]	14.65±0.07[b]
PME (U/ml)	0.262±0.03[a]	0.039±0.001[b]	0.035±0.002[b]
*L**	56.30±0.04[a]	55.68±0.01[b]	55.92±0.05[c]
*a**	1.85±0.05[a]	-0.37±0.02[b]	0.81±0.06[c]
*b**	38.82±0.10[a]	22.08±0.02[b]	25.19±0.06[c]

[1]: Numbers are means of three replicates followed by standard deviation. Means with the same letter are not significant different ($p<0.05$)

During the storage there was an increase of 4-6 log CFU/mL in the bacteria and mold and yeast counts in the unprocessed sample (Figure 4.7.1). The increase in microbial flora was higher than previous shelf-life studies performed by several authors due to the selected storage temperature (8-10ºC). The increase in the PEF and thermal sample during the storage was about 6 log in the bacteria and mold and yeast counts (Figure 4.7.1). The shelf-life of the treated samples was established taking into account the initial microbial flora of the control sample in every treatment (X axis, point 0). On this basis, the microbial count of sample treated

with thermal processing exceed the initial count of control sample after 2 weeks while it took 2.5 weeks for the PEF treated sample. Therefore, the shelf-lives could be stated at 2 and 2.5 weeks at 8-10 ºC for the thermally and PEF processed samples, respectively. In this study PEF sample had a slightly higher shelf-life than thermal sample.

Different authors have obtained quite long shelf-lives after different PEF treatment conditions ranging from 112 days at 4, 22 and 37ºC, 56 and 42 days at 4 and 22ºC and 42 days at 4ºC in an orange juice and 120 days at 2ºC in a blended orange-carrot juice (Yeom et al., 2000, Élez et al., 2006, Jia et al., 1999, Rivas et al., 2006). Min et al., (2003 b) found an increase in the bacteria counts of $1.0x10^2$ and $1.0x10^4$ CFU/mL in yeast and mold counts in a PEF treated sample after 112 days at 4ºC. They argued that the increase of bacterial counts in the PEF treated sample could be due to the relatively low inactivation of ascospores.

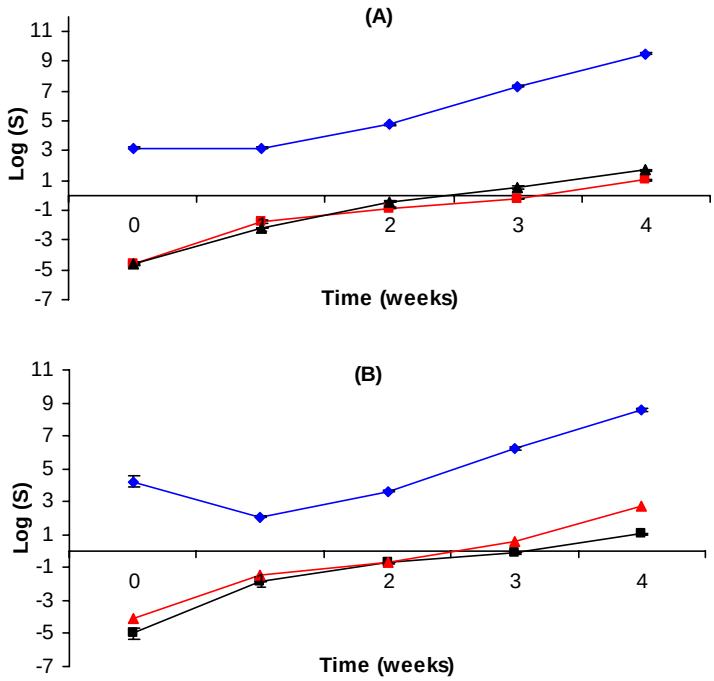

Figure 4.7.1: Growth of microbial flora (A) and mold and yeast counts (B) during the storage (♦) control sample, (▲) thermal sample and (■) PEF sample. The standard deviation was expressed by error bars.

4.7.4.3 Effects of processing and storage on PME activity

The effect of thermal and combined PEF and thermal processing on PME activity in the OJMB is shown in Table 4.7.1. PME activity was inactivated by 89.4 and 90.1 % after the thermal and PEF treatments, respectively, with no significant differences between the two technologies (Table 8.2.28). Different authors have also obtained a high degree of PME inactivation after PEF treatment. Élez et al., (2006) achieved no PME activity and 81.6% inactivation after thermal (90 ºC, 1 min) and PEF (35 kV/cm, 1000 μs) processing respectively. Rivas et al., (2006) found 75.6 and 81% after PEF treatment (25 kV/cm, 280 and 330 μs) and 98% inactivation after thermal treatment (98ºC, 21 s). Yeom et al., (2000) found an inactivation of 88% after 35 kV/cm, 59 μs and 98% after 94.6 ºC for 30 s and there was no activation after 112 days at 4 and 22ºC. The differences in the degree of PME inactivation achieved could be due to the orange variety, harvest season, treatment intensity or type of food used in the study. Despite of the differences, all of these studies apply different treatment intensity for thermal and PEF processing obtaining different PME inactivation degrees being the results among the two technologies not comparable.

During the storage there was a significant decrease in the PME activity in all samples (Table 8.2.29) (Figure 4.7.2). Élez et al., (2006) also found a decrease in the initial PME activity of the untreated sample to around 40% of the initial activity. A phase separation was observed after two weeks in the unprocessed sample indicating the destabilization effects of the PME activity. In the treated samples there were no activation of the enzyme during the storage ($p<0.05$) and there was no phase separation. In the thermal sample a slight precipitation was observed at the bottom, maybe due to the casein precipitation. Several authors have also observed no PME activation during storage after PEF treatment (Élez et al., 2006, Rivas et al., 2006). This fact demonstrates that PEF treatment can achieve a PME irreversible inactivation and a 90% PME reduction is enough to guarantee the stability of the product stored under refrigeration conditions.

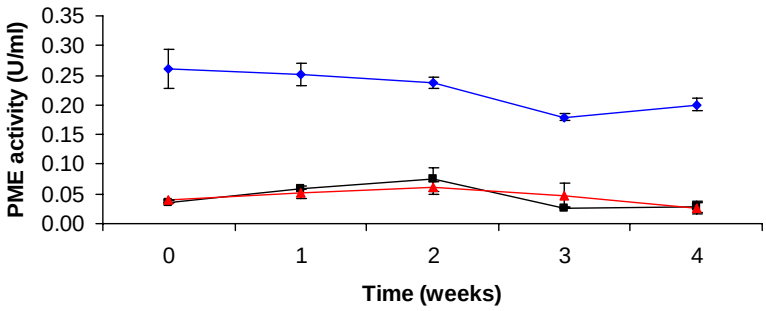

Figure 4.7.2: Change of PME activity during the storage, (♦) control sample, (▲) thermal sample and (■) PEF sample. The standard deviation was expressed by error bars.

4.7.4.4 Effects of processing and storage on color measurement

Table 4.7.1 shows the effect of different treatments on *L**, *a** and *b** parameters. There was a significant decrease in *L**, *a** and *b** parameters after the different treatments (Table 8.2.30, Table 8.2.32, Table 8.2.34). The ΔE of samples was 2.70 after PEF treatment and 2.77 after thermal treatment with a noticeable color difference compared to controls (Eq. 1). Cserhalmi et al., 2006 found no differences between the untreated and PEF treated samples in the total color of orange juice (ΔE=0.47). *L** parameter increased slightly during the first three weeks and decreased in the fourth week in the PEF and thermal treatment samples however the differences were negligible (Table 8.2.31) (Figure 4.7.3).

There were no significant differences in the *a** parameter during the first three weeks increasing in the fourth week in the untreated and PEF samples and no differences in the thermal sample (Table 8.2.33) (Figure 4.7.3). *b** parameter decreased significantly during the storage in the unprocessed and PEF sample but increased after the thermal treatment (Table 8.2.35) (Figure 4.7.3). Rivas et al., (2006) found no differences in the luminosity and saturation after the thermal and PEF treatments in an orange-carrot juice but an increase in the hue angle was observed after each treatment. During the storage, the authors did not observe changes in the color parameters in the PEF treated sample but decreasing in the

thermal treatment. Min et al., (2003 a) found higher *L** and hue angle values during the storage for 196 days at 4ºC of PEF treated samples than that for thermally treated samples. Ayhan et al, (2002) found higher *L** and *b** values after PEF treatment and lower *a** values than fresh orange juice (brighter and more yellowish) and during the storage at 4ºC no effect on color parameters was observed and *L** and *a** values were reduced after 28 days at 22ºC.

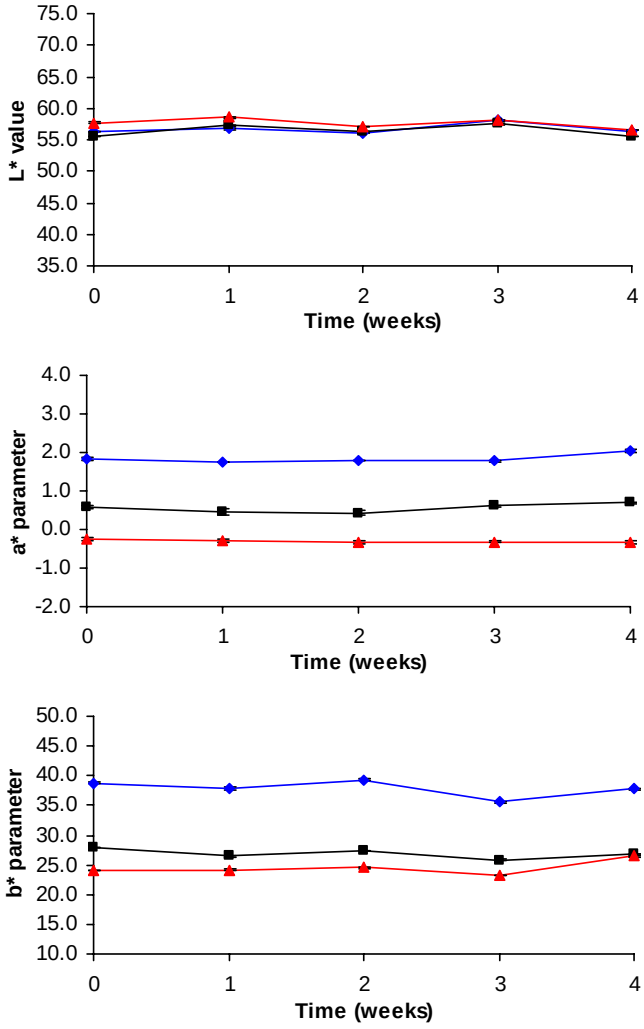

Figure 4.7.3: Change of color parameters with storage time in the unprocessed sample (♦), thermal treated sample (▲) and PEF treated sample (■).The standard deviation was expressed by error bars.

4.7.4.5 Effect of processing and storage on volatile compounds content

The effect of thermal and combined PEF and thermal processing on volatile compounds concentration in the OJMB is presented in Table 4.7.2. There were no differences in the nonanal, caryophyllene, valencene and dodecanal content after PEF and thermal treatment (Table 8.2.36). The average loss of volatile compounds content was -1.68 and 20.95% after the PEF and thermal treatment respectively. Several authors have also observed less volatile compounds loss after PEF treatment. Cserhalmi et al., (2006) found no loss of volatile compounds content in an orange juice after PEF treatment (28 kV/cm, 100 μs) whereas Jia et al., (1999) and Aguilar, et al., (2007) found less volatile compounds loss after PEF than thermal treatment in an orange and apple juice respectively. They contented that the different sensitivity of the volatile compounds to the PEF and thermal treatments may be due to the molecular weight and boiling point differences with the lower ones more easily lost during the treatments. However the PEF treatments studied achieved less microbial inactivation than did the thermal treatment so the results were not comparable.

The compounds most sensitive to the thermal treatment were β-phellandrene and α-phellandrene whereas limonene, 4-carene and ethyl octanoate were the least sensitive to heat. On the other hand, β-phellandrene and caryophyllene were most sensitive to the PEF treatment. The content of β-myrcene, limonene, 3-carene, 4-carene and ethyl octanoate was increased by the PEF treatment. Different theories could explain this phenomenon. Min et al., (2003 c) found that after PEF treatment some compounds increased in content claiming that the PEF sample had a lower particle size distribution and consequently an increase in the release of the volatile compounds. Ayhan et al., (2002) found an increase in the content of different volatile compounds (limonene, myrcene, valencene and α-pinene) after PEF treatment (35 kV/cm, 59 μs) in an orange juice. They reasoned that these compounds are found in higher concentration in the pulp and could be released after the PEF treatment into the aqueous phase.

During the storage all compounds in the untreated sample decreased in content except limonene and caryophyllene (Figure 4.7.4-A). The average loss was 34.24%. The compounds that were lost to a higher extent were decanal and β-phellandrene. The content of volatile compounds in the thermal sample decreased during the storage except limonene, caryophyllene and α-phellandrene (Figure 4.7.4-B). The average loss was 27.13%. β-phellandrene, nonanal, ethyl octanoate and decanal were lost most during the storage. Regarding to the PEF sample, the average loss was 3.72% (Figure 4.7.4-C). Compounds that increased their content during the storage were α-pinene, β-myrcene, α-phellandrene, 3-carene, 4-carene and caryophyllene whereas the content of decanal and β-phellandrene decreased. Different authors observed a slightly better preservation of volatile compounds in different fruit juices (orange and apple juice) after PEF treatment during the shelf-life (Min et al., 2003 a; Min et al., 2003 c; Ayhan et al., 2002; Yeom et al., 2000). The storage temperature seemed to influence to a great extent the acceleration of the loss of volatile compounds content.

During the shelf-life of the OJMB at 8ºC, the unprocessed sample spoiled after 1 week, whereas the PEF and thermally treated samples remained stable during the entire storage period (4 weeks). A decrease in the color parameters was observed after the different treatments. A slight decrease in pH, Brix and PME values were observed in the unprocessed sample and a slight increase in the PME and no variation in the pH and Brix values were observed in the PEF and thermally treated samples. Both technologies achieved the same enzyme and microbial inactivation. During storage, populations of bacteria, molds and yeasts increased by 4-6 log cycles in the unprocessed sample. In the thermal and PEF treated samples the population increased by 6 log cycles. A decrease in the concentration of volatile compounds was also observed in all samples, although the decrease of the PEF treated sample was smaller. The results showed that with the same microbial and enzyme inactivation PEF processed sample has longer shelf-life with higher standard of quality than thermal processed sample.

Table 4.7.2: Effect of thermal and PEF processing on volatile compounds content of an orange juice-milk beverage.

Compound	Loss (%)	
	PEF	Thermal
α-pinene	9.11[1]±2.61[2a]	36.30±0.28[b]
β-pinene	4.66±1.98[a]	44.14±1.15[b]
β-phellandrene	46.18±0.25[a]	79.32±9.02[b]
β-myrcene	-25.76±8.14[a]	31.74±7.74[b]
Limonene	-5.68±6.42[a]	8.26±4.60[b]
α-phellandrene	3.86±5.50[a]	62.88±0.86[b]
3-Carene	-23.01±13.67[a]	40.91±4.94[b]
4-Carene	-108.94±2.20[a]	7.80±13.12[b]
Nonanal	18.54±7.67[a]	31.11±5.80[a]
Ethyl Octanoate	-26.39±18.36[a]	-161.97±22.85[b]
Decanal	11.80±9.12[a]	28.99±2.01[b]
Caryophyllene	35.93±1.54[a]	39.52±1.79[a]
Dodecanal	14.42±1.85[a]	16.64±7.96[a]
Valencene	21.77±1.23[a]	27.74±3.93[b]
Average loss (%)	-1.68	20.95

[1]: Numbers are means of three replicates followed by standard deviation. Means with the same letter are not significant different ($p<0.05$).

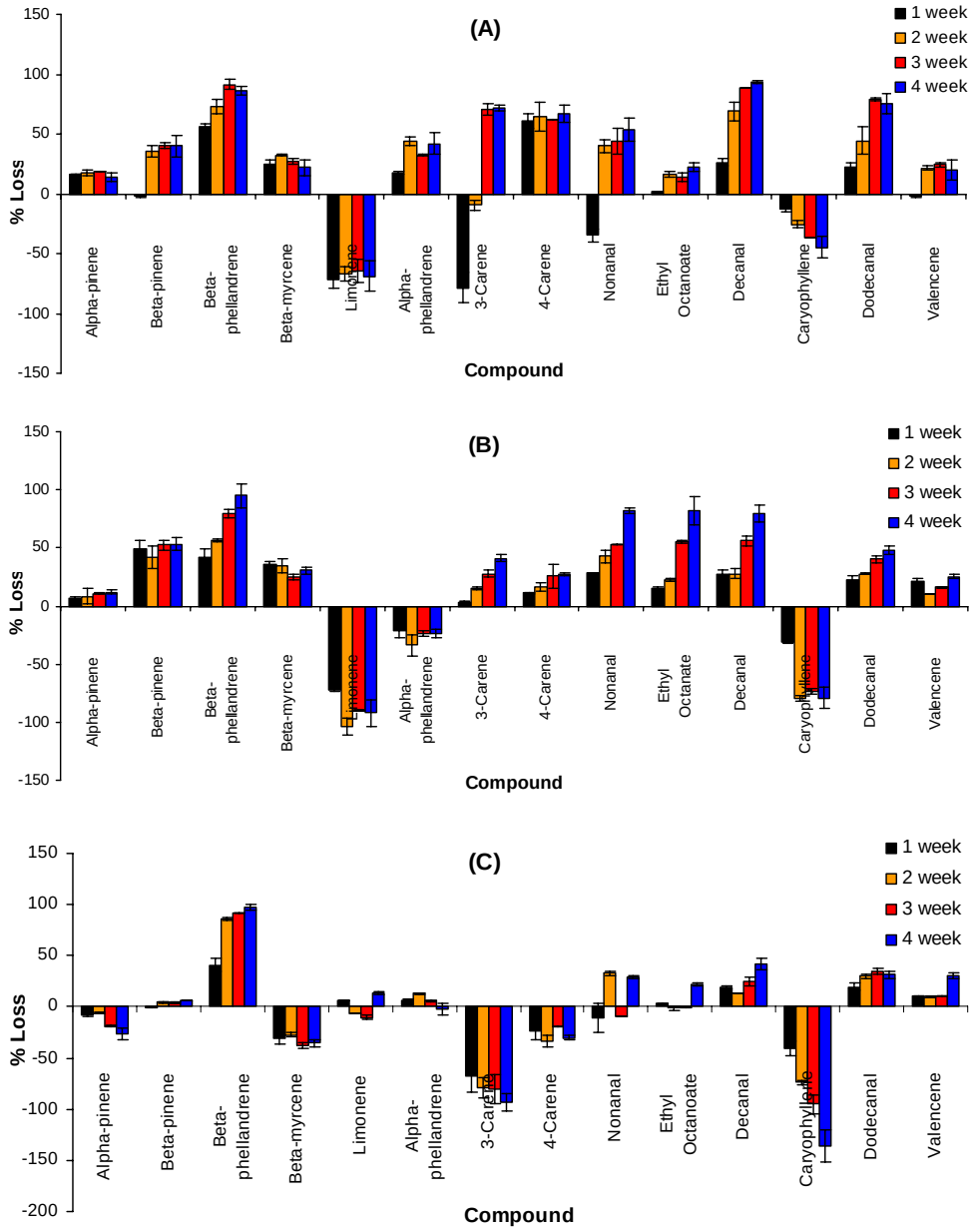

Figure 4.7.4: Change of volatile compounds content during the storage (A) control sample, (B) thermal sample and (C) PEF sample. The standard deviation was expressed by error bars.

5.-GENERAL DISCUSSION

In recent years, a large quantity of new pasteurized liquid foods requiring chilled storage and distribution has appeared on the European market. Among these, fruit-juice mixes, vegetable soups and different sauces are predominant; they are produced from food ingredients of very different natures, leading to complex compositions. Within these products fruit juices and milk based beverages offer an innovating flavor providing essential nutritional components such as water-soluble vitamins and natural antioxidants from juice and calcium and vitamin-D from milk, among others.

In order to apply a preservation technology (HHP, PEF or thermal) to a new product it is necessary to find the adequate formulation based on the consumer acceptability that allows developing inactivation kinetic studies. The new formulation must suppose a substantial nutritional and sensorial improvement respect to the products of such nature commercialized nowadays. Different product formulations based on the orange juice content were studied. A more suitable physicochemical characteristics, better consumer sensory evaluation and higher microbial inactivation degree was obtained in the 50% orange juice sample. This new formulation represents an increase in the vitamin-C and antioxidants supply.

One of the most important aspects in fruit juice preservation is enzyme activity. Enzymes are responsible for juice quality losses and the structure and treatment resistance differs among them. In the orange juice, pectin methyl esterase (PME) is mainly responsible for its destabilization. Its resistance to thermal and HHP treatment is higher than the majority of the microorganisms found in the juice. This fact justifies the characterization of the PME kinetic behavior against the different new preservation technologies (PEF and HHP). PME inactivation study in the orange juice-milk based product corroborated the enzyme treatment resistance. Intense treatment conditions were necessary to inactivate it (90ºC, 1 min and 700 MPa at 55ºC for 2 min). The presence of two fractions (92% labile and 6-8% stable) with different stability to the treatment was confirmed. A biphasic model was able to describe enzyme inactivation curves. Kinetic constants,

activation energy and activation volume values obtained for each fraction allowed to characterize its behavior against the combined HHP and thermal treatment.

By comparing PME kinetic constants and activation energy values obtained in the beverage with other PME inactivation kinetic studies in orange juice, it was observed higher values in the orange juice-milk system so therefore, higher treatment resistance demonstrating a protective effect. This fact alerted over the need to study the substrate effect in the thermal and HHP PME inactivation. There were significant differences in PME inactivation in the different orange matrices (juice-milk beverage, orange juice and purified PME in buffer at basic pH). At same pH values lesser thermal and HHP PME inactivation was obtained in the beverage than in the orange juice matrix. In the purified enzyme differences were also observed being more pressure resistant and less thermostable than the rest of matrices. The inactivation mechanism of the different technologies, pH value or purifying level, are the possible main causes of the differences in the inactivation degree obtained.

In the PME inactivation study by PEF treatment in the orange juice-milk system it was observed that at low treatment temperature (25 ºC) some enzyme activation effect was found as indicated by an increase in PME activity (between 11-60%). Application of a mild PEF treatment could increase the permeability of the orange pulp by facilitating the release of the bounded PME. After PEF treatment, PME activity measured as “free” enzyme, increased. By increasing the temperature, the inactivation reached a maximum of 91% inactivation after 30 kV/cm, 65 ºC (final temperature 80 ºC) and 50 μs demonstrating the synergetic effect between the temperature and PEF treatment.

Other important food quality aspect is the aroma. The effect of different preservation technologies (PEF, HHP and thermal) on the volatile compounds concentration was studied. The different treatments were equivalent in the intensity applied (30 kV/cm, 50 μs and 65ºC -PEF-, 650 MPa and 50ºC-HHP- and 85ºC, 1 min for thermal treatment) by obtaining a 90% of PME inactivation. In the thermal treatment between 16 and 43% of volatile compounds content was lost. After PEF

treatment, losses varied between -13 and 30% and an increase in the content of certain compounds was produced possibly due to their release from the pulp. In the HHP processing the same phenomenon was presented with an increase in the content of certain volatile compounds but treatment losses (between -14 and 42%) were similar to those of thermal treatment. In conclusion, PEF technology was the one which best preserved the original aroma of the fresh product so this technology was decided to be used in the microbial inactivation studies.

One of the main problems related to the microbial spoilage effects in fruit juice based products is the development of acid-tolerant microorganisms. Within them *Lactobacillus* genus is one of the most important due to its capability to grow in these kind of products, being essential to know its behavior against PEF treatment. For that purpose, the effect of different PEF processing variables (electric field strength, treatment time, temperature and pulse width) on the inactivation of *Lactobacillus plantarum* was studied.

An increase in electric field and treatment time generally produces higher inactivation degree. However, this behavior is not always demonstrated. Every microorganism has a critical electric field and increasing it membrane electroporation is produced leading to the death of the microorganism. The membrane electroporation depends on the cellular wall characteristics (Gram+ or Gram-) and cell size and shape (rod-shape and greater size cells are more susceptible to the treatment). The critical electric field could be transformed in critical energy applied that corresponds to a minimal energy level enough to produce electroporation. Within the present study it was observed that an increasing in the energy applied produced a rapid decreasing in the initial survivors number corresponding to the most susceptible group of cells. A higher energy applied did not produce any significant inactivation corresponding to a fraction of cells resistant to the treatment. It was necessary to overpass a higher energy level to obtain microorganism inactivation. Finally the most PEF resistant fraction remained unaffected. This circumstance makes that the survival curve do not present a linear behavior against energy applied.

An increase in the initial treatment temperature produced higher inactivation with lower energy consumption. This is produced by an increase in the membrane permeability making it more susceptible to the PEF treatment. Based on that, PEF and mild temperature combination could be a good strategy to improve treatment effectiveness and reduce the energy applied up to 60%. An increase in pulse width did not produce any significant inactivation effect.

An optimization study based on the energy applied has the main objective of designing a treatment that obtains a higher inactivation degree with lesser energy consumption. The influence of different process variables on *L. plantarum* inactivation study showed that a treatment based on high electric fields (40 kV/cm), short treatment times (100 μs), pulse width (~2 μs) and mild temperature (30-50ºC) produced the highest inactivation degree with the lowest energy consumption. Comparing the energy applied in this study with other studies in simpler substrates it could be observed that the energy applied was 10 times higher in the beverage substrate. By comparison with the HTST thermal treatment (High Temperature Short Time) the optimum PEF treatment could be HEFST (High Electric Field Intensity Short Time).

Once it was observed that the electric field, treatment time and temperature were the three most influential variables on PEF inactivation of *L. plantarum*, it was decided to study PEF inactivation kinetics based on them. Weibull model was able to properly describe *L. plantarum* survival curves against PEF treatment and $\overline{tcw}$ parameter was an index of the microorganism treatment resistance. On the other hand, simpler composition food provided lower $\overline{tcw}$ value indicating a protective effect in the *L. plantarum* treatment resistance. An increase in the initial temperature also favored PEF inactivation by a decrease in $\overline{tcw}$ value.

In orange juice processing raw material physicochemical characteristics vary greatly during the year. Among them, pH is probably the most influential factor in the product microbiological safety and enzyme activity on the product stability. Stabilization of an orange juice-milk product during the shelf-life requires

the use of an adequate stabilizer (pectin). On the other hand, within the latest years, several *Salmonella* outbreaks in fresh orange juice have appeared showing the acid-tolerance of certain *Salmonella* strains such as *S. typhimurium*. This fact alerted sanitary authorities over the duty to control certain new microorganisms to improve the microbiological safety of these kind of foods.

Due to the lack of information about the effect of PEF treatment on microbiological safety of this product, it is important to study the influence of the physicochemical variables on pathogen inactivation. A pH reduction increased *S. typhimurium* PEF inactivation. Weibull model was useful to describe microorganism behavior through $\overline{tcw}$ parameter and z_{pH} parameter was also useful to estimate the $\overline{tcw}$ sensitivity against pH. A variation in the stabilizer concentration (pectin) did not produce any significant effect on the inactivation degree, thus, such characteristic does not present a microbiological safety problem on these type of products. Through this study it was demonstrate the importance of physicochemical characteristics in a PEF treatment design.

Once microbiological and quality aspects were established, the shelf-life of the product at refrigerated conditions (8-10ºC) after PEF and an equivalent thermal treatment (same PME inactivation) was studied. After the thermal treatment, Brix degrees, color and volatile compounds concentration significantly diminished being the overall loss lower in the PEF treatment. During the storage, pH and Brix degrees diminished in both treatments due to a logical microorganism growing within the temperature range (8-10ºC). PEF and thermal treatment obtained the same microbial flora reduction. However, the product shelf-life after PEF treatment was slightly higher (2.5 weeks) than the thermally treated sample. Enzyme activity did not increase during the storage showing an irreversible inactivation. Volatile compounds content diminished during the storage in a higher degree in the thermally treated sample. This fact showed that PEF processing could achieve the same degree of microbial and enzyme inactivation as thermal treatment, but

preserving better the original characteristics during the storage of an orange juice-milk beverage.

6.-CONCLUSIONS

1. The complex composition of the beverage offered a protective effect on microbial and enzyme inactivation.
2. PME HHP and thermal inactivation appeared to be biphasic with the appearance of two fractions with different treatment stability.
3. Substrate, purifying level or pH affected PME HHP and thermal resistance being more thermostable as endogenous form in the beverage and more pressure resistant as purified form in the buffer.
4. PEF and HHP low intensity treatments produced PME activation (11-60%) and increase the volatile compounds concentration (14%).
5. PEF processing was the one that best preserved the original volatile compounds concentration of the beverage.
6. PEF treatment based on high electric field, short treatment time and mild temperature could achieve a high microbial inactivation degree with lower energy consumption (60% reduction).
7. Weibull model was able to describe *L. plantarum* and *S. typhimurium* inactivation behavior and the effect of physicochemical characteristics (pH and pectin concentration) after PEF treatment.
8. $\overline{tcw}$ parameter could be considered an index of microorganism resistance to PEF treatment.
9. A decrease in the pH value increased *S. typhimurium* PEF inactivation while pectin concentration did not have any significant effect.
10. Shelf-life at 8-10ºC of PEF treated sample was 2.5 weeks with no enzyme reactivation, similar color and microbial inactivation and greater volatile compounds content than thermally treated sample.
11. PEF technology could achieve the same degree of microbial and enzyme inactivation as thermal treatment, but preserving better the original characteristics during the storage of an orange juice-milk beverage.

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8.-ANEXES

8.1 COMMUNICATIONS BASED ON THESIS WORK

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Quality and Safety Aspects of PEF Application in Milk and Milk Products

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The articles published to date on the possibilities of applying the new PEF technology to milk and milk products are summarized in a review that presents them in chronological order and grouped on the basis of the factor studied (microorganism, enzyme, quality parameter, or shelf-life). An accompanying table shows details of the equipment and process corresponding to each article in chronological order.

Keywords milk, milk products, pulsed electric field, review

INTRODUCTION

Among the "New Emerging Technologies," also known as "Nonthermal Technologies," the application of high intensity Pulsed Electric Fields (PEF) is considered a very promising alternative to the processes for pasteurizing certain liquid foods, including milk (whole, skimmed, with different concentrations of milk fat, and simulated milk ultrafiltrated—SMUF) and milk products (yogurt and fortified yogurt drinks). The high inactivation levels achieved in a series of spoilage and pathogenic microorganisms that can develop in the foods quoted, with very short treatment times and minimal impact on quality and nutrition factors, suggest that it is possible to obtain microbiologically safe foods of high quality and with a long shelf-life in refrigeration.

REVIEW

The study of the application of PEF in milk (skimmed, whole, and with different concentrations of milk fat) and milk derivatives (yogurt, simulated milk ultrafiltrated—SMUF) is basically directed at studying the inactivation of numerous microorganisms inoculated in the various products. Authors of this article have tried to reproduce the treatment conditions and results given by the authors of the different studies. When a treatment condition is not well-defined it is because it was not mentioned in the referred manuscript.

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Inactivation of Escherichia coli

Various authors have focused on studying *Escherichia coli*. Dunn and Pearlman (1987) used *Escherichia coli* ATCC 10536 in pasteurized, homogenized milk and compared it with an untreated sample. The inactivation was 99.91%. After 4d the count in the untreated sample was 4.7×10^7, whereas in the pulse-treated sample the count was 3.1×10^3. These counts represented a mixture of milk bacteria and *E.coli*. Zhang et al. (1994) studied inactivation of *Escherichia coli* in SMUF and developed a heat transfer model to predict the temperature attained during application of pulses. The model was verified by comparing the predictions with the experimental results. They achieved 3 log reductions after 20 pulses and E = 25 kV/cm.

Qin et al. (1995a) used *Escherichia coli* in SMUF (with parallel electrodes and coaxial chamber, both continuous) and skim milk (with parallel electrodes, continuous and static). They achieved 2 to 3 log reductions in discontinuous treatment in skim milk, 3 to 4 in continuous treatment with parallel electrodes both in SMUF and in skim milk, and over 7 reductions in continuous treatment with coaxial chamber in SMUF. Pothakamury et al. (1995b) studied inactivation of *Escherichia coli* in SMUF and obtained 4 to 5 log reductions by applying 60 pulses, E = 16 kV/cm, and 300 μs. They compared these

Review: Application of Pulsed Electric Fields in Egg and Egg Derivatives

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This work overviews works published on the application of pulsed electric fields (PEF) in egg and egg derivatives, grouped by subject, and arranged chronologically in terms of the factor studied (microorganisms, quality aspects, shelf life and structural changes in gel formation properties). The inactivation of microorganisms by PEF in egg is very considerable, 3.5 decimal reductions in egg white were achieved by PEF in *Salmonella enteritidis*, 5.5 log reductions of *Listeria innocua* by means of a synergistic effect of PEF and nisin in liquid whole egg, and 5.6 log reductions of *Escherichia coli* in beaten fresh liquid egg by PEF treatment applied continuously or discontinuously in five steps. The shelf life of PEF-treated fresh liquid egg was extended to 4 weeks in refrigeration, and quality (colour, viscosity and sensory attributes) was not affected by PEF treatment. PEF treatment did not cause notable changes in proteins in a solution of ovalbumin and dialysed fresh egg white. However, some structural changes and functional modifications were observed in fresh egg white as a result of PEF treatment. The texture and microstructure of gels were affected by the application of PEF, and therefore PEF treatment conditions in egg white must be optimised to minimise possible modifications.

Key Words: pulsed electric field, egg, microbial inactivation, preservation

INTRODUCTION

The use of liquid whole egg (LWE) and egg products is widely extended in a large number of food industries and establishments. Of the total number of eggs consumed, more than 30% are in the form of egg derivative products (Amiali et al., 2004). At present, the liquid whole egg preservation method is thermal pasteurisation (60°C, 3 min) but undesirable effects can occur, such as decrease in viscosity, changes in functional properties, and reduction of foaming capability (Calderón-Miranda et al., 1999). Slight overheating can also cause coagulation of conalbumin (the most thermosensible protein) and the formation of a film on the heat exchange surface, damaging the functional properties of the treated eggs (Martín-Belloso et al., 1997). For these reasons, a non-thermal technology such as pulsed electric fields (PEF) is considered a very promising alternative to pasteurisation processes for some liquid foods, including liquid whole egg, egg white, and products made with egg. The high inactivation levels achieved with a series of spoilage and pathogenic microorganisms that can grow in these foods, using very short treatment times and producing a minimal impact on quality and nutritional factors, suggest that it is possible to obtain microbiologically safe foods of high quality and with a long shelf life in refrigeration, compared with the traditional treatments with heat.

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Inactivation of Microorganisms by PEF in Egg Products

There are various studies focusing on PEF inactivation of different target microorganisms in egg such as *S. enteritidis*, *L. innocua*, *E. coli* and *P. fluorescens* (Table 1). *E. coli* was used by Martín-Belloso et al. (1997) to study the effectiveness of 2 types of PEF treatment (continuous and batch), frequency and pulse width, finding no differences in the inactivation achieved between the different types of treatments and frequencies. The inactivation was a function of the number of pulses and the pulse width, and it was greatest with a pulse duration of 4 μs, which may be

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ORIGINAL PAPER

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Effect of temperature and substrate on PEF Inactivation of *Lactobacillus plantarum* in an orange juice–milk beverage

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Abstract The inactivation kinetics of *Lactobacillus plantarum* in an orange juice–milk beverage treated by Pulsed Electric Fields (PEF) were studied. Experimental data were fitted to Bigelow and Hülsheger kinetic models and Weibull frequency distribution function. Results indicate that both Hülsheger model and Weibull function fit well the experimental data being Accuracy factor values (Af) closer to 1 and Mean Square Error (MSE) closer to 0. The $\overline{tcw}$ parameter of the Weibull model can be considered as a kinetic indicator as it expresses the microorganism's resistance to treatment by electric pulses. An increase in temperature favoured the inactivation of *L. plantarum* by PEF as reflected by a decreased in $\overline{tcw}$ value. Under the same conditions to those studied by other authors we reached less inactivation of *L. plantarum* in the beverage used in this study than in substrates with a simpler composition.

Keywords PEF · Orange juice · Milk · *L. plantarum* · Inactivation · Modelling

Introduction

The consumption of fruit juices and drinks based on juice and milk subjected to a low pasteurization and stored under refrigeration has experienced a spectacular increase in the last few years, particularly in developed countries. This is in response to a growing demand for minimally processed foods that are perceived by consumers as healthier. The potential attraction of these products is that they preserve the original characteristics of fresh food whilst offering innovative new flavor mixtures. Over the last few years, non-thermal technologies such as the Pulsed Electric Fields (PEF) have been studied on both, laboratory and industrial scale and can be used in the preservation of food as fruit juices, milk and liquid whole egg [1, 2]. They have the potential to inactivate microorganisms while maintaining the products original characteristics. However, studies showing the capability of PEF as an alternative to heat in the inactivation of microorganism in foods with a complex composition are scarce [3–13].

Lactobacillus plantarum is a spoilage microorganism frequently found in juice based products. *L. plantarum* is a spoilage microorganism frequently found in juices. Between 2.7 and 8.9% of *L. plantarum* on the orange's surface enters the juice during extraction and that this level remained constant overtime [14]. When the juice is mixed with other substrates to produce a more sophisticated or nutritional foodstuff, contamination is transferred and there is the likelihood that *L. plantarum* grows and spoils the product. The inactivation of this microorganism by PEF has been studied in different substrates and foodstuffs [6–9, 15, 16]. However, the nature of the inactivation of the microorganism by pulse treatment has not been studied in foods made by a mixture of fruit juice and milk where different components and their interactions can affect the death of the microorganism. Different authors have tried to model the microorganism inactivation curves through predictive mathematical models that fit approximately to the curve shape which relates the survivor fraction with treatment time at different field intensities. The majority of inactivation curves have been analyzed by predictive models which presume a lineal behavior [17, 18] and several authors have applied it to PEF technology [19–24].

As in many emergent preserving technologies, the relationship between the log of the survival fraction and the treatment time does not follow in many cases a lineal behavior, rendering curves with tails or shoulders that make difficult the application of a lineal model. Trying to give an explanation for such behavior, several authors have assumed that each cell has a different resistance to pulse treatment and that the resulting inactivation curve is the sum of a series of lethal events expressed in the form of distribution. One frequency distribution model widely applied to

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Pulsed electric fields inactivation of *Lactobacillus plantarum* in an orange juice–milk based beverage: Effect of process parameters

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Abstract

This work studies the effect of electric field strength, treatment time, process temperature, and pulse width on the inactivation of *Lactobacillus plantarum* inoculated in an orange juice–milk based beverage. For any given quantity of energy applied, the highest degree of inactivation was achieved with high field intensities and short treatment times. The inactivation curve had different slopes, one up to application of 200–285 $\times 10^3$ J L^{-1}, a second stage up to application of 813–891 $\times 10^3$ J L^{-1} in which the inactivation did not increase significantly, and a third stage up to application of 1069–1170 $\times 10^3$ J L^{-1}. When the process temperature was raised to 55 °C the inactivation increased by 0.5 cycles, achieving an energy saving of up to 60%. No increase in inactivation was achieved when the pulse width was increased from 2.5 $\times 10^{-6}$ to 4 $\times 10^{-6}$ s. The inactivation achieved with *L. plantarum* in this beverage is less than that reported by other authors in foods with a simpler composition.

Keywords: PEF; Inactivation; Beverage; *Lactobacillus plantarum*; Orange juice; Milk

1. Introduction

Mixed fruit juices and milk beverages fortified with vitamins, minerals and fiber are among the most widely consumed functional foods (Pszczola, 2005) and consumption of these products has increased by 30% in Europe in the last 10 years, but only 10% belong to the short shelf-life group that needs a chilled chain for their storage and distribution (MAPA, 2003). Nevertheless, it is thought that the trend will increase in the next few years, partly owing to the application of emerging non-thermal technologies such as high hydrostatic pressure (HHP) and pulsed electric fields (PEF), which find an interesting field for application in these kinds of products because they allow cold pasteurization while at the same time preserving their nutritional components.

Studies on the possibility of applying PEF in fruit juices have proliferated in recent years, concentrating mainly on apple and orange juice and other substrates of a different nature such as soymilk (Li & Zhang, 2004), apple cider (Evrendilek et al., 2000), chocolate milk (Evrendilek, Dantzer, Streaker, Ratanatriwong, & Zhang, 2001), mixed juices (Rodrigo, Martínez, Harte, Barbosa-Cánovas, & Rodrigo, 2001; Rodrigo, Barbosa-Cánovas, Martínez, & Rodrigo, 2003), model beer (Ulmer, Heinz, Gänzle, Knorr, & Vogel, 2002), horchata (Spanish beverage) (Selma, Fernández, Valero, & Salmerón, 2003) and beer with gas (Evrendilek, Li, Dantzer, & Zhang, 2004). However, there are no studies on the application of PEF in the inactivation of microorganisms in juice–milk mixtures such as the beverage under study.

In studies on inactivation of microorganisms by PEF, some works were focused on analyzing the influence of process variables on the inactivation, using buffers or foods with a simple composition as a suspension medium such as skim milk (Martín, Swanson, Chang, Qin, & Barbosa-Cánovas, 1997), phosphate buffer (Wouters, Dutreux, Smelt, & Lelieveld, 1999), NaCl and peptone solution (Aronsson, Lindgren, Johansson, & Rönner, 2001; Arons-

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Inactivation kinetics of pectin methyl esterase under combined thermal–high pressure treatment in an orange juice–milk beverage

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Abstract

The inactivation kinetics of PME in an orange juice–milk beverage system treated by thermal (65–80 °C) and combined thermal (25–65 °C)–high pressure (0.1–700 MPa) processes were fitted using a biphasic model. About 6–8% of the initial activity corresponding to the heat and pressure stable fraction was observed. For complete inactivation a treatment at 90 °C, 1 min or 700 MPa at 55 °C for 2 min was necessary showing the protective effect of the orange–milk media. The extent of inactivation was different in the orange matrices showing that PME was more thermostable in the orange juice–milk based beverage system as compared to the purified enzyme in a buffer system. On the other hand, the purified enzyme in a buffer system showed the highest pressure stability. Parameters such as pH (from acid in the orange juice matrices to basic in the buffer), matrix composition (from less to more complex) and purification level of the enzyme (purified in the buffer or nature in the orange juice) play an important role in the stability of the PME against the different processing technologies studied.

Keywords: Orange juice; Milk; Pectin methyl esterase; Inactivation; High pressure; Thermal

1. Introduction

In recent years, a large quantity of new pasteurised liquid foods requiring chilled storage and distribution have appeared on the European market. Among these, fruit juice mixes, vegetable soups and different sauces are predominant; they are produced from food ingredients of very different natures, leading to complex compositions. The fruit juice mixes include beverages based on a mixture of fruit juices and milk, fortified with vitamins, minerals, fiber and these products represent the most widely consumed functional foods (Pszczola, 2005).

For these systems only limited data on quality and safety aspects are available in open literature and it is essential to evaluate the potential use of alternative technologies, such as High Hydrostatic Pressure (HHP), in order to preserve their freshness, as well as their nutritional and functional properties. Studies on the application of HHP to different juice matrices have recently become available but studies on more complex fruit juice based foods (including fat, proteins and additives such as pectin and sugar in an acid environment) are still lacking.

Pectin methyl esterase (PME) is an important enzyme in orange juice products and its effects on quality aspects such as cloud stability are well known (Cameron, Baker, & Grohmann, 1998; Do Amaral, De Assis, & De Faria Oliveira, 2005). Orange PME is a thermal and high pressure stable enzyme composed by several fractions with different processing stabilities (stable and labile fraction). The orange PME stable fraction is mainly responsible for the destabilization of orange juice (cloud loss) at

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8.2 STATISTICAL ANALYSIS

Table 8.2.1: Effect of juice content on PEF inactivation of *E.coli* at 40 kV/cm.

Log (S) by % juice

Método: 95.0 porcentaje HSD de Tukey % juice	Frec.	Media	Grupos homogéneos
15	4	-1.91	X
25	4	-1.9	X
50	4	-1.7225	X
70	4	-1.195	X

Contraste	Diferencias	+/- Límites
15 - 25	-0.01	0.467063
15 - 50	-0.1875	0.467063
15 - 70	*-0.715	0.467063
25 - 50	-0.1775	0.467063
25 - 70	*-0.705	0.467063
50 - 70	*-0.5275	0.467063

* indica una diferencia significativa.

Table 8.2.2: Effect of temperature on volatile compounds concentration after thermal treatment.

Volatile compounds loss (%) by T

Método: 95.0 porcentaje HSD de Tukey T	Frec.	Media	Grupos homogéneos
60	12	15.9806	X
65	12	19.877	XX
70	12	26.1924	XXX
75	12	27.9513	XXX
80	12	33.051	XX
85	12	39.325	X
90	12	41.0536	X

Contraste	Diferencias	+/- Límites
60 - 65	-3.89642	16.3351
60 - 70	-10.2118	16.3351
60 - 75	-11.9708	16.3351
60 - 80	*-17.0704	16.3351
60 - 85	*-23.3444	16.3351
60 - 90	*-25.073	16.3351
65 - 70	-6.31542	16.3351
65 - 75	-8.07433	16.3351
65 - 80	-13.174	16.3351
65 - 85	*-19.448	16.3351
65 - 90	*-21.1766	16.3351
70 - 75	-1.75892	16.3351
70 - 80	-6.85858	16.3351
70 - 85	-13.1326	16.3351
70 - 90	-14.8612	16.3351
75 - 80	-5.09967	16.3351
75 - 85	-11.3737	16.3351
75 - 90	-13.1022	16.3351
80 - 85	-6.274	16.3351
80 - 90	-8.00258	16.3351
85 - 90	-1.72858	16.3351

* indica una diferencia significativa.

Table 8.2.3: Effect of electric field strength on volatile compounds concentration after PEF treatment at 25, 45 and 65ºC.

Volatile compounds loss by E at 25°C

Método: 95.0 porcentaje HSD de Tukey			
E	Frec.	Media	Grupos homogéneos
15	12	-13.6592	X
20	12	-0.378333	XX
25	12	4.67667	XX
30	12	8.26667	X

Contraste	Diferencias	+/- Límites
15 - 20	-13.2808	21.7504
15 - 25	-18.3358	21.7504
15 - 30	*-21.9258	21.7504
20 - 25	-5.055	21.7504
20 - 30	-8.645	21.7504
25 - 30	-3.59	21.7504

* indica una diferencia significativa.

Volatile compounds loss by E at 45°C

Método: 95.0 porcentaje HSD de Tukey			
E	Frec.	Media	Grupos homogéneos
15	12	5.7925	X
20	12	13.4225	X
25	12	17.2022	X
30	12	20.9108	X

Contraste	Diferencias	+/- Límites
15 - 20	-7.63	20.506
15 - 25	-11.4097	20.506
15 - 30	-15.1183	20.506
20 - 25	-3.77967	20.506
20 - 30	-7.48833	20.506
25 - 30	-3.70867	20.506

* indica una diferencia significativa.

Volatile compounds loss by E at 65°C

Método: 95.0 porcentaje HSD de Tukey			
E	Frec.	Media	Grupos homogéneos
15	12	11.5683	X
20	12	18.5675	XX
25	12	26.3691	X
30	12	30.5125	X

Contraste	Diferencias	+/- Límites
15 - 20	-6.99917	14.6277
15 - 25	*-14.8008	14.6277
15 - 30	*-18.9442	14.6277
20 - 25	-7.80165	14.6277
20 - 30	-11.945	14.6277
25 - 30	-4.14335	14.6277

* indica una diferencia significativa.

Table 8.2.4: Effect of temperature on volatile compounds concentration after PEF treatment at 15, 20, 25 and 30 kV/cm.

Volatile compounds loss by T at 15 kV/cm

Método: 95.0 porcentaje HSD de Tukey

T	Frec.	Media	Grupos homogéneos
25	12	-13.6592	X
45	12	5.7925	XX
65	12	11.5683	X

Contraste	Diferencias	+/- Límites
25 - 45	-19.4517	21.2267
25 - 65	*-25.2275	21.2267
45 - 65	-5.77583	21.2267

* indica una diferencia significativa.

Volatile compounds loss by T at 20 kV/cm

Método: 95.0 porcentaje HSD de Tukey

T	Frec.	Media	Grupos homogéneos
25	12	-0.378333	X
45	12	13.4225	XX
65	12	18.5675	X

Contraste	Diferencias	+/- Límites
25 - 45	-13.8008	18.3794
25 - 65	*-18.9458	18.3794
45 - 65	-5.145	18.3794

* indica una diferencia significativa.

Volatile compounds loss by T at 25 kV/cm

Método: 95.0 porcentaje HSD de Tukey

T	Frec.	Media	Grupos homogéneos
25	12	4.67667	X
45	12	17.2022	XX
65	12	26.3691	X

Contraste	Diferencias	+/- Límites
25 - 45	-12.5255	15.3591
25 - 65	*-21.6925	15.3591
45 - 65	-9.16698	15.3591

* indica una diferencia significativa.

Volatile compounds loss by T at 30 kV/cm

Método: 95.0 porcentaje HSD de Tukey

T	Frec.	Media	Grupos homogéneos
25	12	8.26667	X
45	12	20.9108	XX
65	12	30.5125	X

Contraste	Diferencias	+/- Límites
25 - 45	-12.6442	14.9421
25 - 65	*-22.2458	14.9421
45 - 65	-9.60167	14.9421

* indica una diferencia significativa.

Table 8.2.5: Effect of pressure level on volatile compounds concentration after HHP treatment at 30 and 50ºC.

Volatile compounds loss by P at 30°C

Método: 95.0 porcentaje HSD de Tukey

P	Frec.	Media	Grupos homogéneos
500	12	-14.4427	X
450	12	-14.1837	X
550	12	-12.0736	X
600	12	-3.76867	X
650	12	7.47825	X

Contraste	Diferencias	+/- Límites
450 - 500	0.259	31.8213
450 - 550	-2.11008	31.8213
450 - 600	-10.415	31.8213
450 - 650	-21.6619	31.8213
500 - 550	-2.36908	31.8213
500 - 600	-10.674	31.8213
500 - 650	-21.9209	31.8213
550 - 600	-8.30492	31.8213
550 - 650	-19.5518	31.8213
600 - 650	-11.2469	31.8213

* indica una diferencia significativa.

Volatile compounds loss by P at 50°C

Método: 95.0 porcentaje HSD de Tukey

P	Frec.	Media	Grupos homogéneos
450	12	22.9283	X
500	12	30.3541	X
550	12	32.383	X
600	12	35.9505	X
650	12	42.3475	X

Contraste	Diferencias	+/- Límites
450 - 500	-7.42575	22.1508
450 - 550	-9.45467	22.1508
450 - 600	-13.0222	22.1508
450 - 650	-19.4192	22.1508
500 - 550	-2.02892	22.1508
500 - 600	-5.59642	22.1508
500 - 650	-11.9934	22.1508
550 - 600	-3.5675	22.1508
550 - 650	-9.9645	22.1508
600 - 650	-6.397	22.1508

* indica una diferencia significativa.

Table 8.2.6: Effect of temperature on volatile compounds concentration after HHP treatment at 450, 500, 550, 600 and 650 MPa.

Volatile compounds loss by T at 450 MPa

Método: 95.0 porcentaje HSD de Tukey

T	Frec.	Media	Grupos homogéneos
30	12	-14.1837	X
50	12	22.9283	X

Contraste	Diferencias	+/- Límites
30 - 50	*-37.112	20.9987

* indica una diferencia significativa.

Volatile compounds loss by T at 500 MPa

Método: 95.0 porcentaje HSD de Tukey

T	Frec.	Media	Grupos homogéneos
30	12	-14.4427	X
50	12	30.3541	X

Contraste	Diferencias	+/- Límites
30 - 50	*-44.7968	19.6123

* indica una diferencia significativa.

Volatile compounds loss by T at 550 MPa

Método: 95.0 porcentaje HSD de Tukey

T	Frec.	Media	Grupos homogéneos
30	12	-12.0736	X
50	12	32.383	X

Contraste	Diferencias	+/- Límites
30 - 50	*-44.4566	21.1014

* indica una diferencia significativa.

Volatile compounds loss by T at 600 MPa

Método: 95.0 porcentaje HSD de Tukey

T	Frec.	Media	Grupos homogéneos
30	12	-3.76867	X
50	12	35.9505	X

Contraste	Diferencias	+/- Límites
30 - 50	*-39.7192	20.8567

* indica una diferencia significativa.

Volatile compounds loss by T at 650 MPa

Método: 95.0 porcentaje HSD de Tukey

T	Frec.	Media	Grupos homogéneos
30	12	7.47825	X
50	12	42.3475	X

Contraste	Diferencias	+/- Límites
30 - 50	*-34.8693	18.0579

* indica una diferencia significativa.

Table 8.2.7: Effect of treatment time on survivors fraction of *L. plantarum* after PEF treatment at 35 and 40 kV/cm and 35ºC.

Log (S) by Treatment time at 40 kV/cm and 35°C

Método: 95.0 porcentaje HSD de Tukey

Treatment time	Frec.	Media	Grupos homogéneos
130	3	-2.12333	X
110	3	-1.80333	X
80	3	-1.71667	XX
90	3	-1.7	XX
60	3	-1.63667	XX
40	3	-1.53667	X

Contraste	Diferencias	+/- Límites
40 - 60	0.1	0.209094
40 - 80	0.18	0.209094
40 - 90	0.163333	0.209094
40 - 110	*0.266667	0.209094
40 - 130	*0.586667	0.209094
60 - 80	0.08	0.209094
60 - 90	0.0633333	0.209094
60 - 110	0.166667	0.209094
60 - 130	*0.486667	0.209094
80 - 90	-0.0166667	0.209094
80 - 110	0.0866667	0.209094
80 - 130	*0.406667	0.209094
90 - 110	0.103333	0.209094
90 - 130	*0.423333	0.209094
110 - 130	*0.32	0.209094

* indica una diferencia significativa.

Log (S) by Treatment time at 35 kV/cm and 35°C

Método: 95.0 porcentaje HSD de Tukey

Treatment time	Frec.	Media	Grupos homogéneos
180	3	-1.9	X
130	3	-1.70667	X
100	3	-1.67	X
80	3	-1.61333	XX
60	3	-1.58667	XX
40	3	-1.49333	X

Contraste	Diferencias	+/- Límites
40 - 60	0.0933333	0.133595
40 - 80	0.12	0.133595
40 - 100	*0.176667	0.133595
40 - 130	*0.213333	0.133595
40 - 180	*0.406667	0.133595
60 - 80	0.0266667	0.133595
60 - 100	0.0833333	0.133595
60 - 130	0.12	0.133595
60 - 180	*0.313333	0.133595
80 - 100	0.0566667	0.133595
80 - 130	0.0933333	0.133595
80 - 180	*0.286667	0.133595
100 - 130	0.0366667	0.133595
100 - 180	*0.23	0.133595
130 - 180	*0.193333	0.133595

* indica una diferencia significativa.

Table 8.2.8: Effect of electric field strength on survivors fraction of *L. plantarum* after PEF treatment at 40, 60, 80 and 130 μs and 35ºC.

Log (S) by E at 40 micros and 35°C

Método: 95.0 porcentaje HSD de Tukey			
E	Frec.	Media	Grupos homogéneos
40	3	-1.53667	X
35	3	-1.49333	X

Contraste	Diferencias	+/- Límites
35 - 40	0.0433333	0.0692569

* indica una diferencia significativa.

Log (S) by E at 60 micros and 35°C

Método: 95.0 porcentaje HSD de Tukey			
E	Frec.	Media	Grupos homogéneos
40	3	-1.63667	X
35	3	-1.58667	X

Contraste	Diferencias	+/- Límites
35 - 40	0.05	0.130883

* indica una diferencia significativa.

Log (S) by E at 80 micros and 35°C

Método: 95.0 porcentaje HSD de Tukey			
E	Frec.	Media	Grupos homogéneos
40	3	-1.71667	X
35	3	-1.61333	X

Contraste	Diferencias	+/- Límites
35 - 40	*0.103333	0.0471906

* indica una diferencia significativa.

Log (S) by E at 130 micros and 35°C

Método: 95.0 porcentaje HSD de Tukey			
E	Frec.	Media	Grupos homogéneos
40	3	-2.12333	X
35	3	-1.70667	X

Contraste	Diferencias	+/- Límites
35 - 40	*0.416667	0.147498

* indica una diferencia significativa.

Table 8.2.9: Effect of temperature on survivors fraction of *L. plantarum* after PEF treatment at 35 and 40 kV/cm.

Log (S) by T at 40 kV/cm

Método: 95.0 porcentaje HSD de Tukey			
T	Frec.	Media	Grupos homogéneos
55	6	-2.055	X
35	6	-1.75333	X

Contraste	Diferencias	+/- Límites
35 - 55	*0.301667	0.275271

* indica una diferencia significativa.

Log (S) by T at 35 kV/cm

Método: 95.0 porcentaje HSD de Tukey

T	Frec.	Media	Grupos homogéneos
55	18	-1.97278	X
35	18	-1.66167	X

Contraste	Diferencias	+/- Límites
35 - 55	*0.311111	0.0820346

* indica una diferencia significativa.

Table 8.2.10: Effect of treatment time on survivors fraction of *L. plantarum* after PEF treatment at 35 and 40 kV/cm and 55ºC

Log (S) by Treatment time at 40 kV/cm and 55°C

Método: 95.0 porcentaje HSD de Tukey

Treatment time	Frec.	Media	Grupos homogéneos
130	3	-2.45667	X
110	3	-2.16333	X
90	3	-2.01333	XX
80	3	-1.97	XX
60	3	-1.89667	X
40	3	-1.83333	X

Contraste	Diferencias	+/- Límites
40 - 60	0.0633333	0.20177
40 - 80	0.136667	0.20177
40 - 90	0.18	0.20177
40 - 110	*0.33	0.20177
40 - 130	*0.623333	0.20177
60 - 80	0.0733333	0.20177
60 - 90	0.116667	0.20177
60 - 110	*0.266667	0.20177
60 - 130	*0.56	0.20177
80 - 90	0.0433333	0.20177
80 - 110	0.193333	0.20177
80 - 130	*0.486667	0.20177
90 - 110	0.15	0.20177
90 - 130	*0.443333	0.20177
110 - 130	*0.293333	0.20177

* indica una diferencia significativa.

Log (S) by Treatment time at 35 kV/cm and 55°C

Método: 95.0 porcentaje HSD de Tukey

Treatment time	Frec.	Media	Grupos homogéneos
180	3	-2.06667	X
100	3	-2.06	X
130	3	-1.99667	XX
80	3	-1.99333	XX
60	3	-1.88667	XX
40	3	-1.83333	X

Contraste	Diferencias	+/- Límites
40 - 60	0.0533333	0.180097
40 - 80	0.16	0.180097
40 - 100	*0.226667	0.180097
40 - 130	0.163333	0.180097
40 - 180	*0.233333	0.180097
60 - 80	0.106667	0.180097
60 - 100	0.173333	0.180097
60 - 130	0.11	0.180097
60 - 180	0.18	0.180097
80 - 100	0.0666667	0.180097
80 - 130	0.00333333	0.180097
80 - 180	0.0733333	0.180097
100 - 130	-0.0633333	0.180097
100 - 180	0.00666667	0.180097
130 - 180	0.07	0.180097

* indica una diferencia significativa.

Table 8.2.11: Effect of pulse width on survivors fraction of *L. plantarum* after PEF treatment at 40 and 35 kV/cm and 35ºC.

Log (S) by Pulse width at 40 kV/cm and 35°C

Método: 95.0 porcentaje HSD de Tukey Pulse width	Frec.	Media	Grupos homogéneos
2.5	18	-1.75278	X
4	11	-1.69	X

Contraste	Diferencias	+/- Límites
2.5 - 4	-0.0627778	0.13804

* indica una diferencia significativa.

Log (S) by Pulse width at 35 kV/cm and 35°C

Método: 95.0 porcentaje HSD de Tukey Pulse width	Frec.	Media	Grupos homogéneos
4	12	-1.74583	X
2.5	18	-1.66167	X

Contraste	Diferencias	+/- Límites
2.5 - 4	0.0841667	0.129114

* indica una diferencia significativa.

Table 8.2.12: Effect of pulse width on survivors fraction of *L. plantarum* after PEF treatment at 40 and 35 kV/cm and 55ºC.

Log (S) by Pulse width at 40 kV/cm and 55°C

Método: 95.0 porcentaje HSD de Tukey Pulse width	Frec.	Media	Grupos homogéneos
2.5	18	-2.05556	X
4	12	-1.96	X

Contraste	Diferencias	+/- Límites
2.5 - 4	-0.0955556	0.161506

* indica una diferencia significativa.

Log (S) by Pulse width at 35 kV/cm and 55°C

Método: 95.0 porcentaje HSD de Tukey Pulse width	Frec.	Media	Grupos homogéneos
4	12	-2.04667	X
2.5	18	-1.97278	X

Contraste	Diferencias	+/- Límites
2.5 - 4	0.0738889	0.133183

* indica una diferencia significativa.

Table 8.2.13: Effect of electric field strength on *D* value of Bigelow model for *L. plantarum*.

D value by E

Método: 95.0 porcentaje HSD de Tukey

E	Frec.	Media	Grupos homogéneos
40	2	51.165	X
35	2	68.019	X
25	2	130.205	X
15	2	294.74	X

Contraste	Diferencias	+/- Límites
15 - 25	*164.535	16.171
15 - 35	*226.721	16.171
15 - 40	*243.575	16.171
25 - 35	*62.186	16.171
25 - 40	*79.04	16.171
35 - 40	*16.854	16.171

* indica una diferencia significativa.

Table 8.2.14: Effect of electric field strength on $\overline{tcw}$ value for *L. plantarum*.

tcw value by E

Método: 95.0 porcentaje HSD de Tukey

E	Frec.	Media	Grupos homogéneos
40	2	7.185	X
35	2	9.45	X
25	2	43.155	X
15	2	61.01	X

Contraste	Diferencias	+/- Límites
15 - 25	*17.855	10.7613
15 - 35	*51.56	10.7613
15 - 40	*53.825	10.7613
25 - 35	*33.705	10.7613
25 - 40	*35.97	10.7613
35 - 40	2.265	10.7613

* indica una diferencia significativa.

Table 8.2.15: Effect of temperature on $\overline{tcw}$ value for *L. plantarum*.

tcw value by T

Método: 95.0 porcentaje HSD de Tukey

T	Frec.	Media	Grupos homogéneos
55	3	4.11333	X
35	3	7.33	X

Contraste	Diferencias	+/- Límites
35 - 55	*3.21667	0.918097

* indica una diferencia significativa.

Table 8.2.16: Effect of electric field strength on *D* value of Bigelow model for *S. typhimurium*.

D value by E

Método: 95.0 porcentaje HSD de Tukey

E	Frec.	Media	Grupos homogéneos
40	2	149.015	X
35	2	234.81	X
25	2	454.25	X
15	2	1454.65	X

Contraste	Diferencias	+/- Límites
15 - 25	*1000.4	65.6374
15 - 35	*1219.84	65.6374
15 - 40	*1305.63	65.6374
25 - 35	*219.44	65.6374
25 - 40	*305.235	65.6374
35 - 40	*85.795	65.6374

* indica una diferencia significativa.

Table 8.2.17: Effect of electric field strength on *tc* value of Hülsheger model for *S. typhimurium*.

tc value by E

Método: 95.0 porcentaje HSD de Tukey

E	Frec.	Media	Grupos homogéneos
40	2	1.365	X
35	2	4.6	X
25	1	22.56	X
15	1	54.87	X

Contraste	Diferencias	+/- Límites
15 - 25	*32.31	7.34749
15 - 35	*50.27	6.36311
15 - 40	*53.505	6.36311
25 - 35	*17.96	6.36311
25 - 40	*21.195	6.36311
35 - 40	3.235	5.19546

* indica una diferencia significativa.

Table 8.2.18: Effect of electric field strength on $\overline{tcw}$ value for *S. typhimurium*.

tcw value by E

Método: 95.0 porcentaje HSD de Tukey

E	Frec.	Media	Grupos homogéneos
40	2	24.605	X
35	2	38.375	X
25	1	111.111	X
15	1	484.51	X

Contraste	Diferencias	+/- Límites
15 - 25	*373.399	60.1472
15 - 35	*446.135	52.089
15 - 40	*459.905	52.089
25 - 35	*72.736	52.089
25 - 40	*86.506	52.089
35 - 40	13.77	42.5305

* indica una diferencia significativa.

Table 8.2.19: Effect of type of microorganism on $\overline{tcw}$ value.

tcw value by Microorganism at 15 kV/cm

Método: 95.0 porcentaje HSD de Tukey

Microorganism	Frec.	Media	Grupos homogéneos
E. coli	2	5.1	X
L. plantarum	2	61.01	X
S. typhimurium	1	484.51	X

Contraste	Diferencias	+/- Límites
E. coli - L. plantarum	*-55.91	17.7562
E. coli - S. typhimurium	*-479.41	21.7468
L. plantarum - S. typhimurium	*-423.5	21.7468

* indica una diferencia significativa.

tcw value by Microorganism at 25 kV/cm

Método: 95.0 porcentaje HSD de Tukey

Microorganism	Frec.	Media	Grupos homogéneos
E. coli	2	2.83	X
L. plantarum	2	43.155	X
S. typhimurium	1	111.11	X

Contraste	Diferencias	+/- Límites
E. coli - L. plantarum	*-40.325	11.7816
E. coli - S. typhimurium	*-108.28	14.4295
L. plantarum - S. typhimurium	*-67.955	14.4295

* indica una diferencia significativa.

tcw value by Microorganism at 35 kV/cm

Método: 95.0 porcentaje HSD de Tukey

Microorganism	Frec.	Media	Grupos homogéneos
E. coli	2	2.19	X
L. plantarum	2	9.45	X
S. typhimurium	2	38.375	X

Contraste	Diferencias	+/- Límites
E. coli - L. plantarum	-7.26	20.7949
E. coli - S. typhimurium	*-36.185	20.7949
L. plantarum - S. typhimurium	*-28.925	20.7949

* indica una diferencia significativa.

tcw value by Microorganism at 40 kV/cm

Método: 95.0 porcentaje HSD de Tukey

Microorganism	Frec.	Media	Grupos homogéneos
E. coli	2	1.11	X
L. plantarum	2	7.185	X
S. typhimurium	2	24.605	X

Contraste	Diferencias	+/- Límites
E. coli - L. plantarum	*-6.075	4.42459
E. coli - S. typhimurium	*-23.495	4.42459
L. plantarum - S. typhimurium	*-17.42	4.42459

* indica una diferencia significativa.

Table 8.2.20: Effect of pH on $\overline{tcw}$ value at 15 and 40 kV/cm.

tcw by pH at 40 kV/cm

Método: 95.0 porcentaje HSD de Tukey

pH	Frec.	Media	Grupos homogéneos
3.5	2	16.66	X
4	2	40.03	XX
4.5	2	61.365	X

Contraste	Diferencias	+/- Límites
3.5 - 4	-23.37	24.9119
3.5 - 4.5	*-44.705	24.9119
4 - 4.5	-21.335	24.9119

* indica una diferencia significativa.

tcw by pH at 15 kV/cm

Método: 95.0 porcentaje HSD de Tukey

pH	Frec.	Media	Grupos homogéneos
3.5	2	249.05	X
4	2	371.4	X
4.5	2	488.74	X

Contraste	Diferencias	+/- Límites
3.5 - 4	*-122.35	120.964
3.5 - 4.5	*-239.69	120.964
4 - 4.5	-117.34	120.964

* indica una diferencia significativa.

Table 8.2.21: Effect of pectin concentration on $\overline{tcw}$ value at 15 and 40 kV/cm.

tcw by pectin concentration at 40 kV/cm

Método: 95.0 porcentaje HSD de Tukey

% pectina	Frec.	Media	Grupos homogéneos
0.1	2	37.37	X
0.6	2	45.5	X
0.3	2	61.365	X

Contraste	Diferencias	+/- Límites
0.1 - 0.3	-23.995	25.8898
0.1 - 0.6	-8.13	25.8898
0.3 - 0.6	15.865	25.8898

* indica una diferencia significativa.

tcw by pectin concentration at 15 kV/cm

Método: 95.0 porcentaje HSD de Tukey

% pectina	Frec.	Media	Grupos homogéneos
0.1	2	402.67	X
0.3	2	488.74	X
0.6	2	516.925	X

Contraste	Diferencias	+/- Límites
0.1 - 0.3	-86.07	151.009
0.1 - 0.6	-114.255	151.009
0.3 - 0.6	-28.185	151.009

* indica una diferencia significativa.

Table 8.2.22: Effect of electric field strength on. $\overline{tcw}$ value at 0.1, 0.3 and 0.6%

tcw by E at 0.1%

Método: 95.0 porcentaje HSD de Tukey

E	Frec.	Media	Grupos homogéneos
40	2	37.37	X
15	2	402.67	X

Contraste	Diferencias	+/- Límites
15 - 40	*365.3	34.556

* indica una diferencia significativa.

tcw by E at 0.3%

Método: 95.0 porcentaje HSD de Tukey

E	Frec.	Media	Grupos homogéneos
40	2	61.365	X
15	2	488.74	X

Contraste	Diferencias	+/- Límites
15 - 40	*427.375	52.5437

* indica una diferencia significativa.

tcw value by E at 0.6%

Método: 95.0 porcentaje HSD de Tukey

E	Frec.	Media	Grupos homogéneos
40	2	45.5	X
15	2	516.925	X

Contraste	Diferencias	+/- Límites
15 - 40	*471.425	182.85

* indica una diferencia significativa.

Table 8.2.23: pH value after PEF and thermal treatment.

pH value by Treatment

Método: 95.0 porcentaje HSD de Tukey

Treatment	Frec.	Media	Grupos homogéneos
PEF	3	4.15667	X
Blank	2	4.305	X
Thermal	3	4.38	X

Contraste	Diferencias	+/- Límites
Blank - PEF	*0.148333	0.0443892
Blank - Thermal	*-0.075	0.0443892
PEF - Thermal	*-0.223333	0.0397029

* indica una diferencia significativa.

Table 8.2.24: pH value during storage of PEF, thermal and untreated sample.

pH value by Week PEF sample

Método: 95.0 porcentaje HSD de Tukey			
Week	Frec.	Media	Grupos homogéneos
4	3	4.16333	X
2	3	4.20333	X
3	3	4.23333	X
0	3	4.34667	X
1	3	4.35333	X

Contraste	Diferencias	+/- Límites
0 - 1	-0.00666667	0.0310313
0 - 2	*0.143333	0.0310313
0 - 3	*0.113333	0.0310313
0 - 4	*0.183333	0.0310313
1 - 2	*0.15	0.0310313
1 - 3	*0.12	0.0310313
1 - 4	*0.19	0.0310313
2 - 3	-0.03	0.0310313
2 - 4	*0.04	0.0310313
3 - 4	*0.07	0.0310313

* indica una diferencia significativa.

pH value by Week thermal sample

Método: 95.0 porcentaje HSD de Tukey			
Week	Frec.	Media	Grupos homogéneos
2	3	4.11667	X
4	3	4.13	X
3	3	4.13667	X
1	3	4.24333	X
0	3	4.28	X

Contraste	Diferencias	+/- Límites
0 - 1	*0.0366667	0.0240368
0 - 2	*0.163333	0.0240368
0 - 3	*0.143333	0.0240368
0 - 4	*0.15	0.0240368
1 - 2	*0.126667	0.0240368
1 - 3	*0.106667	0.0240368
1 - 4	*0.113333	0.0240368
2 - 3	-0.02	0.0240368
2 - 4	-0.0133333	0.0240368
3 - 4	0.00666667	0.0240368

* indica una diferencia significativa.

pH value by Week Untreated sample

Método: 95.0 porcentaje HSD de Tukey			
Week	Frec.	Media	Grupos homogéneos
4	3	4.01667	X
3	3	4.15	X
2	3	4.16	X
1	3	4.18333	X
0	2	4.305	X

Contraste	Diferencias	+/- Límites
0 - 1	*0.121667	0.0477713
0 - 2	*0.145	0.0477713
0 - 3	*0.155	0.0477713
0 - 4	*0.288333	0.0477713
1 - 2	0.0233333	0.0427279
1 - 3	0.0333333	0.0427279
1 - 4	*0.166667	0.0427279
2 - 3	0.01	0.0427279
2 - 4	*0.143333	0.0427279
3 - 4	*0.133333	0.0427279

* indica una diferencia significativa.

Table 8.2.25: Brix degrees after PEF and thermal treatment.

Brix degrees by Treatment

Método: 95.0 porcentaje HSD de Tukey

Treatment	Frec.	Media	Grupos homogéneos
PEF	3	14.6667	X
Blank	3	15.0667	X
Thermal	3	15.1	X

Contraste	Diferencias	+/- Límites
Blank - PEF	*0.4	0.186731
Blank - Thermal	-0.0333333	0.186731
PEF - Thermal	*-0.433333	0.186731

* indica una diferencia significativa.

Table 8.2.26: Brix degrees during the storage of PEF, thermal and untreated sample.

Brix degrees by Week PEF sample

Método: 95.0 porcentaje HSD de Tukey

Week	Frec.	Media	Grupos homogéneos
3	3	14.3667	X
2	3	14.6	X
1	3	14.6	X
4	3	14.6	X
0	2	14.65	X

Contraste	Diferencias	+/- Límites
0 - 1	0.05	0.34164
0 - 2	0.05	0.34164
0 - 3	0.283333	0.34164
0 - 4	0.05	0.34164
1 - 2	0.0	0.305572
1 - 3	0.233333	0.305572
1 - 4	0.0	0.305572
2 - 3	0.233333	0.305572
2 - 4	0.0	0.305572
3 - 4	-0.233333	0.305572

* indica una diferencia significativa.

Brix degrees by Week Thermal sample

Método: 95.0 porcentaje HSD de Tukey

Week	Frec.	Media	Grupos homogéneos
4	3	13.9	X
3	3	14.8	X
2	3	14.9333	XX
1	3	14.9333	XX
0	3	15.0667	X

Contraste	Diferencias	+/- Límites
0 - 1	0.133333	0.208164
0 - 2	0.133333	0.208164
0 - 3	*0.266667	0.208164
0 - 4	*1.16667	0.208164
1 - 2	0.0	0.208164
1 - 3	0.133333	0.208164
1 - 4	*1.03333	0.208164
2 - 3	0.133333	0.208164
2 - 4	*1.03333	0.208164
3 - 4	*0.9	0.208164

* indica una diferencia significativa.

Brix degrees by Week Untreated sample

Método: 95.0 porcentaje HSD de Tukey			
Week	Frec.	Media	Grupos homogéneos
4	3	13.9	X
3	3	14.8	X
2	3	14.9333	XX
1	3	14.9333	XX
0	3	15.0667	X

Contraste	Diferencias	+/- Límites
0 - 1	0.133333	0.208164
0 - 2	0.133333	0.208164
0 - 3	*0.266667	0.208164
0 - 4	*1.16667	0.208164
1 - 2	0.0	0.208164
1 - 3	0.133333	0.208164
1 - 4	*1.03333	0.208164
2 - 3	0.133333	0.208164
2 - 4	*1.03333	0.208164
3 - 4	*0.9	0.208164

* indica una diferencia significativa.

Table 8.2.27: Microbial inactivation after PEF and thermal treatment.

Log S by Treatment for microbial counts

Método: 95.0 porcentaje HSD de Tukey			
Treatment	Frec.	Media	Grupos homogéneos
PEF	3	-4.59333	X
Thermal	4	-4.45414	X

Contraste	Diferencias	+/- Límites
PEF - Thermal	-0.139197	0.154954

* indica una diferencia significativa.

Table 8.2.28: PME activity after PEF and thermal treatment.

PME activity by Treatment

Método: 95.0 porcentaje HSD de Tukey			
Treatment	Frec.	Media	Grupos homogéneos
PEF	3	0.0356667	X
Thermal	3	0.0393333	X
Blank	4	0.2615	X

Contraste	Diferencias	+/- Límites
Blank - PEF	*0.225833	0.0477405
Blank - Thermal	*0.222167	0.0477405
PEF - Thermal	-0.00366667	0.0510367

* indica una diferencia significativa.

Table 8.2.29: PME activity during the storage of PEF, thermal and untreated sample.

PME activity by Week PEF sample

Método: 95.0 porcentaje HSD de Tukey

Week	Frec.	Media	Grupos homogéneos
3	3	0.027	X
4	4	0.02825	X
0	3	0.0356667	X
1	2	0.0595	X
2	2	0.0895	X

Contraste	Diferencias	+/- Límites
0 - 1	*-0.0238333	0.0237228
0 - 2	*-0.0538333	0.0237228
0 - 3	0.00866667	0.0212183
0 - 4	0.00741667	0.0198479
1 - 2	*-0.03	0.025987
1 - 3	*0.0325	0.0237228
1 - 4	*0.03125	0.0225054
2 - 3	*0.0625	0.0237228
2 - 4	*0.06125	0.0225054
3 - 4	-0.00125	0.0198479

* indica una diferencia significativa.

PME activity by Week Thermal sample

Método: 95.0 porcentaje HSD de Tukey

Week	Frec.	Media	Grupos homogéneos
4	3	0.026	X
0	3	0.0393333	XX
3	3	0.0476667	XX
1	4	0.05275	X
2	4	0.06	X

Contraste	Diferencias	+/- Límites
0 - 1	-0.0134167	0.0250277
0 - 2	-0.0206667	0.0250277
0 - 3	-0.00833333	0.0267558
0 - 4	0.0133333	0.0267558
1 - 2	-0.00725	0.0231712
1 - 3	0.00508333	0.0250277
1 - 4	*0.02675	0.0250277
2 - 3	0.0123333	0.0250277
2 - 4	*0.034	0.0250277
3 - 4	0.0216667	0.0267558

* indica una diferencia significativa.

PME activity by Week Untreated sample

Método: 95.0 porcentaje HSD de Tukey

Week	Frec.	Media	Grupos homogéneos
3	3	0.179	X
4	3	0.200667	XX
2	4	0.22825	XX
1	4	0.25175	X
0	4	0.2615	X

Contraste	Diferencias	+/- Límites
0 - 1	0.00975	0.0432263
0 - 2	0.03325	0.0432263
0 - 3	*0.0825	0.0466898
0 - 4	*0.0608333	0.0466898
1 - 2	0.0235	0.0432263
1 - 3	*0.07275	0.0466898
1 - 4	*0.0510833	0.0466898
2 - 3	*0.04925	0.0466898
2 - 4	0.0275833	0.0466898
3 - 4	-0.0216667	0.0499135

* indica una diferencia significativa.

Table 8.2.30: L* value after PEF and thermal treatment.

L value by Treatment

Método: 95.0 porcentaje HSD de Tukey

Treatment	Frec.	Media	Grupos homogéneos
Thermal	3	55.6833	X
PEF	3	55.9233	X
Blank	3	56.3033	X

Contraste	Diferencias	+/- Límites
Blank - PEF	*0.38	0.0891627
Blank - Thermal	*0.62	0.0891627
PEF - Thermal	*0.24	0.0891627

* indica una diferencia significativa.

Table 8.2.31: L* value during the storage of PEF, thermal and untreated sample.

L value by Week PEF sample

Método: 95.0 porcentaje HSD de Tukey

Week	Frec.	Media	Grupos homogéneos
4	3	55.4133	X
0	3	55.61	X
2	3	56.2067	X
1	3	57.2667	X
3	3	57.54	X

Contraste	Diferencias	+/- Límites
0 - 1	*-1.65667	0.176224
0 - 2	*-0.596667	0.176224
0 - 3	*-1.93	0.176224
0 - 4	*0.196667	0.176224
1 - 2	*1.06	0.176224
1 - 3	*-0.273333	0.176224
1 - 4	*1.85333	0.176224
2 - 3	*-1.33333	0.176224
2 - 4	*0.793333	0.176224
3 - 4	*2.12667	0.176224

* indica una diferencia significativa.

L value by Week Thermal sample

Método: 95.0 porcentaje HSD de Tukey

Week	Frec.	Media	Grupos homogéneos
4	3	56.5667	X
2	3	57.16	X
0	3	57.7	X
3	3	58.0867	X
1	3	58.6067	X

Contraste	Diferencias	+/- Límites
0 - 1	*-0.906667	0.272476
0 - 2	*0.54	0.272476
0 - 3	*-0.386667	0.272476
0 - 4	*1.13333	0.272476
1 - 2	*1.44667	0.272476
1 - 3	*0.52	0.272476
1 - 4	*2.04	0.272476
2 - 3	*-0.926667	0.272476
2 - 4	*0.593333	0.272476
3 - 4	*1.52	0.272476

* indica una diferencia significativa.

L value by Week Untreated sample

Método: 95.0 porcentaje HSD de Tukey

Week	Frec.	Media	Grupos homogéneos
2	3	56.1167	X
0	3	56.3033	XX
4	3	56.4267	X
1	3	56.7833	X
3	3	58.08	X

Contraste	Diferencias	+/- Límites
0 - 1	*-0.48	0.199061
0 - 2	0.186667	0.199061
0 - 3	*-1.77667	0.199061
0 - 4	-0.123333	0.199061
1 - 2	*0.666667	0.199061
1 - 3	*-1.29667	0.199061
1 - 4	*0.356667	0.199061
2 - 3	*-1.96333	0.199061
2 - 4	*-0.31	0.199061
3 - 4	*1.65333	0.199061

* indica una diferencia significativa.

Table 8.2.32: a* value after PEF and thermal treatment.

a value by Treatment

Método: 95.0 porcentaje HSD de Tukey

Treatment	Frec.	Media	Grupos homogéneos
Thermal	3	-0.37	X
PEF	3	0.806667	X
Blank	3	1.85	X

Contraste	Diferencias	+/- Límites
Blank - PEF	*1.04333	0.10952
Blank - Thermal	*2.22	0.10952
PEF - Thermal	*1.17667	0.10952

* indica una diferencia significativa.

Table 8.2.33: a* value during the storage of PEF, thermal and untreated sample.

a value by Week PEF sample

Método: 95.0 porcentaje HSD de Tukey

Week	Frec.	Media	Grupos homogéneos
2	3	0.43	X
1	3	0.47	XX
0	3	0.586667	XX
3	3	0.62	X
4	3	0.696667	X

Contraste	Diferencias	+/- Límites
0 - 1	0.116667	0.127001
0 - 2	*0.156667	0.127001
0 - 3	-0.0333333	0.127001
0 - 4	-0.11	0.127001
1 - 2	0.04	0.127001
1 - 3	*-0.15	0.127001
1 - 4	*-0.226667	0.127001
2 - 3	*-0.19	0.127001
2 - 4	*-0.266667	0.127001
3 - 4	-0.0766667	0.127001

* indica una diferencia significativa.

a value by Week Thermal sample

Método: 95.0 porcentaje HSD de Tukey

Week	Frec.	Media	Grupos homogéneos
2	3	-0.333333	X
4	3	-0.33	X
3	3	-0.316667	X
1	3	-0.306667	X
0	3	-0.24	X

Contraste	Diferencias	+/- Límites
0 - 1	0.0666667	0.0991061
0 - 2	0.0933333	0.0991061
0 - 3	0.0766667	0.0991061
0 - 4	0.09	0.0991061
1 - 2	0.0266667	0.0991061
1 - 3	0.01	0.0991061
1 - 4	0.0233333	0.0991061
2 - 3	-0.0166667	0.0991061
2 - 4	-0.00333333	0.0991061
3 - 4	0.0133333	0.0991061

* indica una diferencia significativa.

a value by Week Untreated sample

Método: 95.0 porcentaje HSD de Tukey

Week	Frec.	Media	Grupos homogéneos
1	3	1.74333	X
3	3	1.77667	XX
2	3	1.81	XX
0	3	1.85	X
4	3	2.04333	X

Contraste	Diferencias	+/- Límites
0 - 1	*0.106667	0.0760109
0 - 2	0.04	0.0760109
0 - 3	0.0733333	0.0760109
0 - 4	*-0.193333	0.0760109
1 - 2	-0.0666667	0.0760109
1 - 3	-0.0333333	0.0760109
1 - 4	*-0.3	0.0760109
2 - 3	0.0333333	0.0760109
2 - 4	*-0.233333	0.0760109
3 - 4	*-0.266667	0.0760109

* indica una diferencia significativa.

Table 8.2.34: b* value after PEF and thermal treatment.

b value by Treatment

Método: 95.0 porcentaje HSD de Tukey

Treatment	Frec.	Media	Grupos homogéneos
Thermal	3	23.97	X
PEF	3	27.8633	X
Blank	3	38.8233	X

Contraste	Diferencias	+/- Límites
Blank - PEF	*10.96	0.154885
Blank - Thermal	*14.8533	0.154885
PEF - Thermal	*3.89333	0.154885

* indica una diferencia significativa.

Table 8.2.35: b* value during the storage of PEF, thermal and untreated sample.

b value by Week PEF sample

Método: 95.0 porcentaje HSD de Tukey Week	Frec.	Media	Grupos homogéneos
3	3	25.8067	X
1	3	26.4267	X
4	3	26.8633	X
2	3	27.4767	X
0	3	27.8633	X

Contraste	Diferencias	+/- Límites
0 - 1	*1.43667	0.246206
0 - 2	*0.386667	0.246206
0 - 3	*2.05667	0.246206
0 - 4	*1.0	0.246206
1 - 2	*-1.05	0.246206
1 - 3	*0.62	0.246206
1 - 4	*-0.436667	0.246206
2 - 3	*1.67	0.246206
2 - 4	*0.613333	0.246206
3 - 4	*-1.05667	0.246206

* indica una diferencia significativa.

b value by Week Thermal sample

Método: 95.0 porcentaje HSD de Tukey Week	Frec.	Media	Grupos homogéneos
3	3	23.2367	X
0	3	23.96	X
1	3	24.2	XX
2	3	24.51	X
4	3	26.4867	X

Contraste	Diferencias	+/- Límites
0 - 1	-0.24	0.332991
0 - 2	*-0.55	0.332991
0 - 3	*0.723333	0.332991
0 - 4	*-2.52667	0.332991
1 - 2	-0.31	0.332991
1 - 3	*0.963333	0.332991
1 - 4	*-2.28667	0.332991
2 - 3	*1.27333	0.332991
2 - 4	*-1.97667	0.332991
3 - 4	*-3.25	0.332991

* indica una diferencia significativa.

b value by Week Untreated sample

Método: 95.0 porcentaje HSD de Tukey Week	Frec.	Media	Grupos homogéneos
3	3	35.5667	X
4	3	37.8033	X
1	3	37.9067	X
0	3	38.8233	X
2	3	39.28	X

Contraste	Diferencias	+/- Límites
0 - 1	*0.916667	0.410975
0 - 2	*-0.456667	0.410975
0 - 3	*3.25667	0.410975
0 - 4	*1.02	0.410975
1 - 2	*-1.37333	0.410975
1 - 3	*2.34	0.410975
1 - 4	0.103333	0.410975
2 - 3	*3.71333	0.410975
2 - 4	*1.47667	0.410975
3 - 4	*-2.23667	0.410975

* indica una diferencia significativa.

Table 8.2.36: Nonanal, caryophyllene, valencene and dodecanal content loss after PEF, and thermal treatment.

Nonanal content loss by Treatment

Método: 95.0 porcentaje HSD de Tukey			
Treatment	Frec.	Media	Grupos homogéneos
PEF	3	18.5367	X
Thermal	3	31.106	X
Contraste		**Diferencias**	**+/- Límites**
PEF - Thermal		-12.5693	15.415

* indica una diferencia significativa.

Caryophillene content loss by Treatment

Método: 95.0 porcentaje HSD de Tukey			
Treatment	Frec.	Media	Grupos homogéneos
PEF	3	35.927	X
Thermal	3	39.522	X
Contraste		**Diferencias**	**+/- Límites**
PEF - Thermal		-3.595	3.7893

* indica una diferencia significativa.

Dodecanal content loss by Treatment

Método: 95.0 porcentaje HSD de Tukey			
Treatment	Frec.	Media	Grupos homogéneos
PEF	3	14.423	X
Thermal	3	16.6443	X
Contraste		**Diferencias**	**+/- Límites**
PEF - Thermal		-2.22133	13.0952

* indica una diferencia significativa.

Valencene content loss by Treatment

Método: 95.0 porcentaje HSD de Tukey			
Treatment	Frec.	Media	Grupos homogéneos
PEF	3	21.764	X
Thermal	3	27.74	X
Contraste		**Diferencias**	**+/- Límites**
PEF - Thermal		-5.976	6.60165

* indica una diferencia significativa.