Effects of Pulsed Electric Field Processing on Microbial, Enzymatic and Physical Attributes of Milk and the Rennet-Induced Milk Gels

A thesis submitted in total fulfilment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Kambiz Shamsi

23/12/2008

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ABBREVIATIONS

AlP	Alkaline phosphatase
AMC	7-amino-4-methylcoumarin
ANOVA	Analysis of variance
^{o}C	Degrees Celsius
CFU	Colony forming units
D	Diameter
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FSANZ	Food Standards Australia New Zealand
g	Grams
g	Pull of gravity (Relative centrifugal force)
h	Hours
HPP	High pressure processing
HTST	High temperature short time
kDa	Kilo Dalton
L	Litres
LAB	Lactic acid bacteria
LDH	Lactate dehydrogenase
LPO	Lactoperoxidase
LSD	Least significant difference
LTLT	Low temperature long time
M	Molar
MFGM	Milk fat globule membrane
mg	Milligrams
min	Minutes
mL	Millilitres
mm	Milllimetres
mM	Millimolar
MPa	Mega Pascals
MW	Molecular weight

μ	Fluid viscosity dynamic
µg	Micrograms
µL	Microlitres
µmol	Micromoles
N	Newtons
NaOH	Sodium hydroxide
nm	Nanometres
OSU	Ohio State University
Pa	Pascals
PEF	Pulsed electric field
PAs	Plasminogen activators
PMSF	Phenylmethanesulphonylfluoride
p-NP	*p*-nitrophenol
POD	Peroxidase
PPO	Polyphenol oxidase
RCT	Rennet coagulation time
RSM	Raw skim milk
ρ	Density
s	Seconds
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SMUF	Simulated milk ultrafiltrate
TG	Triglycerides
TCA	Trichloroacetic acid
U	Units
UHT	Ultra high temperature
USDA	United States Department of Agriculture
V	Fluid velocity
XO	Xanthine oxidase

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ABSTRACT

Pulsed electric field (PEF) is a non-thermal processing technology capable of inactivating microorganisms and enzymes in liquid foods flowing between two electrodes at field intensity of 20 to 80 kV cm^{-1}. In the current study, the effects of PEF treatment in combination with mild heat on microflora and native enzymes in raw bovine milk as well as its effects on rheological and textural properties of rennet-induced gels of milk were investigated.

The effects of PEF treatments at field intensities of 25-37 kV cm^{-1}, final outlet temperatures of 30℃ and 60ºC and treatment time of 19.2 µs on the inactivation of alkaline phosphatase (AlP), Total Plate Count (TPC), *Pseudomonas* and *Enterobacteriaceae* counts were determined in skim milk. For the outlet temperatures of 30 and 60℃, the inlet temperatures were maintained at 20 and 50℃, respectively

At 30ºC, PEF treatments of 28, 31, 34 and 37 kV cm^{-1} inactivated AlP by 24, 25, 31 and 42% and <1 log reduction was observed in TPC and *Pseudomonas* count, while the *Enterobacteriaceae* count was reduced by at least 2.1 logs below the detection limit of 1 colony forming units (CFU) mL^{-1}. At 60ºC PEF treatments of 25, 29, 31 and 35 kV cm^{-1} resulted in 29, 42, 56 and 67% inactivation of AlP and up to 2.4 logs reduction in TPC, while the *Pseudomonas* and *Enterobacteriaceae* counts were reduced by at least 5.9 and 2.1 logs, respectively, to below the detection limit of 1 CFU mL^{-1}. A combined effect was observed between the field intensity and heat in inactivation of both AlP enzyme and the natural microflora in skim milk. Fitting the AlP inactivation data to Hülsheger kinetic model suggested that the

slope of the linear relationship between the field intensity and AlP inactivation at 60℃ was more than twice of that at 30℃. Under these experiments conditions AlP was found to be an unsuitable indicator for PEF treatment efficiency.

The combined effects of field intensity (29 and 35 kV cm^{-1}) and heat (35, 45, 55, 65 and 75℃) on the selected native milk enzymes in skim milk were investigated. The maximum inactivation level belonged to AlP followed by total lipase, XO and plasmin both with and without PEF effects. The inactivation levels showed an increasing trend as the field intensity and temperature was increased. As temperature reached ≥65^{o}C, heat became the dominant inactivating factor, therefore temperatures ≤60^{o}C were suggested as the highest applicable temperature for PEF treatment of milk.

The combined effects of various PEF treatment times and heat on the native milk enzymes through changing either the pulse frequencies (100-400 Hz) or the flow rates (30-240 mL min^{-1}) at a constant field intensity of 31 kV cm^{-1} was investigated at final processing temperatures of 30 and 60℃. The resulting treatment times were 4.8, 9.6, 19.2, 28.8 and 38.4 μs. Changing the pulse frequency and/or flow rate resulted in various levels of enzyme inactivation; flow rate being more effective due to longer exposure time of milk enzyme to heat. As expected, the most sensitive enzyme to PEF treatment was AlP followed by total lipase, XO and plasmin. The milk flow pattern in the PEF system calculated from Reynolds number was laminar in the tubing and transitional in treatment chamber at the highest flow rate of 240 mL min^{-1} resulting in a relative even distribution of heat and field intensity in the treatment chamber.

Rheological (Elastic modulus *G′,* viscous modulus *G″* and rennet coagulation time, RCT) and textural properties (firmness) of rennet-induced gels made from milks (whole and skim) treated at field intensities of 35 to 50 kV cm^{-1} were studied. The size of casein micelles and fat globules was not affected by PEF treatment while high-temperature treatment of milk resulted in a marginal decrease in micelle and fat globule size. The rennet coagulation time (RCT) increased as a result of PEF treatment; however, compared to pasteurisation process it was shorter. Gels made from non-PEF treated control skim milk showed the maximum *G′, G″* and firmness. The PEF treatment of milk at various field intensities and temperatures adversely affected the *G′, G″* and firmness of gels. However, in comparison, the HTST and LTLT pasteurisation processes resulted in much more pronounced adverse effects on rheological and textural properties of the gels. Severe heat-treated (97℃ for 10 min) skim milk formed no gel. Scanning electron microscopy of gels indicated that PEF or heat treatments increased the size of pores in the gel network resulting in a lower *G′, G″* and firmness.

The findings of this study suggested that PEF treatment under the conditions employed is capable of inactivating spoilage microflora of milk at least to the same extent as HTST pasteurization. It can also partially inactivate milk enzymes depending on the enzyme type and temperature applied. It also has less adverse effect on rennet-induced gels of milk compared to LTLT and close to HTST pasteurization methods.

CHAPTER 1

GENERAL INTRODUCTION

Thermal processes used for food preservation can alter the nutritional and sensory qualities of the food (Mertens and Knorr, 1992; Knorr *et al.*, 1994). High-temperature short-time (HTST) pasteurisation has been effectively used for decades as a method of choice to extend the shelf life of milk and to inactivate its pathogenic bacteria; however, it can affect the organoleptic and nutritional properties of milk. The most common organoleptic change in pasteurised milk is the generation of "cooked flavour" (Calderon-Miranda *et al.*, 1999a) while Vitamins B and C and bioactive compounds can also be affected.

The quest for energy conservation by the manufacturers to reduce carbon footprint of the processes involved in food processing and preservation and the increasing consumers' demand for fresh-like quality foods have given rise to the development of innovative nonthermal food processing technologies including ionizing radiation, high-intensity light pulses, high isobaric pressure, electric or magnetic fields, antimicrobial chemicals, polycationic polymers, lytic enzymes as well as pulsed electric fields (Mertens and Knorr, 1992; Pothakamury *et al.*, 1993; Castro *et al.,* 1993).

The pulsed electric field (PEF) technology can be considered as a potential alternative to traditional thermal pasteurisation for milk with the advantages of minimising sensory and nutritional damage, thus providing fresh-like products.

However, more investigation is required to understand the nature of PEF effects to achieve a controlled rate of enzymatic and higher microbial inactivation in order to make PEF technology applicable in the food industry. Most PEF systems used for treatment of dairy or food products have been limited to bench top or small pilot scale systems (Bendicho *et al.,* 2002a). Since 1990s, the interest in PEF application in academic and research centres has increased and until 2006 about 450 papers have been cited in the Food Science and Technology Abstracts (Toepfl, 2006) and between 2006 and 2009 about 60 more papers have been published.

1.1 A Brief History of Pulsed Electric Field Technology

The use of electricity in food processing was introduced in the early 1900's and was first applied for the pasteurisation of milk using a process known as the Electro-Pure method (Anderson and Finkelstein, 1919; Fetterman, 1928; Getchell, 1935). However, this method was in fact a thermal process as the milk was heated up by ohmic resistance (Bendicho *et al*., 2002a). The treatment voltages applied to milk ranged from 220 V to 4200 V and only those researchers who had applied high voltages reported the ability of the process to kill the bacteria "below their thermal death point", the temperature at which a microorganism is inactivated by heat (Beattie, 1916; Beattie and Lewis, 1916 and 1925).

In the late 1940s, electric fields were used by Flaumenbaun to increase the permeability of fruits to facilitate the subsequent extraction of juice, which is currently an important application of PEF technology (Heinz and Knorr, 2001). In 1960, Doevenspeck in Germany patented a PEF equipment and during the same

period, Sale and Hamilton (1967) and Hamilton and Sale (1967) published a short series of articles on microbial inactivation by PEF, which are still considered as valuable references in this field.

In the field of genetic engineering, a method was developed by Zimmermann *et al.* (1974) to promote *in-vitro* cell-to-cell fusion using PEF which increased the permeability in localized zones of the membrane, currently known as the reversible electrical breakdown, electro-permeabilization or electroporation. Hülsheger *et al.* in early 1980s published a series of papers discussing the sensitivity of various bacteria to PEF and also mathematically simulated the effect of the electric fields intensity and treatment time on microbial kills (Hülsheger and Niemann, 1980; Hülsheger *et al.*, 1981 and 1983).

Up to the late 1980s, many researchers continued to develop PEF applications for preservation of foods and several patents were filed, and by 1992 PEF was recognised as a nonthermal preservation technology, a novel method which was able to provide consumers with fresh-like foods and valuable sensory properties (Jayaram *et al.*, 1992; Grahl *et al.*, 1992; Mertens and Knorr, 1992).

The PEF technology as a nonthermal process is able to partially inactivate microorganisms and enzymes in liquid foods such as milk and fruit juices, and is reported to have minimum adverse effect on the sensory attributes of the products. The PEF process is considered to be energy efficient. The microbial inactivation is achieved at ambient or moderately elevated temperatures by the application of short bursts of high intensity electric fields to liquid foods flowing between two

electrodes. Liquid foods (e.g. milk or fruit juices), due to their chemical composition and physical properties and in the presence of electrical charge carriers may have different conductivities resulting in various flux of electrical current through the food (Zhang *et al.*, 1995 and 1996; Barbosa-Canovas *et al.*, 2000).

There are currently various research groups in Australia, Belgium, Canada, China, France, Germany, Iceland, Japan, the Netherlands, New Zealand, Scotland, Spain, Sweden, Switzerland, Taiwan, the United Kingdom and USA working on different stages of PEF applications. In 1995, the CoolPure^{R} PEF process developed by PurePulse Technologies (4241, Ponderosa Ave., San Diego, CA, 92123, USA) was approved by FDA for treatment of pumpable foods. It was the first regulatory effort to introduce PEF process in the food industry (FDA, 2000). In 2005, Genesis Juice Cooperative (325, West Third Suites B, Eugene, Oregon 97401, USA) was the first enterprise in the world to commercially adopt PEF treatment technology for cold pasteurisation of fruit juices (Clark, 2006) although this company has since shifted from PEF technology to high pressure system for unknown reasons. A PEF pilot plant facility was also operational at Ohio State University's Food Science and Technology Department and has recently been relocated to the Eastern Research Centre of United States Department of Agriculture (USDA). Although, there have been many publications on the application of PEF to inactivate microorganisms and enzyme, no industrial PEF plant has so far been established to "pasteurise" milk for public consumption.

1.2 Rationale and Objectives of the Study

During the past two decades there have been many studies on the effects of PEF treatment on bovine milk and dairy products. However, there is a gap in knowledge of the effects of PEF treatment on native milk microflora and enzymes as well as on the functionality of milk fat and proteins. This study was undertaken to shed more light on practical aspects of PEF treatment of milk and push the PEF technology one step closer to industrial application. The main aim of this study was to investigate the effects of PEF on microflora, selected enzymes and functionality of milk proteins and fat with the following objectives:

1) To determine the effects of PEF treatment on milk total plate count (TPC), *Enterobacteriaceae* and *Pseudomonas*
2) To investigate the effects of PEF treatment on selected native enzymes of milk including AIP (as a possible indicator of PEF treatment adequacy), total lipase, xanthine oxidase (XO) and plasmin
3) To investigate the effects of PEF treatment on the rheological and textural properties of rennet-induced milk gels

1.3 An Overview of the Study

This study used a laboratory-scale PEF system to treat raw skim bovine milk at a series of field intensities, energy inputs, treatment times and temperatures to determine their effects on natural microflora, enzymes, and rheological and textural properties of rennet-induced milk gels. There are six chapters in this thesis to address the above objectives. The first chapter provides a general introduction to the thesis, and covers the history of PEF technology development and applications through decades. The second chapter provides a review of the

literature which gives a description of PEF system and its components, application of PEF in food industry and dairy research, mechanism of microbial and enzymatic inactivation by PEF and parameters involved, and the effects of PEF on functionality of fat and proteins in milk. Chapter 3 covers the effects of PEF treatment on native AlP and microflora in milk. In Chapter 4, the combined effects of field intensity, temperature and treatment time on activities of four milk enzymes are studied. Chapter 5 deals with the effects of PEF treatment on rheological and textural properties of the milk gel followed by general discussion and conclusions on PEF treatment of milk and recommendations in Chapter 6. Each experimental chapter has its relevant methodology and materials; therefore no specific chapter is allocated to the general materials and methods in the thesis.

CHAPTER 2

REVIEW OF LITERATURE

2.1 The Pulsed Electric Field System

A typical PEF system consists of the following components: a high-voltage power supply, a pulse generator, a number of energy storage capacitors, treatment chambers (either static or continuous) that house the electrodes, a pump to pass the liquid food through the treatment chambers (if the system is continuous), cooling and heating baths, measurement devices (voltage, current and temperature), and a central process unit to control operations (Fig. 2-1 a). The average electric field strength (*E*) is calculated by dividing the peak voltage (*kV*) by the gap distance (in cm) between the electrodes (*d*) (Zhang *et al.*, 1995).

The configuration of a PEF system is a function of food characteristics, geometry of chambers, product flow-rate, and high voltage switching devices. Some important aspects in pulsed electric field technology are the generation of high electric field intensities, the design of chambers to impart uniform treatment of foods with minimum increase in its temperature, and the design of electrodes that minimize the effect of electrolysis. High field intensities are achieved through storing a large amount of energy from a DC power supply in a capacitor bank, in series with a charging resistor which is then discharged in the form of high voltage pulses (Zhang *et al.*, 1995). Figure 2-1 b shows a typical circuit for producing square pulses.

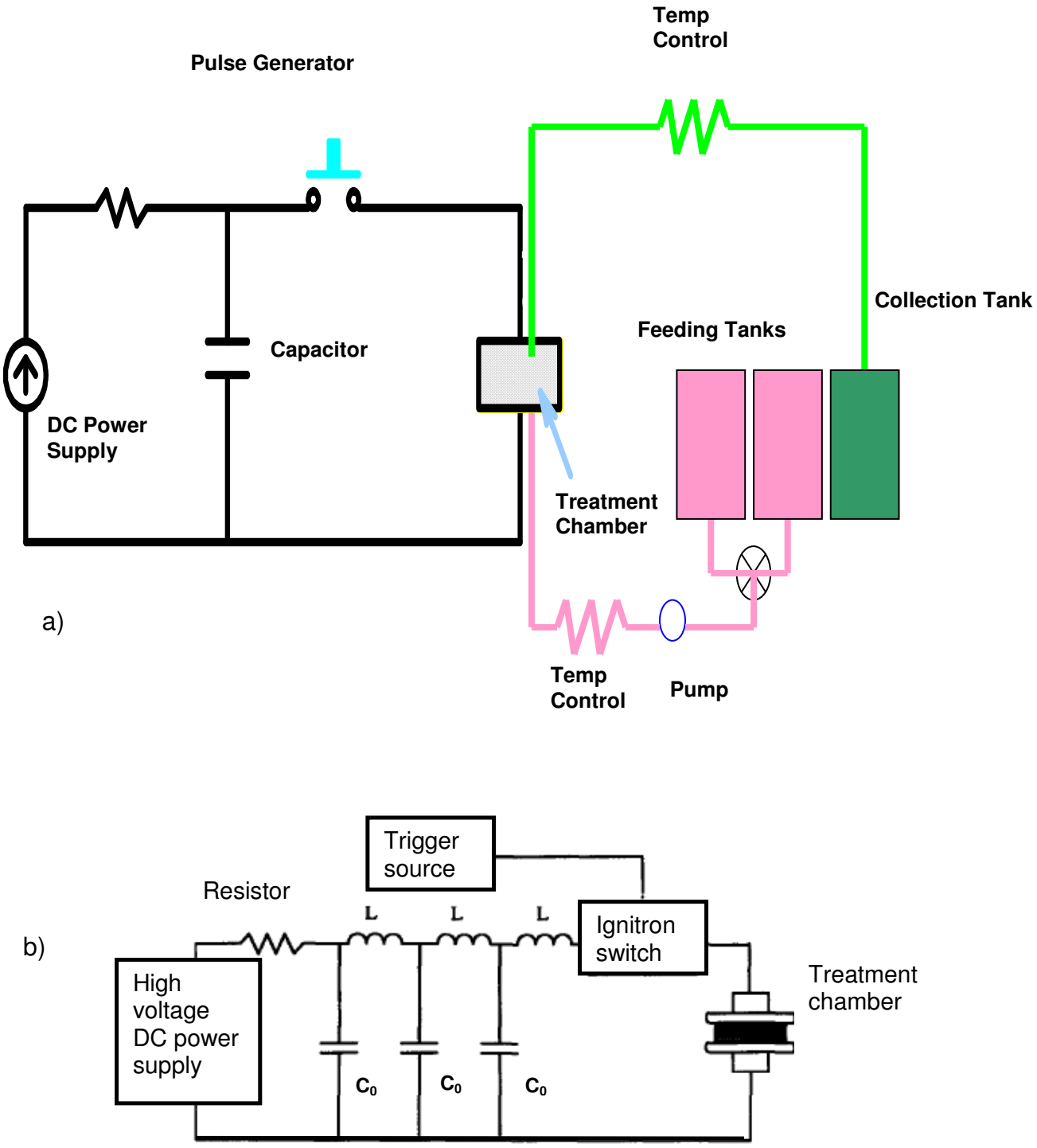


Figure 2-1 a) Schematic diagram of a pulsed electric field System; b) Layout of a square pulse generator using a pulse-forming network of 3 capacitors (C_0) and 3 inductors (L) (Zhang *et al.*, 1995).

Studies on energy requirements have suggested that PEF is an energy-efficient process compared to thermal pasteurization, particularly when a continuous system is used (Qin *et al.*, 1995a).

A typical treatment protocol for pasteurisation of milk might require application of field intensity of 35 kV cm^{-1} for a minimum of 50 µs to achieve a given bacterial reduction (e.g. 1 to 5 logs reduction). The two major factors affecting PEF system design are the conductivity and flow rate of the liquid. Conductivity determines the impedance of the food in the treatment chamber. The electric field is set by the treatment protocol, so the energy required to deliver this field to a litre of food is a direct function of the fluid conductivity. A laboratory-scale PEF system typically processes only a few litres per hour and a pilot-scale system typically operates at tens to hundreds of litres per hour. Commercial systems, however, must be capable of processing thousands to tens of thousands of litres per hour to make it commercially viable (Anon, 2008).

2.1.1 Pulse Generator, Capacitors and Discharge Switches

A pulse generator is made up of high voltage power supply, capacitors, inductors, rectifying circuits and discharge switches and, depending on the design is capable of producing certain types of wave shapes such as square or exponential. The characteristics of these wave shapes are further described in section 2.3.2.2. The capacitors store the energy and release it into the food through an electric discharge switch which turns on and off rapidly. The combination of various capacitors provides a greater storage for energy (Fig. 2.1 b). Many devices may be used as the discharge switch, including a mercury ignitron spark-gap, a gas spark gap, a thyratron, a magnetic switch or a mechanical rotary switch (Zhang *et*

al., 1995). A PEF system can be designed as either bipolar (+ and – voltage pulses) or monopolar (all + or all – pulses) (Gaudreau *et al*., 2008). New generations of the solid-state semiconductor switches is the Insulated Gate Bipolar Transistors (IGBTs) which combines the best features of a metal-oxide semiconductor field-effect transistor (MOFET) input and a bipolar transistor output into a newer power-switching device. Its advantages include rapid switching and small power consumption (Alkhafaji and Farid, 2007).

2.1.2 PEF Treatment Chambers

Many versions of PEF treatment chambers have been designed and evaluated (Fig. 2.2) and each chamber design targets a specific set of product specifications. An increase in the food electrical conductivity increases the peak discharge current and thus the power requirement. The electrodes gap needs to be at least three times the size of the largest particle in food. Thus, in a product containing 6 mm size particles, an 18 mm electrode gap is required (Zhang *et al*., 1995).

A treatment chamber geometry with large electrode surface area and small electrode gap results in a low load resistance of 0.5 to 2 Ω which demands high peak discharge current. Examples of such geometry are parallel plates and co-axial cylinders (Fig. 2-3 a, b, c &d). Conversely, a small electrode surface area and large electrode gap will provide a high load resistance of 50 to 900 Ω; cofield, tubular design is an example (Fig. 2-3 c). In order to make PEF applicable in industry, an optimal design of treatment chambers and processing systems is necessary (Zhang *et al*., 1995).

Treatment chambers, static (Dunn and Pearlman, 1987; Martin-Belloso *et al.* (1997a) (Fig. 2-3e) or continuous have evolved during decades and various researchers have designed and custom-made different types of chambers for various products (Matschke *et al.*, 1998). The continuous flow chambers process liquid foods that are pumped through pulsing electrodes and are therefore more suitable for large-scale operations.

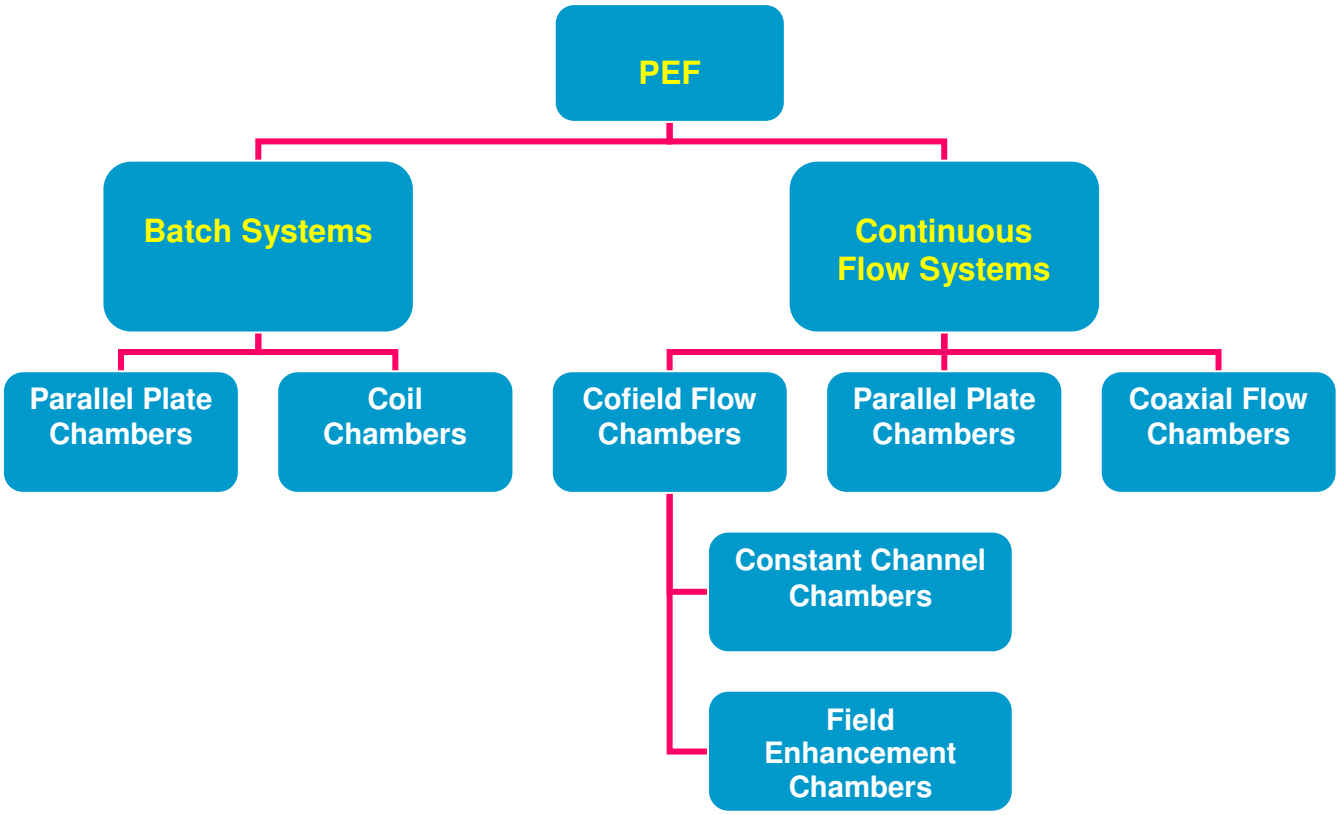
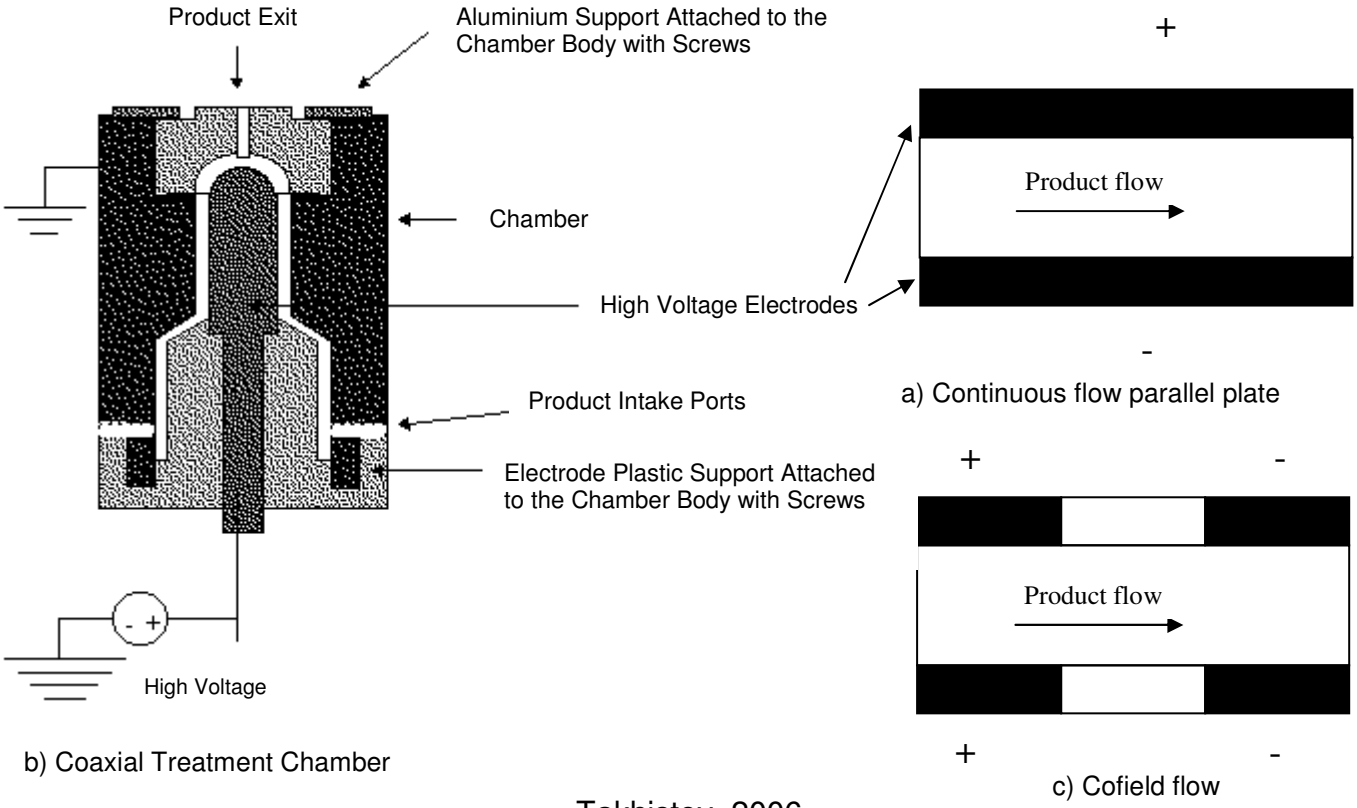


Figure 2-2 Classification of the static and continuous-flow treatment chambers for pulsed electric field treatment



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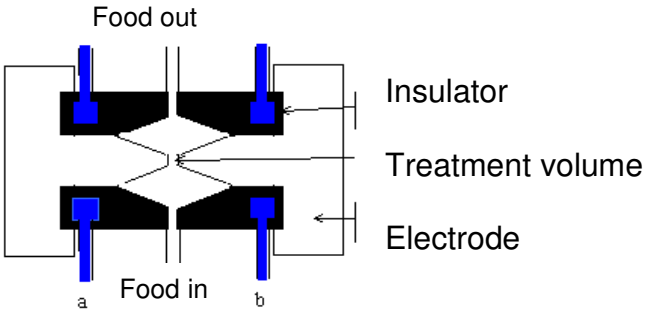
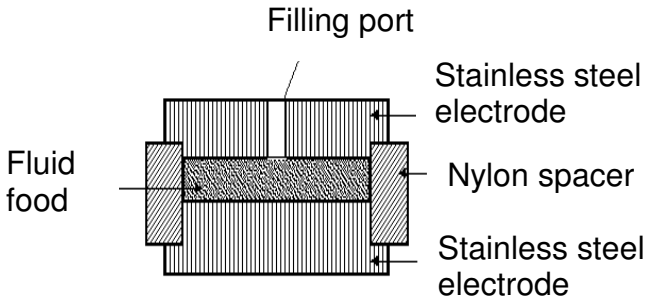


d) Cofield continuous treatment chamber by Yin *et al.* (1997)



e) Cross-section of a PEF static treatment chamber by Dunn and Pearlman (1987)

Figure 2-3 Schematic diagrams of various PEF treatment chambers

However, the cofield chambers in which two stainless steel tubes are separated by insulator and the electric field and the food flow concurrently, are more commonly used. The cofield designs are more reliable since the chance of electrode erosion and local bubble formation that may result in partial discharges is reduced in this chamber (Fig. 2-3 c) (Fox *et al.,* 2007).

In a continuous parallel plate chamber (Fig. 2-3 a) the electrodes are situated in parallel in the treatment chamber while in coaxial treatment chamber (Fig. 2-3 b) one electrode is in the centre and the other electrode is mounted on both sides of the treatment chamber. The cofield flow chamber (used in the current study) provides an optimal balance between the flow and field requirements and maintains consistent field intensities due to concurrent flow of the electrical field and the liquid food (Fig. 2-3 c).

A static chamber designed by Barbosa-Canovas *et al.* (1998) consisted of 2 round-edged, disk-shaped stainless steel electrodes with Polysulfone or Plexiglas as insulation material. The gap between electrodes could be adjusted to either 0.5 or 0.95 cm, the chamber could deliver field intensities of up to 70 kV cm^{-1} and was cooled by water at pre-selected temperatures through jackets built into the chamber. A continuous cofield flow PEF chamber was developed by Yin *et al.* (1997) in which the electric fields were enhanced by using conical insulators to eliminate gas bubble formation within the treatment volume. The conical regions were designed so that the voltage across the treatment zone could be almost equal to the supplied voltage (Fig. 2-3 d).

A coaxial chamber is basically composed of an inner cylinder surrounded by an outer annular cylindrical electrode that allows food to flow between them (Fig. 2-3 b). This treatment chamber has been successfully used in the inactivation of pathogenic and non-pathogenic bacteria, moulds, yeasts, and enzymes in fruit juices, milk, and liquid whole eggs (Martin-Belloso *et al*., 1997b). Toepfl (2006) used a continuous lab-scale chamber which was made up of two parallel stainless steel electrodes with a gap of 0.24 cm and an electrode area of 2 cm^2 for microbial inactivation studies in fruit juices with a maximum flow rate of 83 mL min^{-1}.

Treatment chambers of a few millimetres in diameter are used for laboratory research whereas pilot scale PEF treatment chambers reach a typical size of one centimetre. Fox *et al.* (2007) provided a comprehensive description of treatment chambers' geometry and application. Aspect ratio (the ratio of gap between electrodes to diameter of cylindrical chamber) of the treatment chambers should be larger than 1.5 to guarantee a uniform treatment of all passing fluids (Fiala *et al*., 2001; Morren *et al*., 2003).

2.1.3 Electrodes

The electrodes are located inside the treatment chamber in various positions through which the liquid flows and is exposed to the field intensity. The electrodes are made from electro-chemically inert materials such as carbon, stainless steel, titanium, gold, platinum or metal oxides and need to be replaced at least every 100 h of operation due to chemical erosion caused by the flowing liquids and deposits formation on their surface (Zhang *et al*., 1995). Kitajima *et al.* (2007) developed textile electrode made from a combination of polyester fibre, tungsten wire and

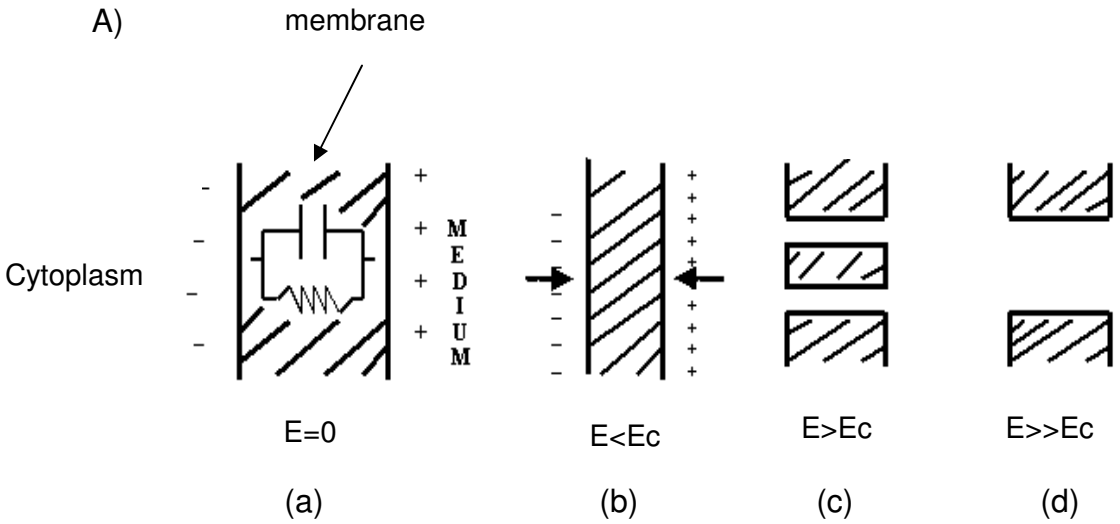
titanium wire which is reported to be resistant to chemical erosion. Figure 2-3 shows positioning of electrodes inside treatment chambers.

2.2 Mechanism of Microbial Inactivation by PEF: Electroporation and Electrical Breakdown

The mechanism underlying the inactivation of microorganisms by PEF is yet to be fully understood and knowledge of the microbial inactivation mechanism is essential in order to design and develop more efficient PEF equipment and define conditions for effective inactivation of microorganisms in food products (Martin-Belloso and Elez-Martinez, 2005a & b; Weaver and Chizmadzhev, 1996). According to Sale and Hamilton (1967) membrane damage is the direct cause of cell inactivation. The inactivation of microorganisms is related mainly to the changes in the cell membrane and its electromechanical instability (Jacob *et al*., 1981; Coster and Zimmermann, 1975).

Two mechanisms have been proposed for the mode of PEF action on microbial membrane: electroporation and electrical breakdown; however, both mechanisms are in fact referring to a phenomenon starting by electroporation resulting in electrical breakdown by which the cell wall is perforated and cytoplasm contents leak out resulting in cell death. The electroporation theory suggests that the main effect of an electric field on microbial cells is to increase the membrane permeability due to membrane compression and poration, and cell inactivation results from osmotic imbalance across the cell membrane (Tsong, 1990). Figure 2-4 (A) shows the mechanism of electrical breakdown and cell poration which was

initially proposed by Zimmermann (1986). He suggested that the membrane can be considered as a capacitor filled with a dielectric medium.



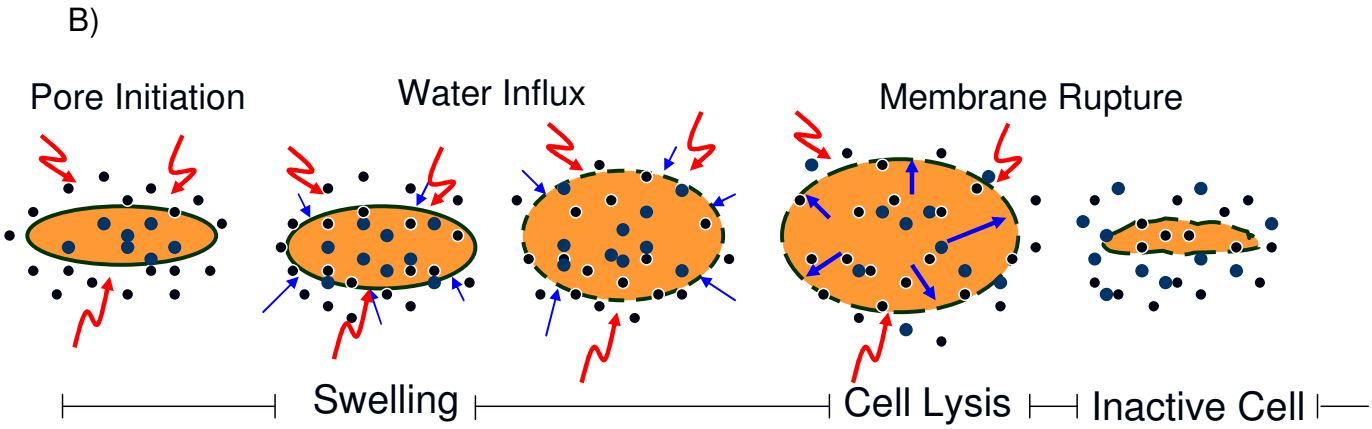


Figure 2-4 A) Schematic diagram of reversible and irreversible breakdown of a microbial cell indicating compression by electroporation when exposed to electric field. The membrane acts as a capacitor and is represented by hatched area. Ec is the critical electric field (a) Intact cell membrane; (b) membrane compression; (c) pore formation with reversible breakdown; (d) irreversible breakdown with formation of large pores (Zimmermann, 1986).

B) Stages of electroporation in a cell membrane through osmosis (Redrawn from Tsong, 1990). The red arrows show the field intensity and blue dots are water molecules.

Based on this theory, when the transmembrane potential is exposed to a higher external field intensity results in membrane damage. According to Chen and Lee (1994) the membrane of a biological cell insulates shell from cytoplasm while the electrical conductivity of the cytoplasm is 6 to 8 times greater than conductivity of the membrane. When the cell is exposed to an electric field, the free charges generated on the membrane surface are attracted to each other due to the difference in the signs (- and +) which causes a compression pressure resulting in a decrease in membrane thickness (Fig. 2-4 A, b). Increasing the field intensity leads to more accumulation of surface charges resulting in a higher electromechanical stress and reversible breakdown of membrane (Fig. 2-4 A, c) (Kinosita and Tsong, 1977; Zimmermann, 1986). The membrane thickness decreases by increasing the field intensity which eventually results in an irreversible breakdown through creating larger pores in the membrane (Fig. 2-4 A, d). If the area of the pores in relation to the membrane surface becomes larger, an irreversible breakdown occurs in the membrane leading to the total destruction of cell (Zimmermann, 1986). In large cells the induced potential is greater which makes them more vulnerable to damage compared to smaller cells (Chen and Lee, 1994).

Osmotic imbalance is a theory through which the electroporation and electrical breakdown has been described (Fig. 2-4 B). The cell exposed to an external electric field is “electroporated” through the leakage of ions and small molecules and thus the membrane becomes permeable to water that causes swelling and eventual rupture (electrical breakdown) and lysis of the cell. Therefore based on the above observations it could be concluded that the inactivation of cell follows a

sequence of a primary electroporation with small pores on the cell membrane followed by a secondary electroporation with larger pores which finally causes electrical breakdown and cell lysis. Large pores are obtained by increasing the intensity of the electric field and pulse duration or reducing the ionic strength of the medium (Schoenbach *et al.*, 1997).

Castro *et al.* (1993) further explained electroporation as a phenomenon in which the high voltage electric field temporarily destabilizes the lipid bilayer and proteins of cell membranes. The plasma membranes thus become permeable to small molecules that cause swelling and eventual rupture of the membrane. Vega-Mercado *et al.* (1996a) proposed that the main effect of the electric field on bacterial cell was to increase membrane permeability due to membrane compression and poration. Kinosita and Tsong (1977) demonstrated that an electric field of 2.2 kV cm^{-1} induced pores of *ca.* 1 nm in diameter in human erythrocytes. They suggested a two-step mechanism for pore formation in which the initial perforation is a response to an electrical potential greater than the Ec (critical field intensity) followed by a time-dependent expansion of the pore size. Large pores are obtained by increasing the intensity of the electric field and pulse duration or by reducing the ionic strength of the medium as shown in Figure 2-4 (A & B).

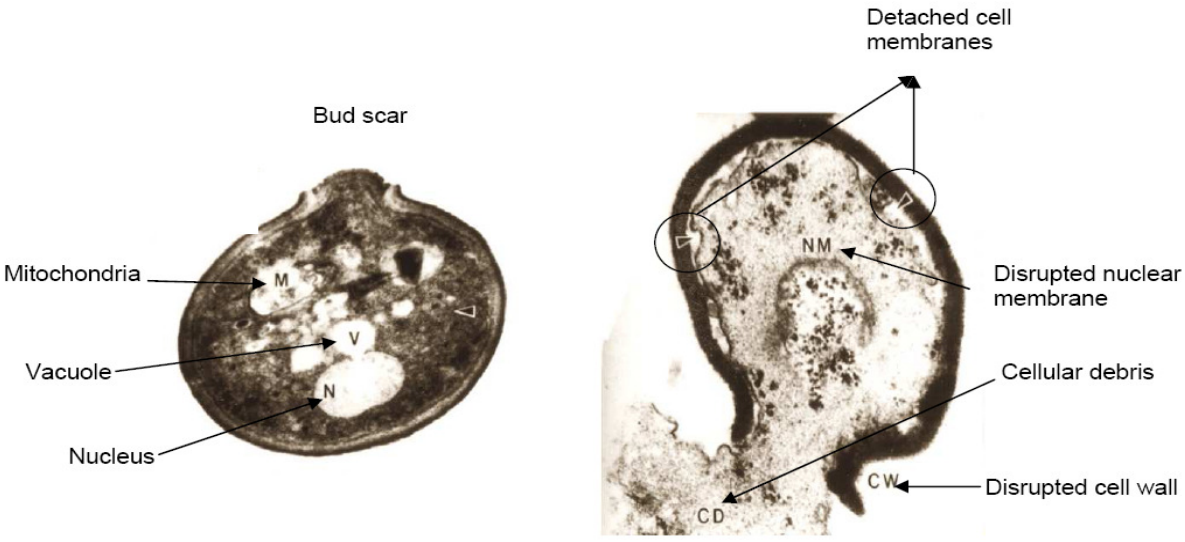
Pothakamury *et al.* (1997) treated a suspension of *Staphylococcus aureus* in simulated milk ultrafiltrate (SMUF) by 64 pulses of 20, 30 and 40 kV cm^{-1} and scanning electron microscopic (SEM) examination showed rough surfaces and small pores in membrane which led to the leakage of cellular contents. This finding was confirmed by Aronsson *et al.* (2001) who studied *Escherichia coli, Listeria*

innocua, Leuconostoc mesenteroides and *Saccharomyces cerevisiae* by means of SEM examination and found a clear difference between untreated and PEF-treated cells (25-35 kV cm^{-1}, 20-40 pulses of 2-4 µs). Harrison *et al.* (1997) and Leadley and Willimas (2006) exposed cells of *S. cerevisiae* and *Bacillus cereus* in apple juice to 64 pulses of 40 kV cm^{-1} and observed the disruption in cell wall (Fig. 2-5).

2.3 Factors Affecting Microbial Inactivation in PEF Treatment

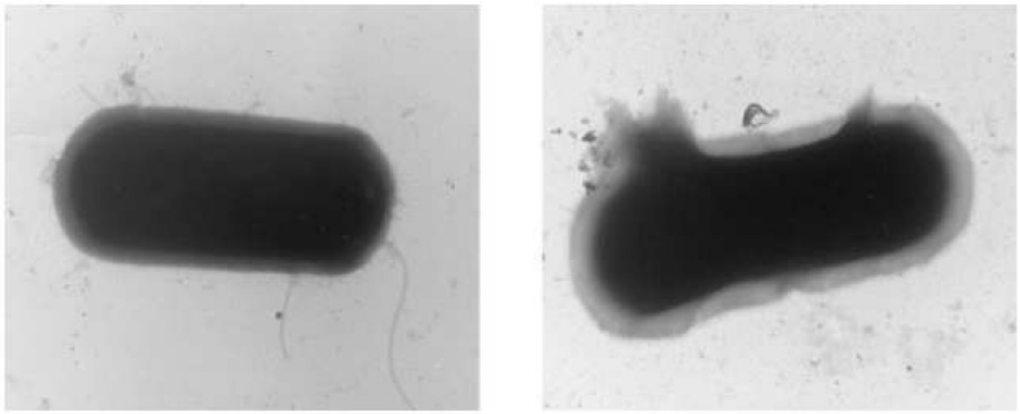
2.3.1 Type and Growth Stage of Microorganisms

The Gram-positive bacteria are more resistant to PEF treatment than Gram-negative ones (Hülsheger *et al.*, 1983). Yeasts are more sensitive to electric fields than Gram-positive bacteria due to their larger size, but they may be more resistant than Gram-negative bacteria (Sale and Hamilton, 1967; Qin *et al.,* 1995a). Some of the early publications on microbial inactivation by PEF treatment are compiled by Barbosa-Canovas *et al.* (1998) and Barbosa-Canovas and Zhang (2001). Sporulated microorganisms are the most difficult ones to inactivate by PEF treatment and even combination of heat around 60°C with 75 pulses of 60 kV cm^{-1} could not inactivate *B. cereus* spores inoculated in SMUF (Pagan *et al.*, 1998; Bendicho *et al.*, 2003a). Growth stage of microorganisms is another factor to be considered in PEF treatment. Logarithmic phase cells are more sensitive to stress than the lag and stationary phase cells. Microbial growth in logarithmic phase is characterised by a high proportion of cells undergoing division, during which the cell membrane is more susceptible to the applied electric fields (Hülsheger *et al.*, 1983). Gaskova *et al.* (1996) reported that the inactivation effect of PEF in the logarithmic phase was 30% greater than in stationary phase.



Untreated cell Cell treated with 64 pulses at 40 kV cm^{-1}

a) Cells of *Saccharomyces cerevisiae* (Harrison, 1997)



Untreated cell Cell treated with 64 pulses at 40 kV cm^{-1} at 25°C

b) Cells of *Bacillus cereus* (Leadley and Williams, 2006)

Figure 2-5 SEM micrographs of PEF-treated *Saccharomyces cerevisiae* and *Bacillus cereus* in apple juice

2.3.2 Processing Parameters

2.3.2.1 Field Intensity

The intensity of the electric field applied to a medium (e.g. milk or fruit juices) is one of the main factors influencing microbial inactivation (Hülsheger and Niemann, 1980). When the cells are exposed to an external electric field with sufficient intensity, electric charges accumulates at the non-conductive microbial membranes (Toepfl *et al.*, 2005). Heinz *et al.* (2002) showed that the critical external field intensity required for microbial inactivation is highly dependent on the cell size as well as the field orientation. Smaller cells require higher field intensity for inactivation. The orientation of the rod-shaped cells along or across the electric field lines also influence the required field intensity (Schoenbach *et al.*, 1997). According to Toepfl *et al.* (2005), *Listeria innocua* requires a minimum of 15 kV cm^{-1} to become inactivated while the larger cells of *S. cerevisiae* are affected at field intensity as low as 2-4 kV cm^{-1}.

2.3.2.2 Pulse Wave Shape and Polarity

Pulse wave shape is another factor affecting the microbial inactivation. Figure 2-6 shows different types of pulse wave shapes produced by the pulse generator in PEF system. Exponentially decaying or square waves are the most common wave shapes used in PEF systems. An exponentially decaying wave rises rapidly to a maximum value and decays slowly to zero and has a long tail with a short pulse width (Fig. 2-6 a & c).

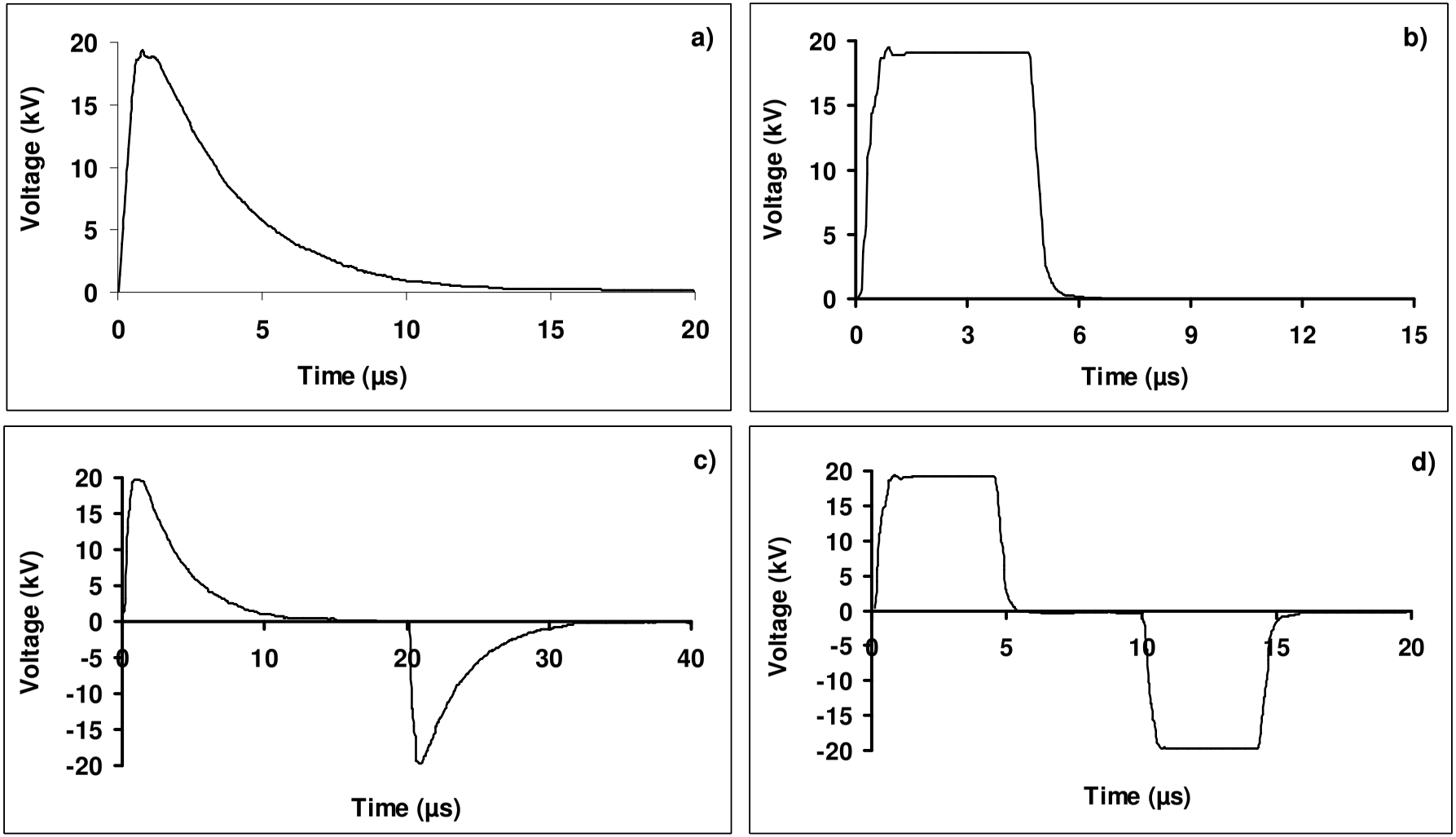


Figure 2-6 Pulse wave shapes commonly used in PEF technology: (a) Exponentially decaying pulse, b) Square pulse, (c) Bipolar exponentially decaying pulse, (d) Bipolar square pulse (Barbosa-Canovas and Sepulveda, 2005).

Square pulses (Fig. 2-6 b & d) maintain the peak voltage for a longer period of time i.e. over 2 μs which makes them more lethal and energy efficient than exponentially decaying pulses (Barbosa-Canovas *et al.*, 1998). Besides, they leave less deposit on the electrode surface due to less electrolysis of food. However, exponentially decaying pulses are easier to generate and change while generation of square pulses is more complex (Zhang *et al.*, 1995; Ho *et al.*, 1995; Qin *et al.*, 1994; Barbosa-Canovas *et al.*, 1998).

Bipolar pulses are reported to be more effective for microbial inactivation than monopolar pulses (Qin *et al.*, 1994). More recent studies, however, have shown no significant differences in effectiveness between mono and bipolar pulses for the inactivation of either Gram positive (*Bacillus cereus, Listeria monocytogenes* NCT 11994) or Gram negatives (*E. coli* NCTC 9001) bacteria in peptone solutions (Beveridge *et al.*, 2002 and 2005), and of *E. coli* O157:H7 inoculated in apple juice (Evrendilek and Zhang, 2005).

2.3.2.3 Treatment Time and Total Specific Pulsing Energy Input

Treatment time *t* is defined as the product of number of pulses by the pulse width (μs) and can be achieved in PEF by either changing the flow rate or the pulse frequency while maintaining the pulse width. It is an important factor in the inactivation process and calculated as: $t = N_p \times N_c \times P_w$

where:

N_p is the number of pulses,

N_c is the number of treatment chambers and,

P_w is the pulse width.

The number of pulses is calculated from: $N_p = t_r \times f$

where:

f is the pulse frequency (Hz) and,

t_r, the residence time (s) in each chamber is determined from $t_r = V/F$

where:

V is the volume of each chamber (mL) and,

F is the flow rate (mL s^{-1}) (Min *et al.*, 2003).

Energy input Q into the food for square pulses is calculated as $Q = v.I.P_w / V$, where v and I are the voltage and current (Zhang *et al.*, 1995).

Based on the above formulae the treatment time and energy input are linearly correlated and increasing each can result in further microbial or enzymatic inactivation. Energy input is considered the main cause of the inactivation of microorganisms and enzymes by PEF (Min *et al.,* 2003). Bendicho *et al.* (2003c) reported that the activity of a protease from *Bacillus subtilis* suspended in SMUF decreased exponentially with increase of input energy which is controlled by treatment time, field intensity, and pulse frequency.

Alkhafaji and Farid (2007) reported that "as the temperature increases, the conductivity of the food and the energy increases due to the increase in the current passing through the liquid. There is a dynamic relationship between electrical conductivity, energy input, and temperature increase, which suggest the need of an accurate control of these parameters during this non-thermal pasteurisation".

More recently, Fernandez-Molina *et al.* (2006) reported that inactivation of *Ps. fluorescens* in skim milk increased with the increase in energy input and treatment time. They achieved a maximum of 2.6 logs reduction after PEF treatment at 38.9 kV cm^{-1}, treatment temperature of 33°C, and an energy input of 128 kJ L^{-1}.

2.3.3 Environmental Parameters

2.3.3.1 Treatment Temperature

Temperature is an inseparable part of PEF treatment since when the electric field is applied to the liquid flowing between the electrodes its temperature rises. Therefore, proper cooling of product (in water bath) between the treatment chambers is used to avoid heat damage and to limit microbial inactivation to PEF effect (Vega-Mercado *et al.*, 1996b). In the current project, the maximum temperature measured immediately at the outlet of final treatment chamber was considered as the treatment temperature due to difficulty of measuring the temperature inside the treatment chamber. Some researchers have shown that PEF treatments at moderate temperatures (50 to 60°C) exhibit additive effects on the inactivation of microorganisms and spores (Dunn and Pearlman, 1987; Rowan *et al.*, 2001; Spilimbergo *et al.*, 2003; Craven *et al.*, 2008). Heinz *et al.* (2003) indicated that using synergistic effects of elevated treatment temperature of 35–65°C on *Escherichia coli* inoculated in apple juice the energy consumption could be reduced from above 100 to less than 40 kJ kg^{-1} for a reduction of 6 log cycles and the need to preheat the juice before treatment provided a possibility to recover the dissipated electrical energy after treatment, leading to a noticeable reduction in operational costs. The enhanced microbial kill by PEF at higher temperatures is

most likely due to the higher fluidity of the phospholipids in the cell membrane at elevated temperatures which make the cell more vulnerable to pore formation by PEF (Jayaram *et al*., 1992).

2.3.3.2 Effect of pH

Vega-Mercado *et al.* (1996b) reported that the PEF-inactivation of *E. coli* in SMUF was greater at pH 5.7 than at pH 6.8. Liu *et al.* (1997) demonstrated that field intensity and benzoic or sorbic acid at pH 3.4 had a synergistic effect on inactivation of *E. coli* O157:H7. The control sample contained 7.19 log CFU mL^{-1} bacteria before treatment which was decreased to 4.26 using benzoic acid and to 4.86 log CFU mL^{-1} in the presence of sorbic acid after treatment at 25°C with 12.5 kV cm^{-1}. Presence of benzoic or sorbic acid (1000 ppm) in the suspending medium at pH 3.4 without field intensity decreased the count by 1.9-2.5 and 0.6-1.1 logs, respectively. Wouters *et al.* (1999) also showed that the PEF treatments of *L. innocua* in phosphate buffer were more effective when the pH of buffer was lowered from 6.8 to 5. The combined effects of low pH and organic acids can enhance the inactivation of spoilage microorganisms with the PEF technology. However, since the above acids are all preservatives, it is possible that the inactivation levels achieved were due to not only the combined effect of pH and PEF treatment but also the chemical properties of the acids. Pagan *et al*. (2005) reported that the effect of pH on the sensitivity of microorganisms to PEF treatment is dependent on the target microflora. The sensitivity of *Yesinia enterocolitica* and *Pseudomonas aeruginosa* to PEF treatment (19-25 kV cm^{-1}) was not affected by the pH of the treatment buffer at 7 or 3.8, however, an extra 4.5 log reduction was obtained with *Listeria monocytogenes* at pH 3.8. *Salmonela* Senftenberg was more resistant to PEF treatment at acidic pH than at neutral pH.

Garcia *et al.* (2005) also reported that *L. monocytogenes* was resistant to PEF at pH 7.0 but more sensitive at pH 4.0. In contrast, *Salmonela* Senftenberg and *E. coli* O157:H7 were sensitive to PEF treatment at pH 7.0 but resistant at pH 4.0.

2.3.3.3 Conductivity and Ionic Strength of the Medium

The electric conductivity of a medium (σ, Siemens m^{-1} or mS cm^{-1}) is an important parameter in PEF treatment. In foods with high electrical conductivities, electric fields with smaller peaks are generated across the treatment chamber which do not have the required killing effects (Barbosa-Canovas *et al.,* 1999). An increase in ionic strength of a liquid leads to increased conductivity, resulting in a decreased microbial inactivation level. Furthermore, large conductivity difference between the medium and microbial cytoplasm weakens the membrane structure due to an increased flow of ionic materials across the membrane. Thus, the inactivation level of microorganisms increases with decreasing medium conductivity (Jayaram *et al.*, 1992).

Different media have been used in PEF studies such as distilled water. Simpson *et al.* (1999) found out that using different media can affect the resistance of microorganisms treated with PEF. For instance, *Salmonella* Typhimurium (CRA 1005) was reported more sensitive than *Listeria monocytogenes* (NCTC 11994) to PEF treatment in distilled water (10, 15 and 20 kV cm^{-1}), 10 mM Tris–maleate buffer, pH 7.4 (15 kV cm^{-1}) and model beef broth (0.75%, w/v; 15 kV cm^{-1}). Therefore, the medium and its properties such as pH and conductivity influence the efficacy of PEF treatment. The media with lower conductivity allows the application of a higher field intensity thus resulting in a higher microbial inactivation (Mittal and Griffiths, 2005; Alkhafaji and Farid, 2007).

Several research groups have reported that by decreasing the conductivity of the treatment media it is possible to increase the inactivation level of *L. brevis, E. coli, S. cerevisiae, S.* Dublin and *L. innocua* (Jayaram *et al*., 1992 and 1993; Grahl and Märkl, 1996; Sensoy *et al*., 1997; Wouters *et al*., 1999). However, Alvarez *et al.* (2000) reported that conductivity did not influence the inactivation of *S.* Senftenberg in citrate-phosphate buffer with pH of 7 while Gaskova *et al.* (1996) found that the inactivation of *S. cerevisiae* was inversely related to the medium conductivity. The differences in reported results arise from the different PEF parameters employed, using various media with different chemical properties and conductivities.

The effectiveness of PEF treatment may be affected by the type of cations present in the medium. Hülsheger *et al.* (1981) treated *E. coli* K12 suspended in different electrolyte solutions of identical conductivities at pH 6-8 with 10 pulses at field intensity of 12 kV cm^{-1} and demonstrated that bivalent cations such as Mg^{+2} or Ca^{+2} had a protective effect on membranes by reducing the sensitivity of treated cells, thus resulting in reduced PEF damage. However, no such protective effects were found with monovalent cations Na^{+} and K^{+}.

2.4 Microbial Inactivation Studies on PEF-Treated Milk and SMUF

The bulk of research output reported in the literature are focused on the impact of PEF treatment on microbial and enzymatic inactivation in milk or SMUF. The SMUF is a salt solution with composition proximity with milk ultrafiltrate. It was first proposed by Jeness and Koops (1962) and now is widely used in dairy research (Bendicho *et al.,* 2002a). The extent of microbial inactivation has been found to be mainly dependent on the electric field intensity, number of pulses applied during

the process, treatment time and energy input level (Qin *et al.,* 1995; Martin-Belloso *et al.,* 1997b; Pothakamury *et al.,* 1997; Bendicho *et al*., 2002a). Table 2-1 lists a summary of some of the PEF studies published over the last decade on the effects of PEF treatment on microbial inactivation in milk and SMUF.

Various studies published on milk treatment by PEF have proven this technology to be effective for the inactivation of moulds, yeasts and vegetative bacterial cells (Hülsheger *et al*., 1983; Jayaram *et al*., 1992; Zhang *et al*., 1994; Zhang *et al*., 1995; Ho *et al*., 1995 and 2000; Pothakamury *et al*., 1996; Liu *et al*., 1997; Marquez *et al*., 1997; Reina *et al*., 1998; Sensoy *et al*., 1997; Evrendilek *et al*., 1999; Barbosa-Canovas *et al*., 2000; Picart *et al*., 2002; Pol *et al*., 2000 and 2001). Various researchers have reported 1 to 6 logs inactivation of different strains of *E. coli* (pathogenic and non-pathogenic) inoculated in UHT milk (1.5% fat), liquid egg, pea soup, apple juice, SMUF, 0.1% NaCl saline, phosphate buffer (pH 7.0) and sodium alginate (Sale and Hamilton, 1967; Hülsheger and Nienmann, 1980; Matsumoto *et al*., 1991; Zhang *et al*., 1994; Pothakamury *et al*., 1995; Liu *et al*., 1997; Barbosa-Canovas *et al*., 2000; Evrendilek *et al*., 2000; Alkhafaji and Farid, 2007).

Dutreux *et al.* (2000a) inoculated *E. coli* and *L. innocua* in pasteurised skim milk and in phosphate buffer (with similar pH and conductivities to milk) and treated them with PEF, with inlet and outlet temperatures of 17^{o}C and 37^{o}C, respectively, a flow rate of 500 mL min^{-1}, pulse frequency of 3 Hz and field intensity of 41 kV cm^{-1}.

Table 2-1 Microbial inactivation in milk and simulated milk ultrafiltrate using pulsed electric field treatment

Microorganisms	Treatment medium	E[b] (kV cm^{-1})	T[c] (°C)	Log reduction	PEF Unit	Treatment Chamber	Wave shape	PF[j]/Pw[k]	References
E. coli	SMUF[a]	36 & 60	40	6.0 & 9.0	Laboratory size (WSU[d] prototype)	Continuous	Exponentially decaying	40 µs	Qin *et al.* 1998
L. monocytogenes Scott A	Milk	25 & 35	25 50	1-4.0	Bench scale continuous (OSU[e]-4A)	Co-field flow	Monopolar square	1,700 Hz	Reina *et al.* 1998
L. innocua	Skim milk	30, 40 & 50	22 28 34	1.7, 2.0 & 2.5	Pilot plant (Physics International[f])	Continuous	Exponentially decaying	3.5 Hz	Calderon-Miranda *et al.* 1999a
E. coli *L. innocua*	Skim milk	41	37	2.3-4.0 & 0.7-3.9	Continuous	Continuous	Not reported	10 - 63 Hz	Dutreux *et al.* 2000
L. innocua	Whole milk	29	35.5	1.13	Batch (Centralp-Enertronic[g])	Batch	Exponentially decaying	1.1-100 Hz	Picart *et al.* 2002
P. fluorescens	SMUF	16.4 & 37.3	50 90	Various	Batch (University of Lleida[h]) & Continuous (WSU)	Static	Exponentially decaying	2 – 3.5 Hz	Bendicho *et al.* 2002b
L. lactis	Skim milk	35	52	3.0	Bench scale continuous (OSU-3C)	Co-field flow	Bipolar square	500 Hz	Michalac *et al.* 2003
L. monocytogenes	Skim milk	15 &30	<50 55	1- 4.5	CoolPure Pulser[i]	Static	Monopolar square	5-50 Hz	Fleischman *et al.* 2004
E. coli O157:H7	Skim milk	24	30	2.0, 1.27 & 1.88	Bench scale continuous (OSU-4A)	Co-field flow	Bipolar & monopolar square	2.8 µs	Evrendilek & Zhang 2005
E. coli ATCC 25922	SMUF	37.2–49.6	<38	>6	Custom made	multi-pass continuous	Bipolar square	870	Alkhafaji & Farid (2007)
Pseudomonas	UHT milk		15 40 45 55	3.1	Lab scale continuous (OSU-4A)	Co-field flow	Monopolar	200 Hz	Craven *et al.* 2008

[a]simulated milk ultrafiltrate; [b]field intensity; [c]temperature; [d] Washington State University; [e]Ohio State University; [f]Physics International, 2700 Merced St, San Leandro, CA, USA; [g]Centralp-Enertronic, F-38070, St. Quentin Fallavier, France; [h]University of Lleida, E-25001 Lleida, Catalonia, Spain; PurePulse Technologies, San Diego, CA, USA; [j]pulse frequency; [k]pulse width

The number of surviving organisms was determined after the application of 0, 3, 10, 20, 35 and 60 pulses (pulse width unknown). Transmission and scanning electron microscopy of the *E. coli* cells subjected to 60 pulses showed changes in the cytoplasm and the cell surface appeared rough and partially destroyed allowing leakage of the cytoplasm.

Rowan *et al.* (2001) investigated the influence of treatment temperature and PEF intensity on the viability of *Mycobacterium paratuberculosis* cells inoculated in 0.1% (w/v) peptone water and in sterilised cow's milk. The viability was assessed through direct viable counts and cell morphology assessed using transmission electron microscopy (TEM). PEF treatment at 50^{o}C with 2500 pulses and 30 kV cm^{-1} reduced the number of viable *M. paratuberculosis* cells by approximately 5.3 logs in 0.1% peptone water and 5.9 logs in milk, while PEF treatment at 5^{o}C reduced the cell numbers only by 1.6 logs. Heating alone at 50^{o}C for 25 min or at 72^{o}C for 25 s resulted in 0.01 and 2.4 log reduction in the numbers of *M. paratuberculosis,* respectively. They concluded that the PEF treatment at 50^{o}C was more effective than thermal pasteurisation for the inactivation of this organism.

Evrendilek and Zhang (2005) investigated the effects of pulse polarity and "pulse delaying time" (the time elapsed between two consecutive crests passing a given point) on the inactivation of *E. coli* O157:H7 in apple juice and skim milk treated at field intensities of 31 and 24 kV cm^{-1}, respectively. Various pulse delaying times of 3 to 1430 µs were applied to both apple juice and skim milk. The pH and electrical conductivity for apple juice were 3.7 and 2.3 mS cm^{-1} and for skim milk 6.7 and 6.2

mS cm^{-1}, respectively. The average temperatures of apple juice before and after PEF treatment were 9±1°C and 29±2°C, respectively, and for skim milk 7±2°C and 30±3°C. A significant difference was observed in *E. coli* O157:H7 numbers in skim milk between mono (1.27 logs) and bipolar (1.96 logs) pulses, but not in apple juice (2.6 and 2.63 logs, respectively) at the pulse delaying time of 20 µs. The difference in inactivation level can be attributed to the difference in pH and ionic composition of skim milk and apple juice.

Fernandez-Molina *et al*. (2005 a, b & c) investigated the shelf life of various PEF-treated skim milks at room temperature or in combination with conventional heating at 60 or 65°C for 21 s on total number of aerobic bacteria and the shelf life of skim milk as well as combining PEF treatment with organic acids to inactivate *Pseudomonas fluorescens* in skim milk. In all three studies, exponentially decaying pulses with field intensities of 30 to 50 kV cm^{-1}, pulse frequency of 4 Hz and treatment temperatures of 40 to 65ºC were used. It was indicated that PEF treatment in combination with heat or organic acids had a greater effect on inactivation process of *Ps. fluorescens* than PEF alone. They concluded that to achieve a higher level of microbial inactivation in milk and a longer shelf life, PEF can be used in combination with heat or organic acids (acetic or propionic acids).

Sepulveda *et al.* (2005) subjected the pasteurised milk to PEF treatment immediately after pasteurisation and after 8 days storage at 4°C using field intensity of 35 kV cm^{-1} and 2 pulses of 2.3 µs duration each. The final temperature was 65°C with a residence time of less then 10 s. It was shown that the application of PEF immediately after thermal pasteurisation could extend the shelf

life of milk up to 60 days at 4°C, while PEF processing after 8 day storage resulted in a longer shelf life of 78 days due to further eradication of enteric and psychrotrophic bacteria by PEF.

The effects of combination of PEF with heat treatments on inactivation of *Salmonella* Enteritidis in skim milk was evaluated by Floury *et al.* (2006a). The selected field intensity was 47 kV cm^{-1} with a pulse frequency of 60 Hz, temperature of 62°C, flow rate of 83 mL min^{-1} and pulse width of 500 ηs. The PEF processing of skim milk resulted in 1.2 logs reduction while the combination of heat and PEF nearly doubled the reduction to 2.3 logs. They concluded that the lethality of the two treatments were mostly "additive" rather than synergistic.

Alkhafaji and Farid (2007) achieved up to 6.6 logs reduction in the number of *E. coli* ATCC 25922 suspended in SMUF using an innovative design of multi-pass PEF treatment chamber with limited increase in liquid temperature or fouling of electrodes. Four multi-pass treatment chambers consisting of two stainless steel mesh electrodes in each chamber were designed. The field intensities of 37 to 49.6 kV cm^{-1} were used with bipolar pulses of 1.7 µs, pulse frequency of 200 Hz. The treatment time of 100 to 900 µs was achieved by recirculating the SMUF through the chambers. The flow rate and final temperature were adjusted to 150 mL min^{-1} and <38°C, respectively. By operating at a condition of equal power when electric field was varied it was noticed that microbial inactivation depended on the electrical field strength and not on power. They also concluded that better treatment can be achieved at higher frequencies/high flow rates.

Sampedro *et al.* (2007) studied the effects of electric field intensity, treatment time, process temperature and pulse width on the inactivation of *Lactobacillus plantarium* inoculated in an orange juice-milk based beverage. The maximum degree of inactivation (2.46 logs) was achieved through combining temperatures of 35 or 55^{o}C, flow rate of 60 mL min^{-1}, bipolar square waves of 2.5 µs, treatment times of 0 to 180 µs, field intensity of 35 to 45 kV cm^{-1} and the pulse frequency of 110 to 356 Hz. In a similar study, Rivas *et al*. (2006) investigated the effects of PEF on *Escherichia coli* ATCC 8739 suspended in an orange juice-milk based beverage. Bipolar square pulses with a pulse width of 2.5 µs, field intensity of 15 to 40 kV cm^{-1} and treatment time of 0 to 700 µs were used and a maximum of 3.83 logs reduction were achieved.

Craven *et al.* (2008) achieved >5 log reduction in the number of inoculated *Ps. fluorescence* in UHT milk inoculated after treatment at 31 kV cm^{-1}, flow rate of 60 mL min^{-1}, treatment time of 19.6 µs, pulse width of 2 µs and pulse frequency of 200 Hz, with an outlet temperature of 55℃. The non-PEF treatment control had only 0.2 log inactivation. Compared with the non-PEF control milk, the shelf life of the PEF-treated milk at 4℃ was extended by at least 8 days to 13 and 11 days for inoculation levels of 10^3 and 10^5 CFU mL^{-1}, respectively.

2.5 Effects of PEF on Enzymes in Milk or SMUF

The impact of PEF on enzymatic inactivation is a matter of controversy since in several cases a high level of inactivation has been reported while in other cases no effect has been observed (Van Loey *et al.*, 2002; Bendicho *et al.*, 2002b; Zhong *et al.*, 2005 and 2007; Mittal and Cross 1997). The mechanism of enzyme

inactivation by PEF is unclear (Giner *et al.*, 2001; Ohshima *et al.*, 2007), but it is believed to be due to unfolding, denaturation, and breakdown of covalent bonds and oxidation-reduction reactions caused by intense electric fields in the protein structure (Barsotti and Cheftel, 1999; Barsotti *et al.*, 2002).

Not all researches appear to have taken into account the temperature effects in PEF treatment which is an important variable in enzyme inactivation. The effects of PEF treatment on the activities of various milk enzymes including AlP, lipases, lactoperoxidase (LPX) and proteases (e.g. plasmin) in milk or SMUF has been reported by several researchers (Castro *et al.*, 2001; Bendicho *et al.*, 2002b; Vega-Mercado *et al.*, 1995); however, no report on PEF treatment of xanthine oxidase in milk or spiked in SMUF has been published.

According to Ho *et al.* (1997) enzyme inactivation requires a more severe PEF treatment than that needed for inactivating microorganisms. The higher the electric field intensity and temperature, the greater reduction in enzyme activity is achievable. Various researchers have reported large variations in the inactivation for different enzymes in milk and SMUF by PEF treatment (Martin-Belloso *et al.*, 2005b). Table 2-2 shows a collection of literature on PEF-mediated enzyme inactivation.

Grahl and Märkl (1996) treated raw milk by applying exponentially decaying pulses of 21.5 kV cm^{-1} in a batch PEF system with a gap of 0.5 cm between electrodes, and reported that LPX and AlP did not show any noticeable inactivation although lipase was inactivated by 60% at energy input of >200k J L^{-1} and maximum

temperature of 50ºC. Van Loey *et al.* (2002) reported that almost 100% of the LPX activity in raw milk was retained after applying 100 pulses of 5 µs at a field intensity of 19 kV cm^{-1}, pulse frequency of 1 Hz and treatment temperature of 25ºC, and that even after energy input of 500 kJ L^{-1} no loss of LPX activity was observed. They also evaluated the susceptibility of AlP in raw milk to PEF treatment of 200 pulses of 40 µs at 10 kV cm^{-1} at a maximum temperature of 70°C and achieved 74% inactivation. They attributed the inactivation of AlP solely to PEF treatment; however, it is obvious that high temperature might have played a role in the inactivation process. Castro (1994) reported that 59-65% of AlP was inactivated in SMUF, skim milk, low-fat milk (2%) and whole milk after PEF treatment at 18.8 - 22 kV cm^{-1}.

The effects of PEF and heat treatment on an extracellular lipase from *Pseudomonas fluorescens* suspended in SMUF have been studied by Bendicho *et al.* (2002b). The treatment chambers used were of parallel and co-axial configurations for batch and continuous flow modes, respectively. Samples were treated with 80 pulses at field intensities of 16 to 37 kV cm^{-1}. A batch-mode PEF equipment was used to expose SMUF to 80 pulses at 27.4 kV cm^{-1} (unknown treatment time) which resulted in 62% drop in lipase activity. However, when SMUF was exposed to PEF treatments of 80 pulses at 37.3 kV cm^{-1} and 3.5 Hz in the continuous flow mode, an inactivation rate of only 13% was achieved. The treatment temperature never exceeded 34˚C. The greater "unexpected" inactivation in batch mode was attributed to the higher energy level input (505 kJ L^{-1}) compared to continuous mode (424 kJ L^{-1}) despite the fact that the field intensity in the former was higher.

Table 2-2: Enzyme inactivation in milk and simulated skim milk ultrafiltrate by pulsed electric field treatment

Enzymes	Treatment medium	E[d] (kV/cm)	T[e] (°C)	Inactivation (%)	Treatment Chamber Type	Waveshape	PF[h]/Pw[i] (Hz)/ (µs)	Reference
[a]AIP	Skim milk	21.8	43.9	65	N/R[g]	Exponential decaying	70 pulses of 400 µs	Castro, 1994
Plasmin	SMUF[c]	15-45	60 and 80	90	Continuous with parallel electrodes	N/R	0.1 Hz	Vega-Mercado *et al.* 1995
AIP, LPX[b] lipase	Whole milk	21.5	<50	60 Trace 60	Batch	Exponential decaying	22 Hz	Grahl and Märkl 1996
AIP LPX	Whole milk	19	70	74 0	Batch	Monopolar square	1 Hz	Van Loey *et al.* 2002
Lipase *(P. fluorescens)*	SMUF	16.4 - 27.4	<34	62.1	Batch & coaxial continuous	Exponential decaying	2-3.5 Hz	Bendicho *et al.* 2002b
Protease (*B. subtilis*)	SMUF/ Skim milk	19.7-35.5	<46	62.7-81	Batch & coaxial continuous	Monopolar square	67, 89 and 111 Hz	Bendicho *et al.* 2003a
Protease (*B. subtilis*)	Skim milk	19.7-37.3	34 and 40	Activation-inactivation[f]	Batch & coaxial continuous	Monopolar square	22 pulses of 67 Hz and 80 pulses of 4 µs at 0.1 Hz	Bendicho *et al.* 2005
	SMUF	16.4-27.4	34 and 40	Activation-inactivation[f]	Batch & continuous coaxial	Monopolar square	22 pulses of 67 Hz and 80 pulses of 4 µs at 0.1 Hz	

[a]Alkaline phosphatase; [b]lactoperoxidase; [c]simulated skim milk ultrafiltrate; [d]field intensity; [e]treatment temperature; [f]depending on the frequency of treatment; [g]not reported; [h]pulse frequency; [i]pulse width

As comparison, HTST and low temperature long time (63℃/30 min LTLT) pasteurisation of samples inactivated only 5 and 20% of lipase, respectively.

2.6 Enzymes and Their Significance in Dairy Technology

2.6.1 Alkaline Phosphatase (EC 3.1.3.1)

Alkaline phosphatase is one of the principal enzymes in milk which is of technological significance in dairy industry since 1935 (Kay and Graham, 1935) as it is used as a legal index indicating the adequacy of milk pasteurisation. Moreover, due to its high heat sensitivity, AlP could be used as an intrinsic time-temperature indicator (TTI). Based on the American and European standards, the legal limit of AlP presence in milk after HTST pasteurisation should be between 0.35 to 0.5 U mL^{-1} (Harding and Garry, 2005). At temperature of 73℃ for 20 s or 75℃ for 15 s AlP was inactivated to below the legal maximum limit (Levieux *et al*., 2007). Inactivation of AlP is reported to start at 60℃ and complete at 85.6℃ (Eckner, 1992; Dhar *et al*., 1996; Lopez-Fandino *et al*., 1996; Painter and Bradley, 1997). However, there is still no indicator showing the adequacy of PEF treatment of milk and the effect of PEF treatment on AlP has been investigated by only few researchers (Castro *et al*., 2001; Grahl and Märkl, 1996) and an inactivation level of up to 65% has been reported (Table 2-2). More studies are needed to find a suitable indicator for PEF treatment.

2.6.2 Lipases (EC 3.1.1.1-)

Lipases, native or of microbial origin, catalyse the development of rancidity in milk. More than 90% of the lipase in milk is associated with the casein micelles which causes little lipolysis. The triglycerides (TG) are inside the fat globules surrounded

and protected by the milk fat globule membrane (MFGM) and when this membrane is damaged, the TGs are liberated and lipolysis occurs rapidly, giving rise to hydrolytic rancidity (Fox and Kelly, 2006). Technologically, lipase is the most significant enzyme in milk due to its role in rendering milk and its derivatives unpalatable and eventually unsaleable. It also plays a positive role in cheese ripening if it is of microbial source since microbial lipase is more resistant to heat than native lipase and can survive HTST pasteurisation. Deeth and Fitz-Gerald (1995) have published an extensive review on milk lipolysis and a study on the effects of PEF on microbial lipase has been reported by Bendicho *et al.* (2002b).

2.6.3 Xanthine Oxidase (EC 1.2.3.2)

It has been recognised for about 80 years that milk contains an enzyme capable of oxidising aldehydes, purines and hypoxanthines. The enzyme is generally referred to as xanthine oxidase (XO) and is associated with the MFGM (Patton and Keenan, 1975). The reaction catalysed by XO was described by McCord (1985) as:

$$\text{Hypoxanthine} + O_2 + H_2O \xrightarrow{XO} \text{Xanthine} + H_2O_2 + O_2 \xrightarrow{XO} \text{Uric Acid} + H_2O_2$$

The heat stability of XO is much dependent on whether it is attached to the fat globules or dissolved in the aqueous phase. Compared to AlP, this enzyme is more heat stable and is reported to be destroyed only by treatment at 80℃ for 10s. Owing to difficulties in measuring its activity, XO has not been considered as an indicator of milk pasteurisation adequacy (Shahani *et al*., 1973; Walstra and Jenness, 1984; Lewis *et al*., 1993; Girotti *et al*., 1999). Xanthine oxidase is more

heat stable in cream than in skim milk. Its significance in milk is related to shelf life of milk (spontaneous rancidity when XO levels are unusually high) and cheese ripening (Fox and McSweeney, 1998). There is so far no reported study on the effect of PEF on this enzyme.

2.6.4 Plasmin (EC 3.4.21.7)

Milk contains at least two proteinases, plasmin (alkaline milk proteinase) and cathepsin D (acid milk proteinase). Plasmin is the most important indigenous proteinase in milk and has been the subject of many studies (Grufferty and Fox, 1988; Bastiana and Brown, 1996). This enzyme is quite heat stable and is only partially inactivated by HTST pasteurisation (72^{o}C for 15 s), but becomes reactivated following the treatment, probably due to heat inactivation of its inhibitors or, more likely, inhibitors of plasminogen activators. Plasmin partially survives UHT sterilisation and contributes to age gelation of directly-heated UHT milk produced from even high-quality raw milk. It is reactivated by heating at 80^{o}C for 10 s at pH 6.8 (Fox and Kelly, 2006). In a study by Manji *et al.* (1986) on the effects of direct or indirect methods of UHT treatment on milk plasmin, it was found that only directly ultrahigh-temperature treated milk underwent age gelation and proteolysis after 84 to 98 days of storage at 22 to 25℃. Richardson and Pearce (1981) determined the amount of plasmin in pasteurised skim milk, sodium caseinate and rennet casein as 0.14, 0.73 and 1 to 8 μg mL^{-1}, respectively. It has been suggested that plasmin activity contributes to the poor cheese making properties of late-lactation milk (Fox and McSweeney, 1998). Few researchers have studied the effect of PEF on plasmin and proteases in milk (Vega-Mercado *et al.*, 1995; Bendicho *et al.*, 2002b).

2.7 Fitting of Microbial and Enzymatic Inactivation Data to a Mathematical Model

The development of mathematical expressions to define and quantify the effects of processing parameters on treatment effectiveness as well as determining critical factors in inactivation kinetics is an important task. Mathematical models can be used to gain insight into possible mechanism of action or to predict the microbial concentration and shelf life of processed products (Lund, 1983; Wouters *et al.*, 2001).

There are several models (Peleg, 1995 and 1999; Peleg and Cole, 1998) to mathematically explain the relationship between microbial or enzymatic inactivation and PEF field intensity or treatment time. The Hülsheger *et al.* (1981 & 1983) model is the most reported one which relates the microbial survival fraction *S* with PEF treatment time *t*. This model [ln N/N_0 or $S = -b_E(E-E_c)$, where N and N_0 are the microbial population before and after PEF treatment, b_E the regression coefficient; E_c the extrapolated critical value for field intensity] is based on the assumed linear relationship between the log survival fraction and field intensity as well as a linear relation between fraction of survivors and treatment time. The ln N/N_0 corresponds to the natural log of the survival fraction and yields a straight line when plotted against treatment time. Table 2-3 shows the Hülsheger model applied to various microorganisms.

The Hülsheger model has been adapted by some researchers (Min *et al.,* 2003) as $\ln(A/A_0) = -b_E(E-E_C)$ to describe the enzymatic inactivation after PEF treatment where A and A_0 are the enzyme activity before and after PEF treatment. Optimum

processing conditions should be established to obtain the maximum inactivation level with the minimum heating effect.

In the current project the Hülsheger's model is used to demonstrate the mathematical relationship between treatment time, total specific pulsing energy, field intensity and enzyme inactivation.

Table 2-3 Kinetic constants of Hülsheger's model for different microorganisms suspended in a Na_2HPO_4/KH_2PO_4 buffer at pH 7.0

Microorganisms	E (kV cm^{-1})	t (ms)	E_c (kV cm^{-1})	t_c (µs)	K	R^2
Escherichia coli (4 h)[1]	4 - 20	0.07 - 1.1	0.7	11	8.1	0.97
Escherichia coli (30 h)[1]	10 - 20	0.07 - 1.1	8.3	18	6.3	0.97
Klebsiella pneumonia	8 - 20	0.07 - 1.1	7.2	29	6.6	0.95
Pseudomonas auriginosa	8 - 20	0.07 - 1.1	6.0	35	6.3	0.98
Staphylococcus aureus	14 - 20	0.07 - 1.1	13.0	58	2.6	0.97
Listeria monocytogenes I	12 - 20	0.07 - 1.1	10.0	63	6.5	0.97
Listeria monocytogenes II	10 - 20	0.07 - 1.1	8.7	36	6.4	0.98
Candida albicans	10 - 20	0.14 - 1.1	8.4	110	2.2	0.96

(Hülsheger *et al.*, 1983); E, field intensity; E_c, critical field intensity; t, treatment time; t_c, critical treatment time (minimum treatment time needed to inactivate); K, kinetic constant; R^2, correlation coefficient of regression line; [1]Incubation time

2.8 Effects of PEF Treatment on the Functionality of Milk Proteins and Fat Globules

There are very few studies on the effects of PEF on functionality of proteins in milk and no reported study on the effects of PEF on fat globules since most of the studies have focused on microbial and enzymatic inactivation. However, since the protein and fat functionality affects the yield and physical characteristics of the final product, it is of great importance to expand research in this area. Cheese is the most common dairy product and thermal or nonthermal treatment of cheese milk can directly or indirectly affect the final physical and sensory properties of cheese. Curd formation is a critical step in cheese making process as it could affect the yield, physical properties and appearance of the resulting cheese. The rennet coagulation time as well as the enzymatic activity are economically important and could affect the quality and ripening period of the cheese (Lucey, 2003). Elucidation of the protein functionality in milk has been attempted by many protein chemists; however, a full knowledge on the mechanism of PEF effects on milk enzymes, fat and protein is still lacking. A few studies reported on the effects of PEF on various cheese physical attributes are dealt with in the following sections.

In a comparative study, Sepulveda *et al.* (2000) compared the quality attributes of cheddar cheese made from PEF-treated and pasteurised milk samples. The pulsing rate was set at 3.3 Hz with 30 exponentially-decaying pulses of 35 kV cm^{-1} while milk temperature was kept below 30^{o}C. The flow rate was 600 mL min^{-1} and the treatment chamber used was coaxial. The raw milk cheddar cheese was used as the control and textural and sensory attributes were determined in all samples.

The cheese from the PEF-treated and pasteurised (HTST and LTLT) milk showed increased hardness and springiness compared with the raw milk cheddar.

Wüst *et al.* (2004) assessed the physical attributes of cottage cheese made from PEF-treated skim milk. The treatment was conducted by applying 9.6 bipolar square pulses of 2 μs at field intensities of 25 and 28 kV cm^{-1} with flow rate of 120 mL min^{-1} at a treatment temperature of <45°C. It was found that increasing both field intensity (from 25 to 28 kV cm^{-1}) and pulse frequency (from 200 to 400 Hz) decreased the firmness of the cottage cheese gel, and that field intensity and pulse frequency had a small but increasing effect on the yield of cottage cheese compared to cheeses made from raw or pasteurised skim milk. Compared to treatment of milk at pulse frequency of 200 Hz, the "raw milk" odour was removed from the samples treated at 400 Hz.

Floury *et al.* (2006b) found that the PEF treatments at the field intensities of 45 or 55 kV cm^{-1} with pulse widths of 500 and 250 ηs (square monopolar pulses) respectively, decreased the clotting time. At a total treatment time of 2.1-3.5 μs, a significant drop in casein micelle size was observed while the viscosity of milk decreased and the coagulation properties were enhanced.

There are many studies on the effects of pasteurisation and various heat treatments on milk constituents (including caseins and fat globules) with sound and consistent results; however, to reach a sound understanding of how PEF affects the functionality of proteins and fat globules a standard PEF system and experimental procedure as exists and regulated for pasteurisation is essential.

CHAPTER 3[1]

INACTIVATION OF MICROFLORA AND ALKALINE PHOSPHATASE IN BOVINE MILK BY PULSED ELECTRIC FIELD

ABSTRACT

The effects of PEF treatments at field intensities of 25-37 kV cm^{-1} and final temperatures of 30℃ and 60ºC on the inactivation of alkaline phosphatase (AlP), Total Plate Count (TPC), *Pseudomonas* and *Enterobacteriaceae* counts were determined in raw skim milk. At 30ºC, PEF treatments with 28 to 37 kV cm^{-1} resulted in 24-42% inactivation in AlP and <1 log reduction in TPC and *Pseudomonas* count, while the *Enterobacteriaceae* count was reduced by at least 2.1 logs below the detection limit of 1 colony forming units (CFU) mL^{-1}. The PEF treatments of 25 to 35 kV cm^{-1} at 60℃ resulted in 29-67% inactivation in AlP and up to 2.4 logs reduction in TPC, while the *Pseudomonas* and *Enterobacteriaceae* counts were reduced by at least 5.9 and 2.1 logs, respectively, to below the detection limit of 1 CFU mL^{-1}. Fitting the AlP inactivation data to kinetic model suggested that the slope of the linear relationship between the field intensity and AlP inactivation at 60℃ was more than twice of that at 30℃. A combined effect was observed between the field intensity and temperature in inactivation of both AlP enzyme and the microflora in raw skim milk.

Based on the results of this study a paper was published in the journal of *Innovative Food Science and Emerging Technologies* 9 (2008): 217-223.

3.1 INTRODUCTION

The study on the effects of PEF on the inactivation of AlP in milk to date has been limited to only few researchers who have determined the AlP activity in spiked SMUF or milk after treating with exponentially decaying pulses (Barbosa-Canovas *et al*., 1999; Castro *et al*., 2001; Castro, 1994; Van Loey *et al*., 2002; Grahl and Märkl, 1996) with the aim of observing the effects of field intensity on the structure of enzyme and inactivation level. The indigenous AlP is technologically significant in dairy industry since it is used as an indicator of milk pasteurisation adequacy. However, no study has to date investigated the possibility of using AlP as an indicator of PEF treatment adequacy.

Likewise, most of the studies performed on the effects of PEF on microbial inactivation have been conducted on spiked milk or SMUF with isloated microorganisms rather than the indigenous microflora of milk.

The main objective of this chapter was to investigate the effects of field intensity and total specific pulsing energy on the inactivation of milk total plate count (TPC), *Pseudomonas* and *Enterobacteriaceae* as well as AlP after treating skim milk with various field intensities in combination with mild heat.

3.2 MATERIALS AND METHODS

3.2.1 Raw Skim Milk

The fresh, raw, cold-skimmed milk used in all experiments in this thesis was provided by Warrnambool Cheese & Butter Factory (5331 Great Ocean Road, Allansford, 3277 VIC, Australia,). The conductivity (σ) of milk samples as

measured with a conductivity meter (Model MC-84, TPS Pty Ltd, Brisbane, Australia) was 4.59 and 5.11 mS cm^{-1} at 30 and 60°C, respectively. The pH of milk used for the study in this chapter was measured at 24°C using a bench top pH meter (PHM210, MetroLab, Radiometer Pacific, Copenhagen, Denmark) as 6.66. In each chapter the pH and conductivity of milk is measured and reported separately. The chemicals used for all the studies in this project were of analytical grades and purchased from Sigma-Aldrich (Unit 2 14 Anella Ave, Castle Hill, NSW 2154, Australia).

3.2.2 PEF Treatments

All PEF trials in this and the following chapters have been replicated at least twice in two different occasions to obtain reproducible results. During each PEF trial 3 samples were collected and each sample was tested in duplicate for microbial, enzymatic or functionality analyses. Therefore, for each PEF trial 6 sets of results were reported as the mean ± standard deviation (SD) of that trial. To avoid mixing the PEF-treated samples between each trial, an interval of 4 minutes was allowed between each PEF trial for collecting the samples.

A laboratory scale PEF system (modified from OSU-4, Ohio State University, Columbus, OH, USA, Fig. 3-1a & b) was used for all PEF treatments. The system was equipped with 4 cofield treatment chambers connected in series with a throughput of up to 15 L h^{-1}. Its pulse generation capacity was up to 12 kV that provided bipolar or monopolar square pulses with a pulse rate of 0 to 2000 Hz and pulse width between 1 to 10 µs. A signal generator (Model 9310 Quantum Composers, Bozeman, Mont., USA) controlled pulse width, pulse delay time and pulse frequency. The diameter of each treatment chamber was 2.3 mm that

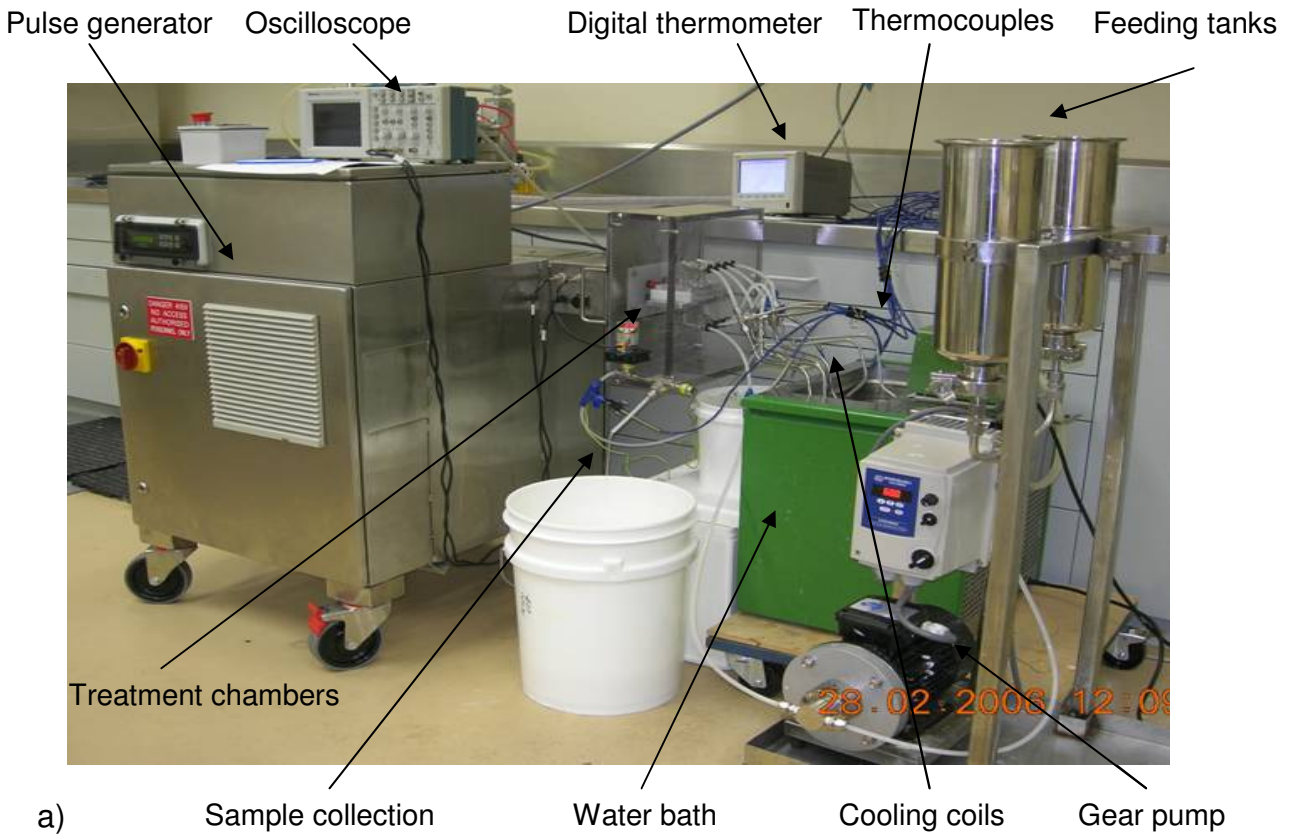
housed 4 stainless steel electrodes with a gap of 2.9 mm (Fig. 3-1b). Treatment chambers were connected to stainless steel coils (internal diameter: 4 mm, external diameter: 6 mm, length: 400 mm). The coils were submerged in temperature-controlled water baths with refrigeration capacity (Thermomix BU, Braun Melsungen, Germany, temperature accuracy ± 0.3°C), one prior to the 1st treatment chamber for pre-warming, one between the outlet of each chamber and the inlet of the next chamber, and one after the 4th treatment chamber (immersed in ice bath) for immediate cooling of samples. The inlet and outlet temperatures of each treatment chamber were monitored and recorded by 2 type-T thermocouples (Fisher Scientific, Pittsburgh, PA) inserted inside stainless steel housings (total of 12 thermocouples) and connected to a 12-channel temperature recorder (ABB SM1000, Amplicon, UK). The thermocouples were grounded to prevent noise and interference. The outlet temperature of the fourth treatment chamber was reported as the final temperature.

The product handling system comprised a gear pump (Micropump GA-V23, Leinburg, Germany) and two stainless steel feeding tanks connected using a 3-way valve. Voltage and current outputs were recorded using a 2-channel digital oscilloscope (Tektronix, Model TDS 380, USA). Figure 3-1 (a & b) shows PEF equipment based at Food Science Australia (671 Sneydes Rd., Werribee, VIC, 3030) and its treatment chamber which is modified from the OSU-4 model so that the product leaving the first treatment chamber passes through cooling coils and the cooled product enters the 2nd chamber. This cooling system applies to the third and fourth treatment chambers as well and the temperature in each stage can be

monitored through the thermocouples attached to each stage. The outlet milk passes through a coil submerged in ice bath for final chilling.

At the start of each trial, the product line of the PEF unit was washed and sanitised immediately before and after each treatment using a cleaning-in-place (CIP) procedure at a maximum pump speed of 15 L h^{-1} consisting: 1.5 L warm (40°C) water, followed by 1.5 L hot (80°C) 5% Techsil solution (Ecolab, 30% caustic soda), 1.5 L hot water rinse, 1.5 L 0.3% Oxonia sanitiser (Ecolab, 10% peroxyacetic acid) solution followed by 1.5 L hot water for final rinse. A sample of rinse water was collected and tested for sterility of the line prior to each microbial inactivation experiment.

In order to avoid cross-contamination and the need for washing and sanitising between each treatment, for each treatment temperature, the equipment was operated with the highest field intensity followed by lower intensities. For example, the input voltage always started from 9.5 down to 6.5 kV since the higher voltage was assumed to have more lethal effect on microorganisms (Table 3-1). The tip of the final outlet tube was sterilised by alcohol.



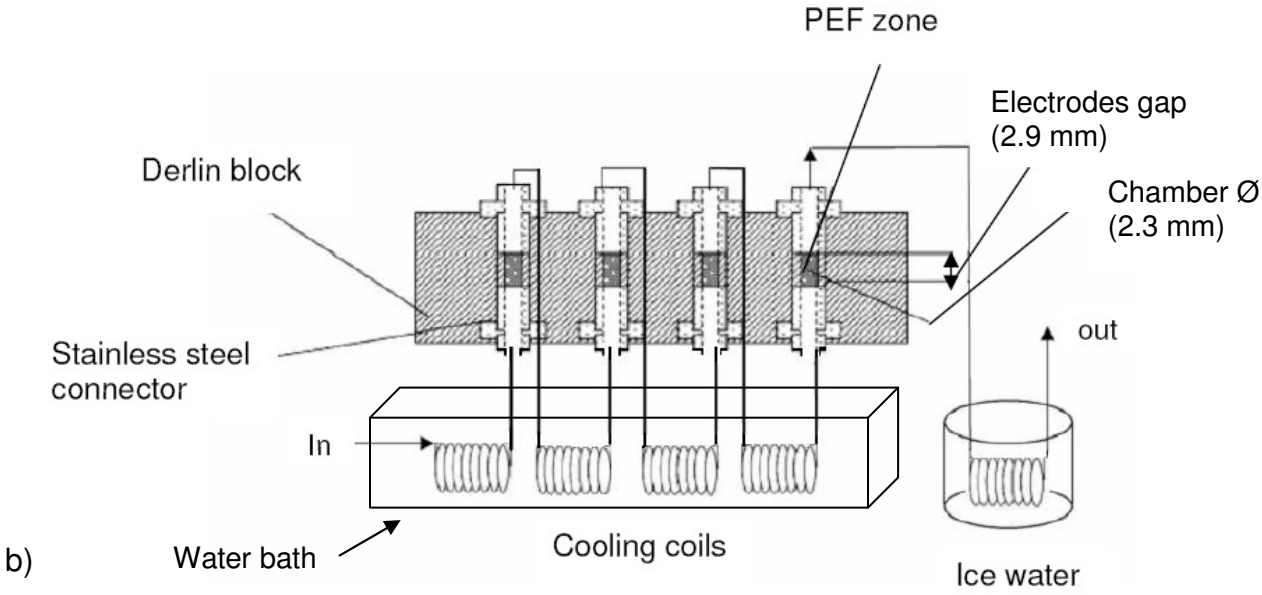


Figure 3-1 a) The OSU-4 pulsed electric field unit at Food Science Australia
b) Schematic drawing of cofield treatment chamber of OSU-4 PEF system at FSA (Re-drawn and modified from Evrendilek and Zhang, 2005)

The treatment time was calculated (for calculation see section 2.3.2.3) based on the geometry of treatment chamber and its diameter of 2.3 mm, effective volume (12 µL), flow rate (60 mL min^{-1}), pulse width (2 µs), pulse frequency (200 Hz) and electrodes' gap of 2.9 mm. For this study, the total PEF treatment time and number of square monopolar pulses were 19.2 µs and 9.6 (in all four treatment chambers), respectively. The PEF treatment was conducted at 25 to 37 kV cm^{-1} with final temperatures (at the outlet of the 4^{th} treatment chamber) of 30 or 60°C. Depending on the field intensities used and the temperature of the skim milk fed into the system (5-8°C), the inlet temperatures were adjusted to 15-20°C for the 30°C trials, and to 45-50°C for the 60°C trials by setting the water bath 30 and 60°C. The inlet temperatures at 30°C for treatment chambers of 1, 2, 3 and 4 were 27, 34, 35 and 33°C and for 60°C were 50, 60, 61 and 60°C, respectively. The sterile (UHT) skim milk was used to start up the system, and samples of the milk were collected for sterility testing. The calculation of treatment time and total specific pulsing energy has been given in chapter 2, section 2.3.2.3.

When the PEF system reached stability in temperature and field intensity, the inlet valve was switched to raw skim milk. The PEF treated samples were collected aseptically in triplicate in sterile 30 mL capped plastic bottles and chilled immediately in an ice bath. Collected samples were evaluated in duplicate for AlP enzyme activity and microbial counts. To prevent air bubble formation in the treatment chamber and process interruption, all trials were conducted with a back-pressure between 15 and 20 psi. Table 3-1 shows the electrical parameters of PEF treatment applied for the experiments in this chapter.

Table 3-1 The PEF treatment conditions of skim milk

Outlet T (℃)	Input voltage (kV)	Field intensity (kV cm^{-1})	Current (A)	Energy input (kJ L^{-1})
	6.5	28	20	65
	7.5	31	24	87
30	8.5	34	28	112
	9.5	37	36	139
	6.5	25	24	65
	7.5	29	28	87
60	8.5	31	36	112
	9.5	35	44	139

The pulse frequency and flow rate were maintained at 200 Hz and 60 mL min^{-1} giving a fixed treatment time of 19.2 μs, respectively. The electrical conductivity of skim milk at 30 and 60℃ was 4.53 and 5.11 mS cm^{-1}, respectively.

The control samples were prepared by passing the milk (with the same flow rate of 60 mL min^{-1}) through the PEF treatment chambers and the water baths with the pulse modulator switched off (E=0). The water bath was adjusted to simulate the temperatures of PEF treated samples. The total exposure time of milk to final treatment temperature was approximately 5 seconds based on the flow rate and volume of tubing between the outlet of the 4^{th} treatment chamber and the ice bath.

3.2.3 Heat Treatment of Milk

As comparisons to PEF treatment, samples of raw skim milk were pasteurised and high-heat treated using a temperature-controlled water bath. For HTST pasteurisation, duplicate aliquots of 25 mL raw skim milk were transferred into a 50 mL beakers and placed in a water bath at 90ºC with constant stirring. Once the milk temperature reached 72ºC (90 s come-up time) the beaker was taken out and a stopwatch was activated to count the 15 s holding time. Care was taken to prevent milk temperature drop during this period. The beaker containing pasteurised milk was then placed in an ice bath. For LTLT pasteurisation, a 100 mL beaker consisting 50 mL raw skim milk was placed in water bath at 63ºC for 30 min with constant stirring and then placed in an ice bath. For high-heat treatment 100 mL raw skim milk in a 200 mL beaker was placed inside a steam bath at 100^{o}C with constant stirring and once the milk temperature reached 97ºC it was held for 10 min then transferred into the ice bath. This milk sample was used for negative control.

3.2.4 Alkaline Phosphatase Activity Assay

The activity of AlP was determined by a method modified from the Australian Standard Method AS 2300.1.10 (Standards Association of Australia, 1988). A bicarbonate buffer solution (pH 9.8) was prepared by dissolving 3.5 g anhydrous sodium carbonate (Na_2CO_3) and 1.5 g sodium bicarbonate ($NaHCO_3$) in Milli Q water in a 1 L volumetric flask and made up to volume. The pH was adjusted to 9.8 by adding few drops of 1 M NaOH. For the positive and negative controls, raw and high-heat treated skim milk was used, respectively. Aliquots (50 µL) of high-

heat treated, PEF-treated, and untreated skim milk were diluted 50, 100 and 200 times with this buffer solution.

Aliquots (500 µL) of Alkaline Phosphatase Liquid Substrate System (Sigma-Aldrich no. N7653) were pipetted into duplicate 1.7 mL microtubes (MCT-175-C, Axygen Scientific Inc., 33210 Central Avenue, Union City, California 94587, USA) and warmed up for 5 min in a water bath at 37°C followed by transferring 100 µL aliquots of each diluted milk sample into the tubes and incubating for 30 min at 37°C. The samples were then removed from water bath and cooled in ice bath for 5 min to stop the enzyme reaction. Then 250 µL of the top layer was pipetted into 96-well microtest plates (Sarstedt Australia Pty. Ltd., 16 Park Way Technology Park, Mawson Lakes, SA 5095, Australia) and the absorbance was measured at 410 nm using a spectrophotometer (UV) (SpectraMax Plus 384, Molecular Devices Corporation, Sunnyvale, CA, USA). One unit of AlP activity was defined as the activity which releases 1 µmol of *p*-nitrophenyl phosphate per minute at pH 9.8 and 37°C (Fadiloglu *et al*., 2004). The amount of *p*-NP released in the assay was calculated using a standard curve established in each experiment using freshly prepared *p*-NP solutions (0-25 µg mL^{-1}) in bicarbonate sodium buffer (pH 9.8). A typical standard curve of *p*-NP is presented in Appendix 1.

$$\text{Enzyme (U mL}^{-1}) = \frac{p\text{-NP (µg mL}^{-1}) \times \text{Va}}{139.12\ (\text{MW of } p\text{-NP}) \times \text{Vm} \times \text{It} \times \text{D}}$$

Where: Va = volume of assay (0.6 mL), Vm = volume of milk (0.01 mL) and D = dilution factor (50, 100 and 200 times) and It = incubation time (30 min)

The residual activity of AlP was calculated from the following formula:

$$\text{Residual activity (\%)} = 100 - \frac{\text{Activity of the treated enzyme solution}}{\text{Activity of the untreated enzyme solution}} \times 100$$

3.2.5 Preparation of Standard Curve

In a 100 mL volumetric flask, 0.25 g *p*-nitrophenol (Sigma-Aldrich no. 1048, $O_2NC_6H_4OH$) was weighed and dissolved in bicarbonate buffer solution (pH 9.8), and made up to volume. The solution was then diluted to 25, 20, 15, 10, 5 and 0 µg mL^{-1} *p*-nitrophenol with buffer solution and was pipetted into micro test plates and the absorbance was measured at 410 nm. The relationship between absorbance and concentration was found to be linear (Appendix 1) based on which the regression coefficient between the concentration of *p*-nitrophenol in buffer solution (µg mL^{-1}) and absorbance was calculated.

3.2.6 Microbial Tests

All microbiological media and supplements were obtained from Oxoid Australia Pty Ltd. (20 Dalgleish Street, Thebarton, Adelaide, South Australia 5031, Australia).

The 0.1% peptone diluent was prepared by dissolving 1 g Bacteriological Peptone (Oxoid LP0037) in 1 L distilled water and heating to boiling temperature in a flask, followed by cooling to 48℃ in a water bath. The diluent was then autoclaved at 121℃ for 15 min and after cooling to room temperature, 9 mL aliquots were

aseptically transferred into test tubes. The PEF-treated, untreated and control milk samples were serially diluted in 0.1% peptone diluent. One mL aliquots of diluted sample were transferred to media in petri dishes, incubated accordingly and the number of CFU were calculated by multiplying the number of colonies by dilution factor divided by volume (mL) of sample used. Only those petri dishes whose number of colonies was between 30 and 300 were selected for enumeration.

The TPC was determined using Plate Count Agar (PCA) (Oxoid CM0019), prepared by suspending 17.5 g of PCA in 1 L of distilled water and boiling to dissolve the agar completely. The agar solution was then cooled down to 48°C, the pH was adjusted to 7.0±0.2 by 1 M NaOH and transferred into Schott bottles and autoclaved at 121°C for 15 min. The medium was then cooled down to 50°C and 16 mL aliquots were transferred into sterile petri dishes (Techno-Plas, 62-66 Mark Anthony Drive, Dandenong, South VIC 3175, Australia). After inoculation, the petri dishes were incubated at 30°C for 72 h.

The enumeration of *Pseudomonas* bacteria was conducted by spread plate method. The medium was prepared by dissolving 24.2 g of Pseudomonas Agar Base (Oxoid CM0559) in 500 mL of distilled water in a round-bottom volumetric 1 L flask and adding 5 mL glycerol. The medium was brought to boil to dissolve the agar completely and then was cooled down to 48°C and the pH was adjusted to 7.1±0.2. The medium was then sterilised by autoclaving at 121°C for 15 minutes and allowed to cool down to 50°C in a water bath. The content of 1 vial of *Pseudomonas* C-F-C Supplement SR103 (Oxoid CM0559) was aseptically mixed with 1 mL sterilised Milli-Q water and 1 mL ethanol and added to the medium to

make it selective for *Pseudomonas* species. Aliquots of 16 mL were distributed into sterile petri dishes (Techno-Plas, 62-66 Mark Anthony Drive, Dandenong, South VIC 3175, Australia) and incubated at 25℃ for 48 h after inoculation.

For *Enterobacteriaceae* enumeration pour plate method with overlay was used to create an anaerobic environment. To prepare the medium, 38.5 g Violet Red Bile Glucose Agar (VRBGA-Oxoid CM0485) was suspended in 1 L distilled water and brought to boil and continued to boil for 2 minutes to dissolve the agar completely. No further sterilisation was conducted as per instruction on the agar label. The agar solution pH was aseptically adjusted to 7.4±0.2 by adding few drops of 1 M sterilised (121ºC for 15 min) NaOH. A thin layer of cooled molten medium was poured over the inoculated base layer and allowed to set before incubation. The petri dishes were incubated at 30℃ for 24 h after inoculation.

3.2.7 Statistical Analysis of Data

Statistical analyses were performed by applying one-way ANOVA to determine the significance of the 95% confidence interval and correlation coefficient using Minitab software (Version 12, Minitab Inc., State College, PA, USA). The means and standard deviations were the results of 6 replicates from each of the two PEF trials.

3.3 RESULTS

3.3.1 Temperature Dependency of Milk Conductivity and Its Influence on the Field Intensity

The field intensities achieved were influenced by change of conductivity of skim milk at 30 and 60℃ regardless of the input voltage (Table 3-1). Skim milk's conductivity at 30℃ was 4.59 mS cm^{-1}, that raised to 5.11 mS cm^{-1} at 60℃. However, the total specific pulsing energy input was the same (65 kJ L^{-1}) for both PEF treatments as the pulsing energy input is a function of input voltage rather than the temperature. An increase in the food electrical conductivity increases the peak discharge current and thus the power requirement will be higher (Zhang *et al.,* 1995).

3.3.2 Microbial Inactivation by PEF Treatment and Thermal Pasteurisation

Treatment of raw skim milk at various field intensities (25 – 37 kV cm^{-1}) resulted in partial inactivation of microflora. The initial TPC and viable count of *Pseudomonas* and *Enterobacteriaceae* was 6.1, 5.9 and 2.1 log CFU mL^{-1}, respectively. At 30℃ and field intensities of 28, 31, 34 and 37 kV cm^{-1}, the inactivation levels of TPC and *Pseudomonas* was < 1 log CFU mL^{-1}. At 60℃, without PEF treatment the log reductions for TPC, *Pseudomonas* and *Enterobacteriaceae* were 2.3, 2.4 and 1.6, respectively. When PEF was applied, the field intensities of 25, 29, 31 and 35 kV cm^{-1} had a little effect on TPC while achieving an additional 1–3.5 logs reduction on *Pseudomonas*, and 0.6 log reduction on *Enterobacteriaceae* count to below the detection limit of 1 CFU mL^{-1}. It is evident that the inactivation of the *Pseudomonas* population increased with the increase in the field intensity and temperature, with a

combined treatment of 35 kV cm^{-1} and 60°C achieving >5.9 logs reduction. Figure 3-2 a & b shows the viable count for all microorganisms.

Both LTLT and HTST pasteurised samples produced 2.5 logs reduction in TPC, 6.0 logs reduction in *Pseudomonas* count, and 2.1 logs reduction in *Enterobacteriaceae* count to below the detection limit of 1 CFU mL^{-1}. There was no significant difference (p> 0.05) between the 35 kV cm^{-1} 60°C PEF treatments and the LTLT or HTST thermal pasteurisations in the inactivation of *Pseudomonas* and *Enterobacteriaceae*.

3.3.3 AlP Inactivation

The initial residual activity of AlP was 1.8 U mL^{-1}. Treatment of raw skim milk in various field intensities (25–37 kV cm^{-1}) resulted in partial inactivation of AlP. Figures 3-2 a & b show the residual activity of native AlP in raw skim milk after PEF treatments at 30 and 60°C. At 30°C, PEF treatment at field intensities of 28, 31, 34 and 37 kV cm^{-1} produced 24, 25, 31 and 42% inactivation, respectively. At 60°C, the field intensities of 25, 29, 31 and 35 kV cm^{-1} resulted in 29, 42, 56 and 67% inactivation, respectively, while without the PEF treatment the inactivation was only 22%. As a control, raw skim milk was thermally pasteurised by HTST and LTLT methods, and 98% of the AlP was inactivated in both pasteurised samples.

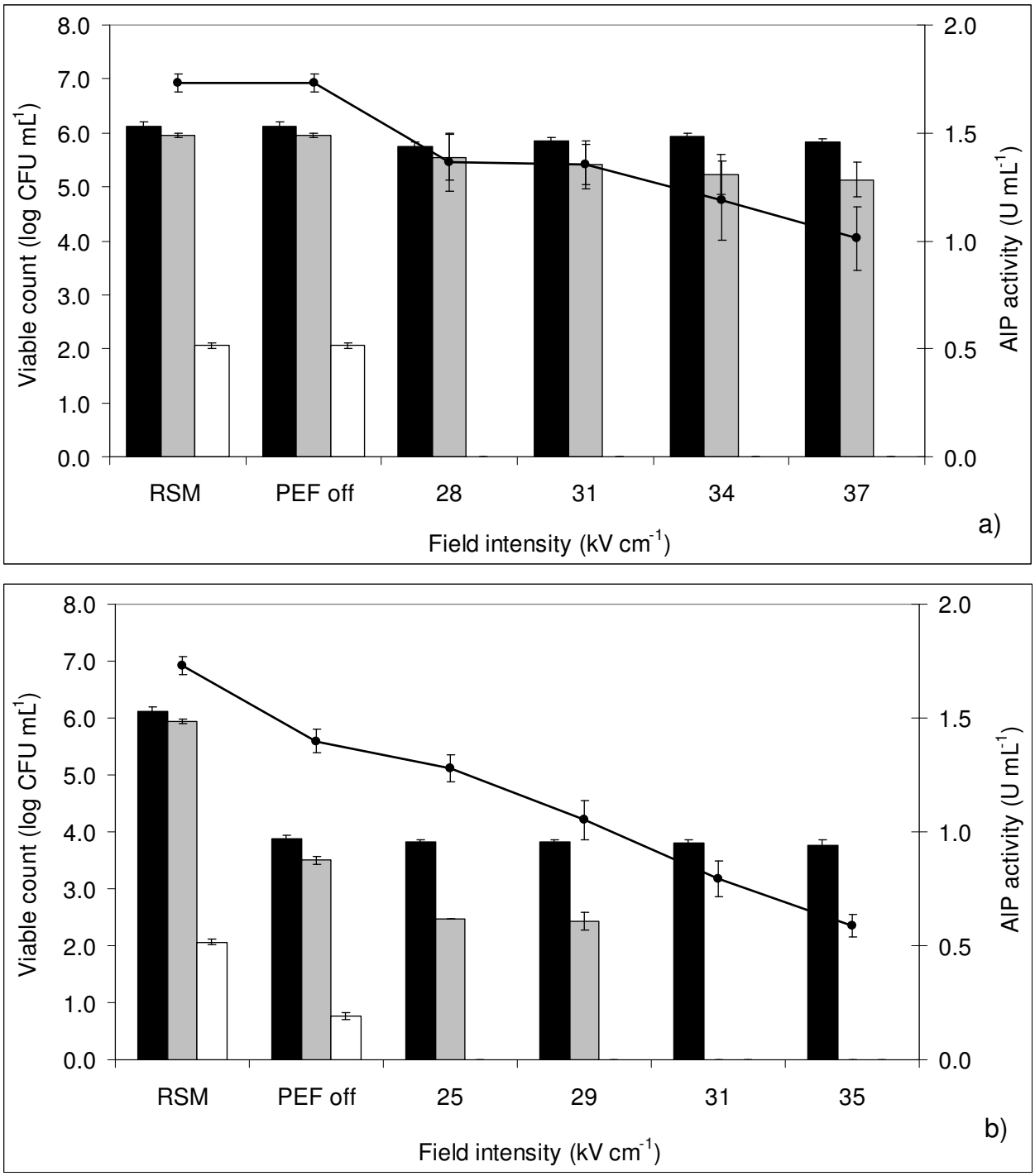


Figure 3-2 Effect of pulsed electric field with final temperature of 30°C (a) and 60°C (b) on microbial flora and native alkaline phosphatase activity in raw skim milk at different field intensities (kV cm^{-1}). PEF conditions: frequency of 200 Hz, monopolar pulses of 2 µs with total treatment time of 19.6 µs. RSM: raw skim milk

■ Total plate count, □ *Pseudomonas*, ☐ *Enterobacteriaceae*, ●— Alkaline phosphatase

3.3.4 Fitting AlP Inactivation Data to Hülsheger Kinetic Model

Figure 3-3 shows the relationship between AlP inactivation and field intensity through the Hülsheger *et al.* (1981) kinetic model. This equation was originally used for microbial inactivation, however, it has been used by some researchers to indicate the mathematical relationship between enzyme inactivation, field intensity and treatment time (Min *et al.*, 2003). The residual activity (RA) of AlP obtained after each PEF treatment was defined as: $RA = A/A_0$, where A and A_0 are the residual activity of AlP after and before PEF treatment. The experimental data were fit to the Hülsheger first-order inactivation kinetic model as follows:

$ln(A/A_0) = -b_E(E-E_C)$, where b_E is a proportionality constant or slope which represents the rate of AlP inactivation as a function of field intensity E. The E_C is the critical field intensity required for the inactivation of AlP.

The treatment time, flow rate and pulse frequency were kept constant at 19.2 µs, 60 mL min^{-1} and 200 Hz, respectively. The correlation between the AlP inactivation and field intensity indicated a strong relationship which meant that higher field intensity would result in higher enzyme inactivation. The slope of the line showed a strong linear relationship between the field intensity and AlP inactivation at both 30 and 60℃ with an R^2 of 0.897 and 0.9701, respectively. The steep of the slope indicates the degree of PEF effect.

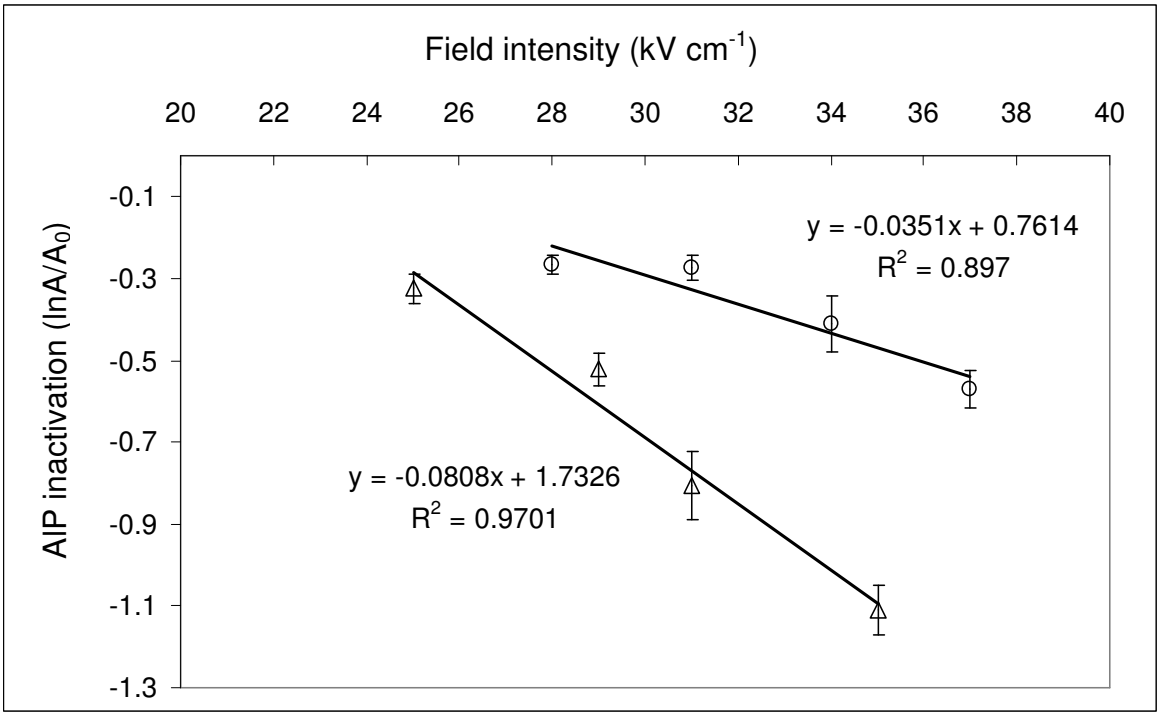


Figure 3-3 The correlation between various PEF field intensities and alkaline phosphatase inactivation in skim milk at 30℃ (O) and 60℃ (Δ).

3.4 DISCUSSION

The energy input (Q) is a function of voltage (V), current (A), pulse width (P_w), number of pulses (N_p) and volume of chamber (v) (see Chapter 2, section 2.3.2.3). The electric pulses are discharged into the fluid food and result in temperature rise due to Joule heating and if there is no cooling system, the outlet temperature could rise above 90ºC for a product entering the chamber at room temperature (Zhang *et al*., 1995). In this study raising the input voltage from 6.5 to 9.5 kV (Table 3-1) increased the total specific pulsing energy input from 65 kJ L^{-1} to 139 kJ L^{-1} which resulted in an increase in the level of microbial and AlP inactivation as shown in Figure 3-2. Bendicho *et al*. (2002b) found a strong relationship (R^2 = 0.89) between the energy input and inactivation of a microbial lipase in SMUF. The range of energy applied was 0 to 500 kJ L^{-1} which achieved a maximum 62% inactivation in lipase. The range of dissipated energy in the experiments in this chapter lies in the range applied by Bendicho *et al.* (2003b), which led to a maximum inactivation of 67% in AlP. Increasing the energy input can result in higher enzyme inactivation (Schilling *et al*., 2008).

Fernandez-Molina *et al.* (2006) also found that increasing the level of energy input resulted in a greater inactivation of *Pseudomonas fluorescens* and *Listeria innocua*. The field intensity applied was between 31 to 39 kV cm^{-1} at a temperature <20°C. The range of energy level was 0 to 270 kJ L^{-1}. For both microorganisms a maximum of 2.6 to 3.0 logs CFU mL^{-1} inactivation was achieved.

The efficacy of microbial inactivation by PEF is affected by a number of factors including field intensity, pulse characteristics, temperature and presence of different types of microflora. Raw milk contains a relatively wide range of microorganisms including Gram positive and Gram negative bacteria, yeasts and moulds, and a range of bacterial spores. Typically some of the Gram positive thermoduric bacteria and spores will survive pasteurisation and PEF processes, and be enumerated in TPC of the treated product.

There was a significant difference ($p< 0.05$) between the inactivation levels of TPC at 30 (Fig. 3-2a) and 60℃ (Fig. 3-2b); however, this difference was mostly due to the effect of heat rather than field intensity. Therefore, the resistance to PEF treatment at 30℃ may be due to the survival of Gram positive bacteria.

In the current study, the *Pseudomonas* population decreased with an increase in the field intensity and treatment temperature. A combined treatment of 60℃ and 35 kV cm^{-1} resulted in >5.9 logs reduction. The additional 1 - 3.5 logs reduction on the native *Pseudomonas* number in raw skim milk resulted from the PEF treatment at 60℃ compared with that at 30℃ which demonstrated the enhanced microbial kill of PEF at mild heating conditions. This observation was also reported for *Salmonella* and *E. coli* inactivation in other food systems (Heinz *et al*., 2003; Bazhal *et al.,* 2006; Amiali *et al.,* 2007). The inactivation of the native *Pseudomonas* population obtained in the current study is consistent with results of previous studies where defined *Pseudomonas fluorescens* cultures were inoculated into milk and milk-based test systems as the target microorganism (4.2 log by 22 kV cm^{-1} at 45-50℃, Grahl and Märkl, 1996; 2.6 logs by 50 kV cm^{-1} at

28℃, Fernandez-Molina *et al.* 2001; 2.2 logs by 35 kV cm^{-1} 52℃, Michalac *et al.*, 2003). Craven *et al.* (2008) inoculated UHT milk samples with *Pseudomonas* and treated them at 55℃ with 31 kV cm^{-1} (139.4 kJ L^{-1}, flow rate of 60 mL min^{-1}, treatment time of 19.6 µs, pulse width of 2 µs and pulse frequency of 200 Hz) and achieved >5 logs reduction in the number of *Pseudomonas*. When the PEF was switched off little inactivation was achieved (only 0.2 log) by heating the milk to 55℃ indicating the efficiency of PEF in inactivation process.

Enterobacteriaceae was more inactivated by PEF and heat than TPC and *Pseudomonas* since it was totally inactivated at both 30 and 60℃ (Fig. 3-2) However, compared to *Pseudomonas* (with initial count of 5.9 logs CFU mL^{-1}), the initial number of *Enterobacteriaceae* was nearly 4 logs lower which contributed to its total elimination. Ferrer *et al*. (2007) and Juffs and Deeth (2007) also reported that the initial microbial load affected the number of survivors after PEF treatment.

Based on the microbial results, the combination of heat and PEF treatment has obviously resulted in a higher inactivation level of microorganisms since the electroporation process (Zimmermann, 1986) is boosted by heat damage of the cell wall leading to accelerated and increased death rate. As the temperature increases above that at which growth of microorganism ceases, the cell wall is damaged which could be irreversible and leading to death of cell (Juffs and Deeth, 2007).

There was no significant difference (p> 0.05) between the PEF treatment at 35 kV cm^{-1} and 60℃ and the LTLT or HTST pasteurisation in inactivation of

Pseudomonas and *Enterobacteriaceae.* These results suggest that the combination of heat (60°C) and 35 kV cm^{-1} is as effective as thermal pasteurisation in inactivating microflora in milk. In the current study, the maximum inactivation level of TPC achieved was much below the legal limit which signifies that the studied milk is well "pasteurised".

Regression analysis of the experimental data obtained from the kinetic model showed a good correlation (R^2 = 0.897 at 30°C, R^2 = 0. 0.9701 at 60°C) between the natural log of the remaining fraction of AIP and field intensity at both temperatures studied. The high R^2 values indicate that the first-order inactivation model is valid for describing the inactivation of AIP by PEF within the range of field intensities. The proportionality constant (b_E) was 0.0351 for 30°C and 0.0808 for 60°C, respectively which suggests that increasing the temperature has an enhancing effect on enzyme inactivation, as demonstrated by a 2.3 times increase in the slope of line (b_E value) when the PEF treatment temperature increased from 30 to 60°C (Fig. 3-3).

As comparisons, raw skim milk was thermally pasteurised by HTST and LTLT methods, and up to 98% of the native AIP was inactivated in both pasteurisation methods. These results indicated that under the these experiments conditions AIP cannot be used as an indicator of PEF treatment adequacy as the maximum inactivation achieved by PEF treatment at 60°C was only 67% vs. 98% inactivation achieved with pasteurisation (Fig. 3-2 a & b). Based on the IDF definition of pasteurised milk, the phosphatase test of a HTST-treated milk should be negative which means that no yellow colour should be released into the assay (IDF, 1984).

As results indicated, the inactivation of AlP was strongly correlated with field intensity and temperature.

Compared with the relatively low level of inactivation of native AlP in milk by PEF reported by Grahl and Märkl (1996) (5% after treatment at 21.5 kV cm^{-1} at 45-50℃) and Van Loey *et al.* (2002) (no significant inactivation after treatment at 20 kV cm^{-1} at 20℃), the higher levels of AlP inactivation obtained in the current study (24-42% inactivation at 30℃, and 29-67% at 60℃) were most likely due to the higher field intensities used (28 to 37 kV cm^{-1} at 30℃, and 25 to 35 kV cm^{-1} at 60℃). However, the field intensities of 28 and 31 kV cm^{-1} resulted in no significant difference (p>0.05) between the inactivation levels, while the difference in inactivation levels at 28 & 31 with 34 & 37 kV cm^{-1} was significant (p<0.05).

Giner *et al.* (2000) and Bendicho *et al.* (2002b) showed that the main factors affecting the enzyme inactivation are field intensity and treatment time, both being related to total specific energy input. In this study, the treatment time was kept constant at 19.6 µs for all treatments while the field intensity was varied. The results of increased AlP inactivation with increasing field intensity were consistent with the findings of Castro *et al.* (2001) who reported that inactivation of AlP was proportional to the electric field intensity. The increased PEF-induced inactivation of AlP at elevated temperature obtained in the current study is in agreement with Van Loey *et al.* (2002) who also observed higher rates of inactivation of AlP and lactoperoxidase by increasing the processing temperature.

Enzymes are stabilised by weak non-covalent forces, such as hydrogen bonds and hydrophobic interactions, and the application of high electric field pulses may have affected the three-dimensional structure of the globular protein in AlP (Ho *et al.*, 1997). Castro (1994) related the PEF inactivation of AlP to degradation of the enzyme's secondary structure and alteration of the entire globular configuration of AlP. Alkaline phosphatase has been shown to be more heat sensitive than other milk enzymes such as lactate dehydrogenase (LDH), gamma-glutamyltransferase (GGT) and aspartate aminotransferase (AST) (Lombardie *et al.*, 2000). The treatment at 60°C (for 5 sec) alone (E=0) had a small effect on AlP activity; however, when combined with PEF treatment at 35 kV cm^{-1} it resulted in a greater degree of AlP inactivation.

3.5 CONCLUSIONS

Total specific pulsing energy and input voltage were directly interrelated and increasing the input voltage from 6.5 to 9.5 kV increased the energy input from 65 to 139 kJ L^{-1}. The PEF treatment at 35 kV cm^{-1} and 60°C was as effective as pasteurisation for the inactivation of microflora in raw skim milk, including *Pseudomonas* and *Enterobacteriaceae*; however, the TPC inactivation was mostly due to the effect of heat rather than PEF treatment. Under the experimental conditions used in this study (30 and 60°C and 25 to 37 kV cm^{-1}) the PEF was shown to be partially efficient in inactivating AlP (23 – 67%). A significant correlation was found between the field intensity and inactivation level of AlP at both temperatures, with the effect at 60°C being more than twice that of 30°C. Under these experimental conditions, the AlP may seem to be an unsuitable indicator for PEF treatment of milk.

The study on AlP as well as other major indigenous enzymes in milk including XO, lipase and plasmin will be expanded in the next chapter by applying various PEF treatment times, field intensities and temperatures.

CHAPTER 4

THE COMBINED EFFECTS OF PULSED ELECTRIC FIELD TREATMENT TIME, FIELD INTENSITY AND HEAT ON THE INACTIVATION OF MILK ENZYMES

ABSTRACT

This study was performed through two sets of trials. In the first set, the combined effects of two field intensities (29 and 35 kV cm^{-1}) with various temperatures (35, 45, 55, 65 and 75°C) on selected native milk enzymes [i.e. alkaline phosphatase (AlP), total lipase, xanthine oxidase (XO) and plasmin] was investigated. The pulse frequency and treatment time selected were 320 Hz and 31 µs for the field intensity of 29 kV cm^{-1} and 200 Hz and 19.2 µs for 35 kV cm^{-1}. The pulse width, flow rate and total specific pulsing energy input for all experiments were maintained at 2 µs, 60 mL min^{-1} and 163 kJ L^{-1}. With the PEF system switched off, all enzymes were inactivated to different extents at 55°C and above; AlP being the most sensitive enzyme followed by total lipase. When PEF system was switched on, a higher level of enzyme inactivation was achieved at both field intensities indicating the combined effects of field intensity and heat on enzyme inactivation up to 60°C, beyond which the heat effect dominated the enzyme inactivation.

In the second set of trials, the effects of various PEF treatment times achieved by changing either the pulse frequencies or flow rates at final processing temperatures of 30 and 60°C on the same native milk enzymes were studied. Pulse frequencies of 100, 200, 300 and 400 Hz resulted in treatment times of 9.6, 19.2, 28.8 and 38.4 µs at a flow rate of 60 mL min^{-1}, whereas flow rates of 30, 60,

120 and 240 mL min^{-1} gave treatment times of 38.4, 19.2, 9.6 and 4.8 µs respectively at a pulse frequency of 200 Hz. The selected field intensity for this part of the study was 31 kV cm^{-1} with monopolar pulses of 2 µs each. Changing the pulse frequency/or flow rate decreased levels of enzyme inactivation; flow rate being more effective. The milk flow pattern through the PEF system calculated from Reynolds number was laminar in the tubing and transitional in treatment chamber at the highest flow rate of 240 mL min^{-1}.

4.1 INTRODUCTION

Although PEF is considered a nonthermal process in which the electric field itself is assumed as the cause of enzymatic or microbial inactivation, processing heat generated by applied field intensity has an impact on the efficiency of the process. It is important to note that PEF is referred to as a nonthermal technology due to the alternative origin of the inactivating parameters (i.e. field intensity or treatment time), and not for the complete absence of thermal effects. As a matter of fact, processing temperature is one of the most relevant processing parameters in PEF technology, surpassed in importance only by the intensity of the applied electric field and the treatment time (Sepulveda, 2003). However, the exposure time of product to heat is kept to minimum before and after the liquid enters the treatment chamber as well as inside the treatment chamber to minimise the heat damage to the product.

There have been many studies (sections 2.3 and 2.5) on the combined effect of PEF and heat on microbial or enzymatic activities in various food systems such as milk, juices or SMUF. The thermal enhancement of PEF treatments can be considered as additive within the boundaries of moderately low and high

temperatures (30 to 60℃ for few seconds) (Craven *et al*., 2008). After this limit is surpassed (as in this study), it is difficult to differentiate between the thermal and nonthermal effects of the process and it is claimed that the thermal effect governs the preservation process (Barsotti and Cheftel, 1999; Dunn, 2001).

Many indigenous enzymes in milk including AlP, lipase, XO and plasmin are technologically significant because of their roles in tracing the thermal history of milk and its shelf life. Microbial lipase and milk XO play a positive role in cheese ripening while they can also render the milk rancid and unpalatable since they both survive HTST pasteurisation. Plasmin survival in UHT milk accelerates the proteolysis resulting in age gelation (Fox and McSweeney, 1998; Girotti *et al*., 1999).

Most PEF studies reported in the literature have focused on the inactivation of microorganisms and individual milk enzymes and so far no study has comprehensively reported the effects of PEF on major native milk enzymes such as AlP, total lipase, XO and plasmin. In most studies, SMUF is spiked with plasmin (Vega-Mercado *et al*., 1995), or microbial lipase (Bendicho *et al*., 2003b) or AlP (Castro *et al*., 2001). In addition, no study was found on the effects of PEF on XO in milk or SMUF. Therefore, from industrial point of view, it is important to investigate how PEF affects these enzymes in bovine milk rather than in spiked model systems under various treatment conditions.

The current study aimed to optimise PEF process conditions including field intensity, treatment time, heat and total specific pulsing energy input to achieve

maximum levels of enzyme inactivation in bovine milk and to establish the relationship between those parameters.

The objective of the first set of trials was therefore to determine the effects of various treatment temperatures (35, 45, 55, 65 and 75ºC) alone or in combination with two field intensities (29 and 35 kV cm^{-1}) on the four native milk enzymes i.e. AlP, total lipase, XO and plasmin. In the second set of trials, the effects of different PEF treatment times and total specific pulsing energy (achieved by changing either the pulse frequency or the flow rate) on the enzyme inactivation in raw skim milk was investigated. The treatment temperatures of 30 and 60°C were selected based on the results obtained from the first set of trials and previous reported studies on PEF treatment (Craven *et al*., 2008; Calderon-Miranda *et al*., 1999 b; Vega-Mercado *et al*., 1995).

4.2 MATERIALS AND METHODS

4.2.1 Raw Skim Milk

Two different lots of raw cold-skimmed milk were used for conducting two separate PEF experiments: one in January 2007 and another in May 2007. The first lot was used for studying the combined effects of heat and field intensity on enzymes and the second lot was used to determine the effect of treatment time on enzyme inactivation. Methods for measuring milk conductivity and pH are described in Chapter 3 sections 3.2.1.

4.2.2 PEF Treatments

The PEF equipment, configuration and specifications, sanitisation protocol, pulse shape, accessories, the start-up of the system and handling of treated samples as well as monitoring the temperature, flow rate and electric voltage input during the process are described in section 3.2.2.

In the first set of trials, for studying the combined effects of heat and field intensity on enzyme inactivation, the input voltage was set on 7.5 or 9.5 kV resulting in the field intensities of 29 or 35 kV cm^{-1}, respectively. The pulse frequency and treatment time for input voltage of 7.5 kV were set to 320 Hz and 31 µs, while for 9.5 kV, they were set to 200 Hz and 19.2 µs. The pulse width and flow rate were 2 µs and 60 mL min^{-1}, respectively resulting in an identical total specific pulsing energy input of 163 kJ L^{-1} for all experiments regardless of the selected treatment temperatures of 35, 45, 55, 65 and 75℃ and conductivity of milk. The PEF system water bath temperatures were set to various degrees to achieve the target temperatures. To investigate the effect of heat on enzyme inactivation without PEF effects, the pulse generator was switched off and the milk was passed through the system at the same flow rate (60 mL min^{-1}) and the water bath temperatures were controlled to achieve the target temperatures. The thermal profile during the PEF treatment in the chamber and tubes is given in Table 4-1.

Table 4-1 Thermal profile of skim milk during PEF treatment

Field intensity (kV cm^{-1})	Temperature profile (ºC)					
0 (non-PEF control)	Target[1]	35	45	55	65	75
	Water bath[2]	38	48	58	68	77
	T1	13	14	14	15	15
	T2	32	39	44	55	64
	T3	34	40	45	56	65
	T4	39	47	54	65	69
	T5	39	47	54	64	71
	T6	40	49	57	66	73
	T7	37	45	53	63	74
	T8	38	47	56	65	75
	T9	38	46	54	65	75
	T10	29	32	34	30	32
29	Target	35	45	55	65	75
	Water bath	25	38	49	60	72
	T1	12	12	12	12	13
	T2	20	29	37	45	54
	T3	31	38	47	53	62
	T4	26	37	47	57	65
	T5	35	45	54	62	67
	T6	27	39	49	59	69
	T7	34	45	56	64	73
	T8	25	37	46	56	74
	T9	35	45	54	64	75
	T10	20	25	27	30	34
35	Target	35	45	55	65	75
	Water bath	25	38	49	60	72
	T1	12	12	12	13	13
	T2	20	29	37	47	55
	T3	31	38	46	56	62
	T4	26	37	47	56	67
	T5	35	45	54	64	70
	T6	27	40	50	60	72
	T7	34	45	55	65	73
	T8	25	37	46	63	74
	T9	33	44	55	65	75
	T10	20	24	25	32	37

[1]Target temperature refers to the final temperature achieved after the 4th treatment chamber which is measured by thermocouple T9 (Appendix 2). [2]Water bath temperatures were set to those temperatures to achieve the final treatment temperatures. The thermocouples sensitivity was from ± 0.1 to ± 0.5ºC; milk temperature fluctuated by ±1ºC depending on the flow rate and location of thermocouples; the location of T1 to T10 in the PEF system is illustrated in Appendix 2.

In the second set of trials, to study the combined effects of treatment time and heat on enzyme inactivation, the selected input voltage was set at 7.5 kV resulting in a field intensity of 31 kV cm^{-1} (depending on the conductivity of milk). The water bath temperatures were set at 15-20℃ and 45-50℃ to achieve the desired final temperatures of 30℃ and 60℃ in output milk after the fourth treatment chamber. The target treatment times were achieved by either changing the pulse frequency (Hz) or flow rate (mL min^{-1}) according to the equation developed by Zhang *et al*. (1995):

$$t = \frac{V \times P_f \times C_n \times P_w}{F}$$

where *t* is the treatment time; *V* is the total volume of treatment chambers; *F* is the flow rate; P_f is the pulse frequency; C_n is the number of chambers and P_w is the pulse width. At a fixed flow rate of 60 mL min^{-1}, with treatment chamber total volume of 48 µL (12 µL × 4) and monopolar pulse width of 2 µs, changing the pulse frequency from 100 to 200, 300 and 400 Hz resulted in the total treatment times of 9.6, 19.2, 28.8 and 38.4 µs, respectively. Likewise, by adjusting the flow rate to 240 to 120, 60 or 30 mL min^{-1} at a constant frequency of 200 Hz, respective treatment times of 4.8, 9.6, 19.2 and 38.4 µs were achieved (Table 4-2).

The control samples were prepared by pumping the skim milk through the treatment chambers at the same flow rates of 240, 120, 60 and 30 mL min^{-1} while the pulse modulator was switched off and water bath temperatures were adjusted to give the target final temperatures.

Based on the capacity of tubing between the outlet of the 4th treatment chamber and the ice bath, the exposure time of milk to the maximum temperature was calculated to be *ca*. 1.25, 2.5, 5 and 10 s for the above flow rates, respectively; however, the exposure time of milk to a range of temperatures below target temperatures from the point milk entered the coil in the first water bath to ice bath was 25, 50, 100 and 200 s, respectively for the four flow rates used.

Table 4-2 Experimental conditions for combined effects of treatment time and heat on enzyme inactivation

PEF Treatment Conditions		
Pulse frequency (Hz)	Flow rate (mL min^{-1})	Treatment time (μs)
100	60	9.6
200	60	19.2
300	60	28.8
400	60	38.4
200	240	4.8
200	120	9.6
200	60	19.2
200	30	38.4
0 (non-PEF control)	240	0
0	120	0
0	60	0
0	30	0

The field intensity and pulse width for all experiments were 31 kV cm^{-1} and 2 μs respectively at 30 and 60ºC.

4.2.3 Heat Treatment of Milk

The heat treatment of skim milk including the HTST, LTLT and high-heat treatment was conducted as described in Chapter 3, section 3.2.3.

4.2.4 Energy Calculation

The total specific pulsing energy (Q) was calculated using the formula developed by Zhang *et al.* (1995):

$$Q = \frac{V \times I \times N_p \times P_w}{v}$$

Where *V, I, N_p, P_w* and *v* represent the voltage, current, number of pulses, pulse width and total volume of treatment chambers, respectively.

4.2.5 Alkaline Phosphatase Activity Assay

The AlP assay method is described in Chapter 3 section 3.2.4.

4.2.6 Lipase Activity Assay

The activity of total lipase (native and microbial) was determined based on a method modified from Humbert *et al.* (1997). A solution of 0.05 M *p*-nitrophenyl decanoate in dimethyl sulfoxide (DMSO) was used as substrate. A barbital buffer (pH = 7.6) was made by mixing 70 mL barbitone sodium (0.05 M) with 30 mL barbitone (0.05 M). The reaction inhibiting mixture was made by mixing 3 volumes of Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, 0.06 M, pH = 7.6) with 1 volume of phenylmethanesulphonylfluoride (PMSF, 0.06 M in dimethylformamide). Aliquots of 133 µL of each milk sample were mixed with 533

µL barbital buffer (0.05 M, pH = 7.6) in microtubes (MCT-175-C, Axygen Scientific Inc., CA, USA) and incubated at 37°C for 15 min. Then 13.3 µL substrate was added into each sample mixture and incubated at 37°C for another 30 min. In order to terminate the enzymatic reaction and clarify the assays, to each assay tube 107 µL reaction inhibiting mixture and 700 µL sodium dodecyl sulphate (SDS 5%, pH=12) were added and shaken vigorously. The microtubes were then centrifuged at 18,000 g for 10 min at room temperature and 250 µL of the supernatant was pipetted into 96-well microtest plates. Blanks were prepared using the same conditions as the assays except that reaction inhibiting mixture was added to the milk sample before adding the substrate solution. The absorbance was measured at 420 nm using a UV spectrophotometer (described in Chapter 3, section 3.2.4) and its value was used to calculate the concentration of *p*-nitrophenol (*p*-NP) released from the substrate using a standard curve based on a linear relationship between the concentration and absorbance (Appendix 3). The standard curve was established by dissolving 0.021 g *p*-NP in 1000 mL barbital buffer to make a 150 µmol mL^{-1} *p*-NP solution and diluting the solution to 0 – 150 µmol. One unit of enzymatic activity was defined as the amount of enzyme needed to release 1 µmol *p*-NP per min at 37ºC. The following equation was used to calculate the total lipase in milk including native and microbial:

$$\text{Enzyme residual activity (mU mL}^{-1}) = \frac{p\text{-NP (µg mL}^{-1}) \times 1.4863 \text{ (volume of assay mL)}}{139.12 \text{ (MW of } p\text{-NP)} \times 0.133 \text{ (milk mL)} \times 30 \text{ min (incubation time)}}$$

4.2.7 Xanthine Oxidase Activity Assay

The activity of XO was determined spectrophotometrically at 290 nm based on the rate of urate formation from xanthine (Cerbulis and Farrell, 1976). Aliquots of milk (75 µL) were diluted with 375 µL sodium phosphate buffer (0.05 M, pH 7.5) and 300 µL Milli Q water in microtubes and incubated at 30°C for 5 min to equilibrate. The enzyme reaction started by adding 375 µL xanthine substrate (0.13 mM in Milli Q water) to the tubes and incubating for 6 min in a water bath at 30°C followed by adding 375 µL of 20% trichloroacetic acid (TCA) to stop the reaction, and centrifugation at 5,000 × g for 10 min. The supernatants were pippetted into plastic cuvettes (light path = 1 cm) and transferred into the UV spectrophotometer (Chapter 3, section 3.2.4) and the absorbency was measured at 290 nm. The following formula was used to calculate the milliunit of enzyme activity:

$$\text{Residual enzyme activity (mU)} = \frac{\Delta A/\text{min} \times 1000 \times 1.5\ \text{mL} \times 1000}{1.22 \times 10^4 \times \text{sample volume (0.075 mL)}}$$

The ΔA is the absorbency measured at 290 nm after 6 min incubation. A milliunit of activity is expressed as one µmol of urate formed per min at pH 7.5 and 30°C. The molar absorbency of uric acid = $1.22 \times 10^4\ cm^{-1}$ (Cerbulis and Farrell, 1976).

4.2.8 Plasmin Activity Assay

As described in section 2.6.4, the plasmin complex in milk comprises plasmin, plasminogen, its activators and inhibitors. Due to sever heat resistance of the plasminogen system, it is a common practice to measure the actual plasmin in treated milk products. The activity of plasmin in milk samples used in this project was determined using a fluorometric method developed by Richardson and

Pearce (1981). The enzyme assay was performed on skim milk since MFGM contains only small amounts of plasmin and plasminogen compared to casein micelles (Hofmann *et al*., 1979). A substrate was prepared by dissolving 7.5 mg coumarin peptide (N-Succinyl-Ala-Phe-Lys 7-amido-4-methylcoumarin acetate salt) in 2.0 mL DMSO and 8.0 mL 0.05 M-Tris buffer pH 7.5. The solution was kept at –20°C in dark when not used. Aliquots of 750 µL of milk samples were diluted with 250 µL 0.4 M sodium citrate solution and centrifuged at 18,000 × g for 30 min. From the supernatant, 64 µL was pipetted into a plastic 4-side clear cuvette containing 755 µL Tris buffer. After equilibration for 5 min at 25°C, 205 µL of the substrate was added to the cuvette and the fluorescence was monitored after 1 and 6 min using a Hitachi Fluorescence Spectrophotometer (Model F-2000, Tokyo, Japan). The excitation was 380 with a bandpass of 10 nm and emission was 460 nm with a bandpass of 20 nm. A standard curve (Appendix 4) was developed based on the linear relationship between the concentration and absorbance by making a solution of 0 - 0.1 µmol 7-amino-4-methylcoumarin (AMC) in Tris buffer. One AMC unit of plasmin activity releases 1 µmol AMC per min under the standard assay conditions. High-heat treated skim milk (10 min in steam bath after reaching 97°C) and raw skim milk were used as the negative and positive controls, respectively.

$$\text{Residual Enzyme activity (AMC U mL}^{-1}) = \frac{\text{Molar concentration of AMC} \times 1.024 \text{ (assay volume)}}{0.064 \text{ (sub-sample volume mL)} \times 0.75 \text{ (sample volume mL)}}$$

4.2.9 Flow Pattern of Skim Milk through the Treatment Chamber

To determine the pattern of milk flow in the system tubing and treatment chamber, Reynolds number (*Re*) was calculated from the following equation (Ruhlman *et al.*, 2001):

$$Re = \frac{\rho V D}{\mu}$$

where ρ is the density of skim milk (g mL^{-1}); V is the fluid velocity (cm s^{-1}); D is the tubing or chamber diameter (cm) and μ is the fluid viscosity dynamic (pascal-second Pa·s). The viscosity of skim milk at 30 and 60 °C is reported by Ruhlman *et al.* (2001). The calculation of *Re* is given in Appendix 5.

4.2.10 Statistical Analysis of Data

Full factorial design was used to determine the effect of both field intensity and heat on enzyme inactivation. The multiple comparisons of means of each treatment (field intensity and heat) were determined using the least significant difference (LSD) and Tukey's test at the confidence level of 95% and the difference between mean values greater than the $LSD_{(0.95)}$ was determined as significant. Analysis of data was carried out using SPSS (Version 15, SPSS Inc. Chicago, USA) and Minitab softwares (Version 14, Minitab Inc., State College, PA, USA) for comparison purpose.

4.3 RESULTS

4.3.1 Combined Effects of Field Intensity and Heat on Enzyme Inactivation

4.3.1.1 Skim Milk Properties

The January 2007 milk had a pH value of 6.69 at 15°C with a conductivity of 4.56, 4.66, 4.81 and 5.21 mS cm^{-1} at 35, 45, 55 and 65°C, respectively. It was not possible to measure the conductivity at 75°C as the conductivity meter temperature limit was 65°C.

4.3.1.2 Enzyme Inactivation by PEF Treatment

The final temperatures selected for PEF treatment of milk were 35, 45, 55, 65 and 75°C at a fixed flow rate of 60 mL min^{-1}. The exposure time of milk to heat below target temperatures was 100 s resulting in a significantly decreasing trend in the enzyme activity level depending on the enzyme characteristics and type. Table 4-3 shows the activity levels of enzymes before and after PEF treatment and when the PEF was switched off. The enzyme activity level at 35ºC (PEF off, E=0) was taken as the initial activity since at this temperature no inactivation occurs. The inactivation levels of AlP in the absence of PEF effect at 35, 45, 55, 65 and 75°C were 0, 4, 25, 49 and 94%, respectively. At the same temperatures, the inactivation levels of total lipase were 0, 11, 22, 44 and 78%. Under the same conditions, XO was inactivated by 0, 4, 18, 41 and 73% while plasmin inactivation levels were 0, 0, 6, 14 and 28%.

When PEF pulse generator was switched on, a decreasing trend in the level of enzyme activity was observed. At field intensity of 29 kV cm^{-1} and with

temperatures set to 35, 45, 55, 65 and 75℃, AlP was inactivated by 21, 24, 46, 94 and 97%. Under the same conditions, the inactivation levels of total lipase, XO, and plasmin were 22, 33, 44, 78 and 89%; 20, 23, 46, 74, and 88%; 17, 19, 31, 53 and 61%, respectively. When the field intensity was increased to 35 kV cm^{-1}, the inactivation levels of AlP, total lipase, XO and plasmin further increased to 24, 46, 53, 96 and 99%; 33, 44, 56, 89 and 99%; 23, 38, 49, 78 and 89% and 17, 22, 42, 53 and 64%, respectively. As XO is an MFGM-bound enzyme, a sample of raw whole milk was also tested for the activity of XO and showed 20% higher activity level in skim milk (data not reported). However, raw whole milk was not used in this study due to the fact that skim milk produces a more transparent assay for all enzymes after centrifugation.

Table 4-3 Activity levels of alkaline phosphatase (AlP), total lipase, xanthine oxidase (XO) and plasmin at different temperatures with and without PEF treatment

		Residual enzyme activity			
Field intensity ($kV\ cm^{-1}$)	T (ºC)	AlP* ($U\ mL^{-1}$)	Total lipase ($mU\ mL^{-1}$)	XO** ($mU\ mL^{-1}$)	Plasmin ($AMC\ U\ mL^{-1}$)
0 (non-PEF control)	35[1]	$2.50^{a}\pm0.02$	$0.09^{a}\pm0.002$	$82^{a}\pm0.2$	$0.36^{a}\pm0.02$
	45	$2.40^{a}\pm0.03$	$0.08^{a}\pm0.002$	$79^{a}\pm1.1$	$0.36^{a}\pm0.05$
	55	$1.87^{b}\pm0.04$	$0.07^{ab}\pm0.002$	$67^{b}\pm1.1$	$0.34^{a}\pm0.02$
	65	$1.27^{c}\pm0.05$	$0.05^{b}\pm0.002$	$48^{c}\pm0.2$	$0.31^{a}\pm0.01$
	75	$0.15^{d}\pm0.01$	$0.02^{c}\pm0.002$	$22^{d}\pm0.6$	$0.26^{b}\pm0.01$
29	35	$1.98^{b}\pm0.05$	$0.07^{b}\pm0.003$	$66^{b}\pm0.7$	$0.30^{b}\pm0.01$
	45	$1.90^{b}\pm0.12$	$0.06^{b}\pm0.003$	$63^{b}\pm0.5$	$0.29^{b}\pm0.02$
	55	$1.35^{c}\pm0.03$	$0.05^{bc}\pm0.003$	$44^{c}\pm0.9$	$0.25^{c}\pm0.03$
	65	$0.14^{d}\pm0.05$	$0.02^{d}\pm0.003$	$21^{d}\pm0.5$	$0.17^{d}\pm0.04$
	75	$0.07^{d}\pm0.07$	$0.01^{d}\pm0.003$	$10^{e}\pm0.8$	$0.14^{e}\pm0.01$
35	35	$1.90^{b}\pm0.04$	$0.06^{b}\pm0.004$	$63^{b}\pm1.4$	$0.30^{b}\pm0.02$
	45	$1.35^{c}\pm0.02$	$0.05^{b}\pm0.004$	$51^{c}\pm3.1$	$0.28^{b}\pm0.03$
	55	$1.17^{c}\pm0.07$	$0.04^{bc}\pm0.004$	$42^{d}\pm1.8$	$0.21^{c}\pm0.02$
	65	$0.10^{d}\pm0.07$	$0.01^{d}\pm0.003$	$18^{e}\pm0.9$	$0.17^{d}\pm0.04$
	75	$0.02^{d}\pm0.04$	$0.00^{e}\pm0.002$	$9.0^{e}\pm1.0$	$0.13^{e}\pm0.04$

*alkaline phosphatase; **xanthine oxidase; [1]initial activity of all enzymes was measured at 35ºC since no inactivation occurs at this temperature. The different letters in each column show a significant difference ($p<0.05$).

Table 4-4 shows the statistical matrix obtained by full factorial design between field intensities and treatment temperatures. Based on the table, up to 55ºC there was no dominance of heat on the enzyme inactivation while at 65ºC or above, heat dominated the inactivation process of enzymes. Figure 4-1 shows the relationship between the field intensity of 29 and 35 kV cm^{-1}, treatment temperatures and enzyme activity levels. Enzyme inactivation was affected by heat, field intensity or a combination of both. The decreasing trend of enzyme activity levels was more evident for AlP, followed by total lipase, XO and plasmin.

Table 4-4 Statistical matrix based on factorial design indicating the interaction of field intensity and temperature on enzyme inactivation

Field intensity (kV cm^{-1})	T (℃)	AlP*	Total lipase	XO**	Plasmin
PEF off (E=0)	35	0	0	0	0
	45	0	0	0	0
	55	×	×	×	×
	65	××	××	××	××
	75	×××	×××	×××	×××
29	35	+	+	+	+
	45	× +	× +	× +	× +
	55	× +	× +	× +	× +
	65	×× +	×× +	×× +	×× +
	75	××× +	××× +	××× +	××× +
35	35	++	++	++	++
	45	×++	×++	×× ++	×++
	55	×++	×++	×× ++	×++
	65	××++	××++	××××++	××++
	75	×××++	×××++	××××++	×××++

(×) heat effect; (+) PEF effect; *alkaline phosphatase; **xanthine oxidase; the residence time of milk in the highest targeted temperature after 4th treatment chamber was 5 s but the total exposure time of milk to a range of temperatures below target temperature was 100 s.

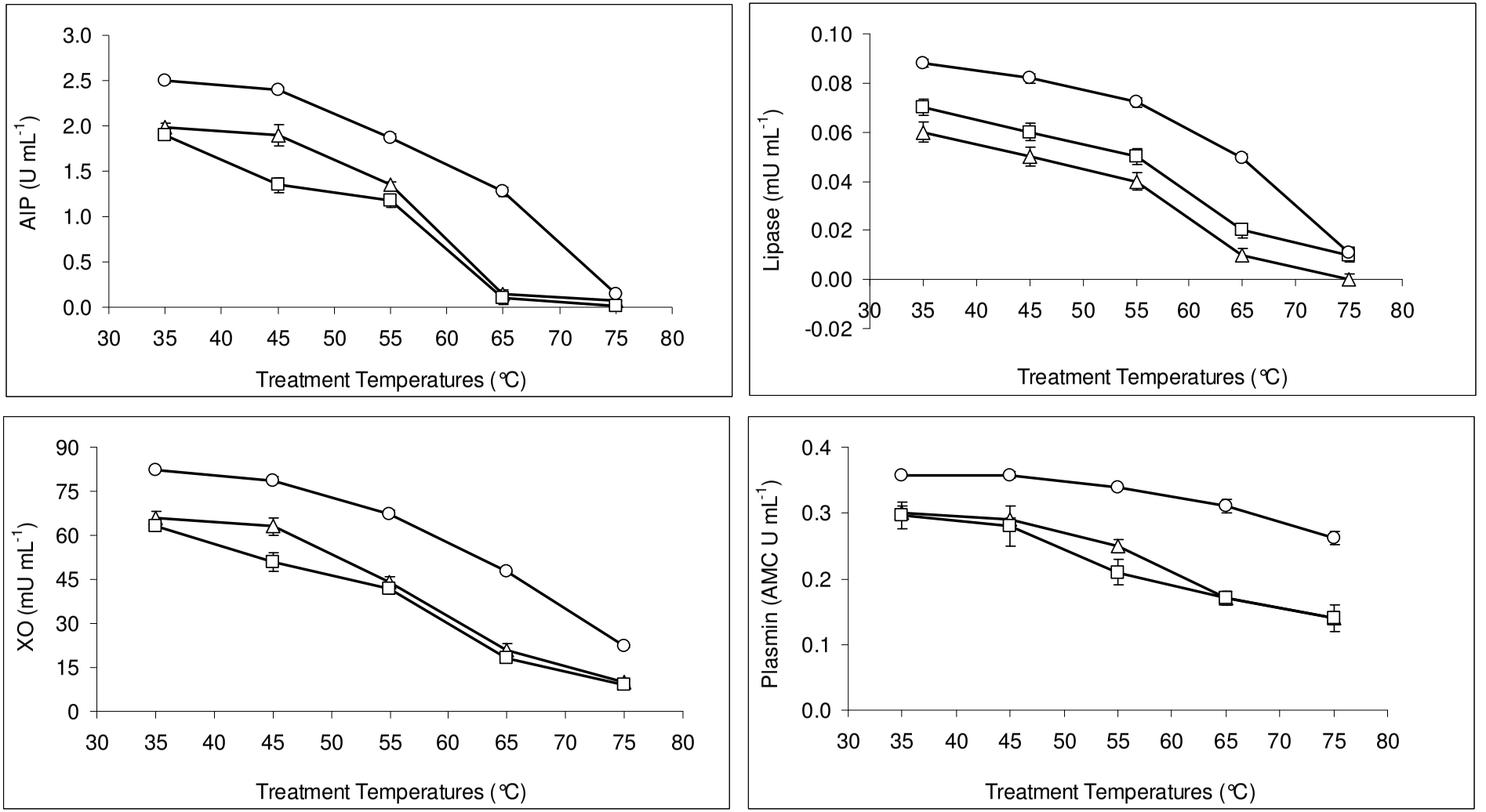


Figure 4-1 Relationship between enzyme activity levels, treatment temperature and field intensity

O PEF off; Δ 29 kV cm^{-1}; □ 35 kV cm^{-1}

The inactivation levels indicated an increasing trend from the lowest field intensity and temperature to the highest. As the processing temperature reached 65°C, it became difficult to differentiate between heat or PEF treatment as the main cause of inactivation. In the previous Chapter, it was found that 33% of AlP residual activity was still left after PEF treatment of milk at 60°C which made it an unsuitable indicator for PEF treatment adequacy. However, under the current optimised experimental conditions, at temperature 65°C and both field intensities, AlP was inactivated over 90% which makes this enzyme a suitable indicator for PEF treatment adequacy. Temperatures above 65°C could be used for further inactivation of XO and plasmin.

4.3.1.3 Fitting the Enzyme Inactivation Data to Hülsheger Kinetic Model

The modified kinetic model of Hülsheger *et al.* (1981) was used to mathematically explain the combined effects of field intensity and heat on enzyme inactivation. The model as described in Chapter 3 section 3.3.4 was originally developed to describe the relation of natural log of survival fraction of microorganisms to the electric field intensity through a proportionality constant in a first-order kinetic manner: $\ln (N/N_0) = -b_E (E-E_c)$,where:

N_0 and N are the number of viable cells before and after PEF treatment, b_E is the proportionality constant, E is the field intensity and Ec is the critical electric field. This model was later adapted by Min *et al*. (2003) to enzyme inactivation by PEF treatment. In the current study, the model was used to analyse the relationship between the PEF treatment temperature (at a constant field intensity and treatment time) and the enzymatic inactivation given the fact that temperature and field intensity are related and applying a definite field at different treatment times

could result in various temperatures. The modified model is Ln $(A/A_0) = -k_T (T-T_c)$ where: A_0 and A are the enzyme activity before and after the PEF treatment, k_T is the proportionality constant in relation to the PEF treatment temperature, T is the PEF treatment temperature and T_c is the critical temperature where the enzymes start becoming inactivated (Table 4-5). The proportionality constant (k_T) for temperature represents the slope of the inactivation curve. The larger k_T value indicates a greater inactivation efficacy correlated with the increase in the PEF treatment temperature (Fig. 4-2). The regression co-efficient (R^2) represents the relative correlation level of the simulation and if greater than 0.7, it reliably explains the level of correlation (Zar, 1999).

Table 4-5 Proportionality constant and regression coefficients of enzyme inactivation by two field intensities as a function of treatment temperatures

Enzyme	Field intensity (kV cm^{-1})	Regression coefficients	k_T
AlP	0	$R^2 = 0.71$	0.0616
	29	$R^2 = 0.87$	0.088
	35	$R^2 = 0.84$	0.1231
Total lipase	0	$R^2 = 0.72$	0.0486
	29	$R^2 = 0.87$	0.0644
	35	$R^2 = 0.89$	0.0571
XO	0	$R^2 = 0.83$	0.0313
	29	$R^2 = 0.91$	0.0554
	35	$R^2 = 0.89$	0.0467
Plasmin	0	$R^2 = 0.85$	0.008
	29	$R^2 = 0.96$	0.0205
	35	$R^2 = 0.93$	0.0193

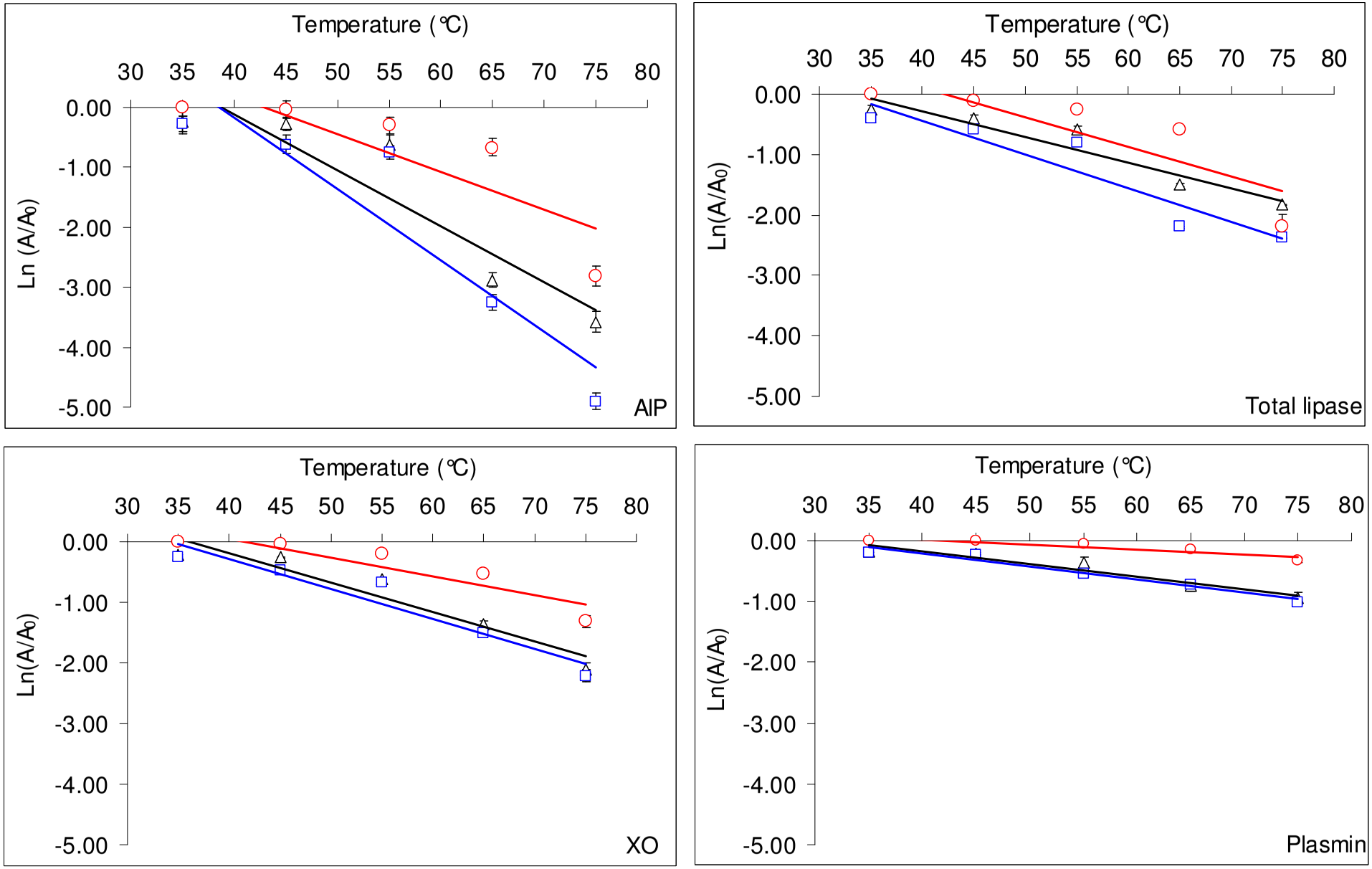


Figure 4-2 Correlation between two field intensities and various temperatures for enzyme inactivation in skim milk. The total specific pulsing energy was 163 kJ L^{-1} for all PEF treatments

O PEF off; Δ 29 kV cm^{-1}; □35 kV cm^{-1}

4.3.2 Combined Effects of Treatment Time and Heat on Enzyme Inactivation

The raw skim milk collected in May 2007 had a pH value of 6.71 at 15°C and an electrical conductivity of 4.51 mS cm^{-1} at 30°C and 5.31 mS cm^{-1} at 60°C. To investigate the effect of heat on enzyme activity level two temperatures of 30 and 60°C were selected as the moderately low and high temperatures based on the results of the first set of trials. First, the PEF was switched off and the milk was passed through the system at flow rates of 240, 120, 60 and 30 mL min^{-1} (resulting in total exposure times of 25, 50, 100 and 200 s to water bath temperature, respectively with final temperatures adjusted to 30 or 60°C (Table 4-1). The activity of AlP at 30°C remained unchanged (1.85±0.15 U mL^{-1}) regardless of flow rates used, while at 60°C it dropped by 28, 31, 36 and 50%, in response to flow rates mentioned above. The level of initial enzyme activity in the skim milk used in this set of trials was 27% lower than AlP activity level in previous set of trials due to difference in milking season (Fox and McSweeney, 1998).

The initial activity level of total lipase in milk at 30°C was 0.102±0.01 (mU mL^{-1}), which remained unchanged regardless of the flow rates employed in the absence of PEF effect (E=0). However, at 60°C, changing the flow rates from 240 to 30 mL min^{-1} resulted in reduced total lipase activity by 20, 26, 30 and 40%. The total lipase levels in the skim milk used for both sets of trials were not significantly (p<0.05) different regardless of difference in milking season.

The initial activity of XO after passing the milk through the system at 30°C with PEF switched off was 84±2.3 mU mL^{-1}, regardless of the flow rates employed.

However, when the temperature was raised to 60℃, the XO inactivation reached 6.0, 7.0, 12 and 30%; the lower flow rate giving a higher inactivation level due to the longer residence time at 60℃. The levels of XO in the skim milk used for both sets of trials were almost the same.

Similar to other three enzymes, the plasmin activity level was determined before PEF treatment at 30 and 60℃ at flow rates of 240, 120, 60 and 30 mL min^{-1} and was found to be 0.12±0.01 AMC U mL^{-1} at all flow rates at 30℃, but was inactivated by 0, 14, 17 and 22% at 60℃ (Table 4-6). The level of plasmin initial activity in the skim milk used for this set of trials was 67% lower than the level of plasmin in the skim milk used for the first set of trials as the milking was done in January 2007 and May 2007, respectively. The level of plasmin can increase 2.3 times in the milk due to seasonal changes and stage of lactation (Politis and Ng Kwai Hang, 1989; Kelly and Fox, 2006).

4.3.2.1 Effects of Pulse Frequency on Enzyme Inactivation

When the PEF system was switched on, at pulse frequencies of 100, 200, 300 and 400 Hz and constant flow rate of 60 mL min^{-1} the inactivation levels of AlP was found to be 30, 33, 37 and 42% at 30℃ that increased to 43, 49, 51 and 56% at 60℃. Under the same conditions, total lipase was inactivated by 28, 33, 40 and 42% at 30℃, and 53, 56, 61 and 66% at 60℃ as a result of the combined effect of increased treatment time and a higher temperature.

The XO inactivation levels under the above conditions were 25, 33, 34 and 36% at 30°C and 45, 48, 51 and 52% at 60℃, while plasmin was inactivated by 14, 18, 23

and 24% at 30℃ and 34, 39, 41 and 43% at 60℃. Increasing the pulse frequency resulted in increased inactivation levels of all enzymes.

4.3.2.2 Effects of Milk Flow Rate on Enzyme Inactivation

At 30℃, the constant pulse frequency of 200 Hz at flow rates of 240, 120, 60 and 30 mL min^{-1} resulted in AlP inactivation by 19, 27, 33 and 37%, while at 60℃ and under the same pulse frequency and flow rates, enzyme activity was further reduced to 33, 38, 49 and 75%, respectively. Under the same conditions, total lipase was inactivated by 19, 26, 34 and 42% at 30℃ and by 33, 42, 59 and 65% at 60℃. Decreasing the flow rate had an inverse effect on enzyme inactivation due to longer residence time of milk in the system.

Xanthine oxidase inactivation levels under these conditions were 8.0, 14, 33 and 37% at 30℃, and 17, 32, 48 and 58% at 60℃ while plasmin inactivation levels at 30℃ were 12, 13, 19 and 28% which increased to 27, 32, 38 and 47% at 60℃, respectively.

Figure 4-3 shows the enzyme activity vs. treatment time achieved by changing either the pulse frequencies or the flow rates. The results show a descending trend at both 30 and 60ºC which indicates that increasing the treatment time during PEF treatments decreases the enzyme activity level.

Table 4-6 Activity of native milk enzymes before and after PEF treatment

T^1 (˚C)	P_f^2 (Hz)	F_r^3 (mL min^{-1})	t^4 (μs)	AlP (U mL^{-1})	Total lipase (mU mL^{-1})	XO (mU mL^{-1})	Plasmin (AMC U mL^{-1})
30*	0	All	0	1.85±0.15^{a}	0.102±0.002^{a}	84±2.7^{a}	0.120±0.001^{a}
	0	240	0	1.34±0.11^{b}	0.081±0.002^{b}	79±0.7^{b}	0.111±0.001^{a}
60	0	120	0	1.27±0.09^{c}	0.074±0.002^{c}	78±1.4^{b}	0.103±0.001^{b}
	0	60	0	1.18±0.02^{d}	0.071±0.003^{c}	66±1.4^{c}	0.099±0.001^{b}
	0	30	0	0.92±0.09^{e}	0.060±0.002^{d}	59±0.8^{d}	0.093±0.001^{c}
	200	240	4.8	1.50±0.05^{ab}	0.083±0.003^{b}	77±1.5^{b}	0.106±0.004^{b}
30	200	120	9.6	1.35±0.06^{b}	0.075±0.001^{c}	72±2.7^{b}	0.105±0.002^{b}
	200	60	19.2	1.24±0.05^{c}	0.067±0.004^{cd}	56±1.1^{d}	0.097±0.003^{b}
	200	30	38.4	1.16±0.07^{d}	0.059±0.002^{d}	53±4.3^{d}	0.086±0.007^{e}
	200	240	4.8	1.24±0.03^{c}	0.068±0.004^{cd}	70±5.2^{b}	0.088±0.001^{e}
60	200	120	9.6	1.14±0.04^{d}	0.059±0.003^{d}	57±7.0^{d}	0.082±0.002^{e}
	200	60	19.2	0.94±0.03^{e}	0.042±0.003^{g}	43±2.2^{e}	0.074±0.001^{ef}
	200	30	38.4	0.46±0.06^{g}	0.036±0.003^{h}	35±1.9^{f}	0.064±0.002^{f}
	100	60	9.6	1.29±0.07^{c}	0.073±0.002^{c}	63±0.7^{c}	0.103±0.001^{b}
30	200	60	19.2	1.24±0.05^{c}	0.068±0.003^{cd}	56±0.4^{d}	0.098±0.001^{b}
	300	60	28.8	1.16±0.02^{d}	0.061±0.003^{d}	57±0.9^{d}	0.097±0.000^{b}
	400	60	38.4	1.07±0.05^{de}	0.059±0.003^{e}	54±2.1^{d}	0.093±0.001^{c}
	100	60	9.6	1.06±0.04^{de}	0.048±0.004^{f}	46±0.8^{e}	0.079±0.002^{d}
60	200	60	19.2	0.94±0.02^{e}	0.045±0.002^{f}	43±0.7^{e}	0.073±0.004^{d}
	300	60	28.8	0.91±0.02^{e}	0.040±0.003^{g}	41±1.1^{e}	0.070±0.003^{d}
	400	60	38.4	0.81±0.09^{f}	0.035±0.003^{h}	40±1.3^{e}	0.068±0.001^{f}

[1]Treatment temperature; [2]Pulse frequency; [3]Flow rate; [4]Treatment time; *Control temperature for determining enzymes' initial activity at all flow rates. The values represent the means of 6 replicates ± SD. The values in each column with different superscripted letters are significantly different (p<0.05).

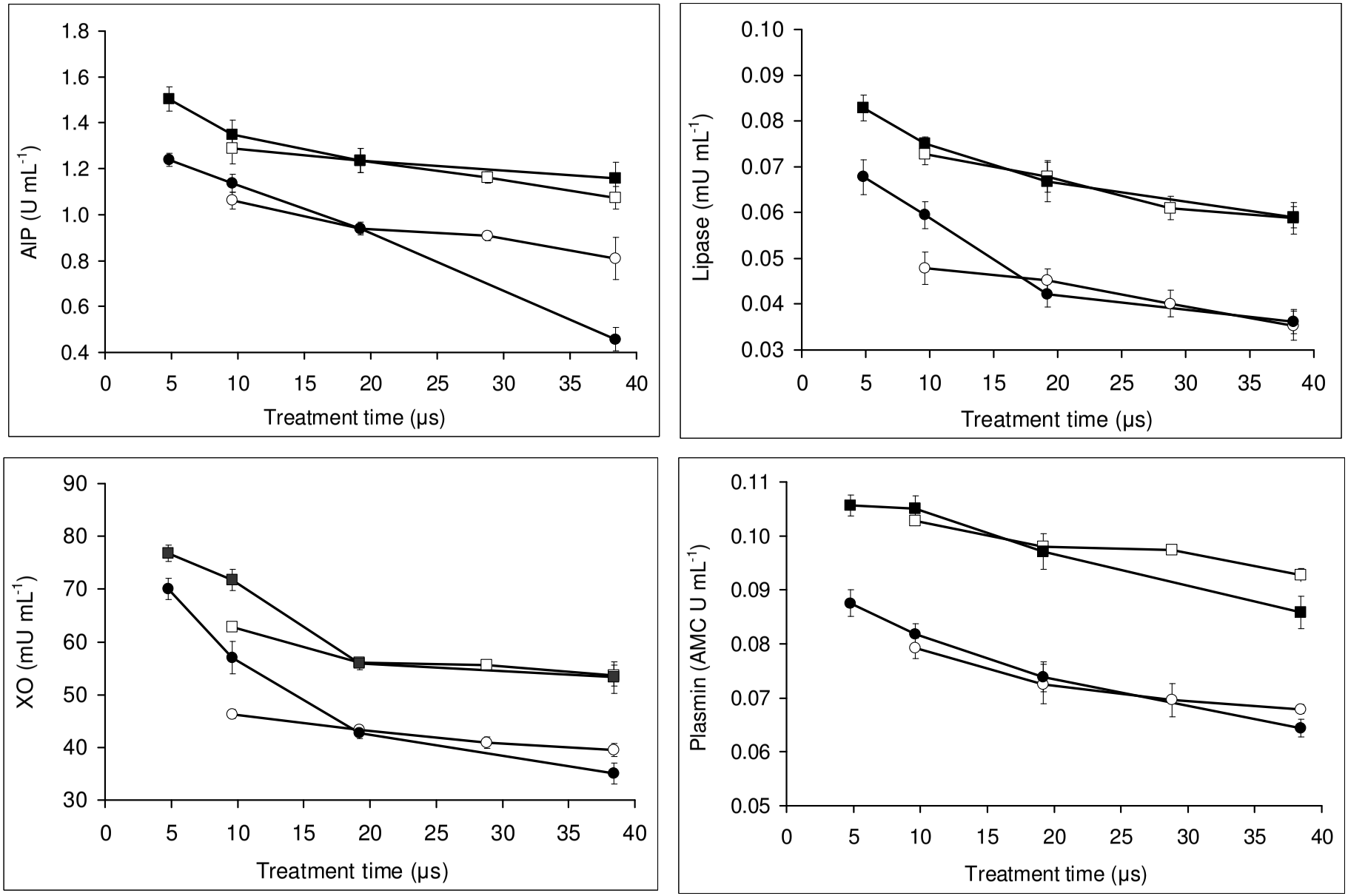


Figure 4-3 Enzyme activity level vs. treatment time achieved by changing either flow rates (mL min^{-1}) or pulse frequencies (Hz)

□ frequency at 30℃; ■ flow rate at 30℃; O frequency at 60℃; ● flow rate at 60℃

4.3.2.3 Fitting the Enzyme Inactivation Data to Hülsheger Kinetic Model

The experimental data were fitted to the Hülsheger *et al.* (1981) first-order inactivation model: $\ln(A/A_0) = -kt$. Figure 4-4 shows the correlation between the natural logs of relative enzyme activity (A/A_0), treatment time and total specific pulsing energy at 30 or 60°C. Treatment time and total specific pulsing energy are linearly correlated which means any increase in treatment time results in increasing the total specific pulsing energy. Therefore, the enzyme inactivation level is linearly dependent on both. The inactivation constant (*k*) and the regression coefficient (R^2) for each set of data are presented in Table 4-7. The regression coefficients > 0.7 are considered reliable for statistical predictions (Zar, 1999). The slope of the trend lines at different temperatures determines the degree of effect of treatment time or total energy on enzyme inactivation. The slope of the trend lines at 60°C was steeper than that at 30°C indicating the additive effect of heat and PEF on enzyme inactivation. It also provides a clue to sensitivity of each enzyme to PEF treatment, i.e. the more sensitive an enzyme is to PEF treatment, the steeper is the slope of the trend line.

4.3.3 Enzyme Inactivation by Pasteurisation and High-Heat Treatment

To give a basis for comparison, raw skim milk samples were pasteurised at 72°C for 15 s and 63°C for 30 min. The inactivation levels of AlP, total lipase and XO with the two methods of pasteurisation were 98 and 99%, 78 and 82%, and 73 and 77%, respectively. Plasmin inactivation level at 72ºC for 15 s was 43%. However, at 63ºC for 30 min a 14% increase in plasmin activity level was observed due to inactivation of plasmin inhibitors (Borda *et al.*, 2004; Fox and Mc Sweeney, 1998).

Table 4-7 Inactivation constants (*k*) and regression coefficients (R^2) of milk native enzymes subjected to various PEF treatment times.

		PEF Treatment Times*			
		Changing flow rate (mL min^{-1})		Changing pulse frequency (Hz)	
Enzymes	T (℃)	*k* (s^{-1})	R^2	*k* (s^{-1})	R^2
AlP	30	0.0083	0.89	0.0132	0.94
	60	0.0301	0.92	0.0085	0.97
Lipase	30	0.0096	0.96	0.0078	0.96
	60	0.0273	0.98	0.0108	0.97
XO	30	0.0161	0.83	0.0054	0.94
	60	0.0217	0.82	0.0065	0.99
Plasmin	30	0.0065	0.96	0.0033	0.95
	60	0.0089	0.82	0.0052	0.87

*Adjusted by changing either pulse frequency or flow rate

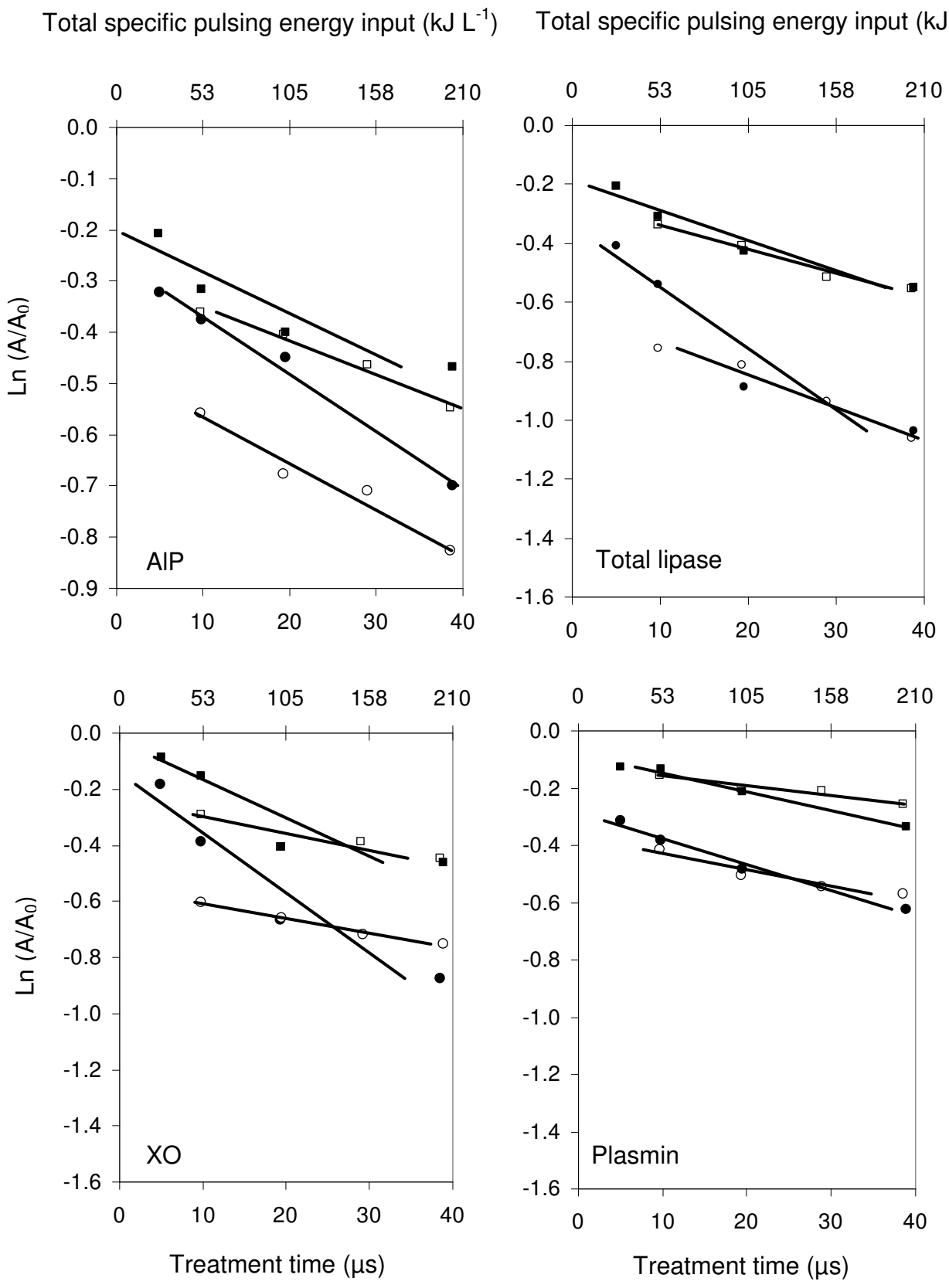


Figure 4-4 The correlation between various PEF treatment times and total specific pulsing energy with milk enzyme inactivation at 30℃ and 60℃. The standard deviation of duplicate samples was from ±0.01 to ±0.1. The pulse frequencies and flow rates were 400, 300, 200 & 100 Hz and 240, 120, 60 & 30 mL min^{-1}, respectively.

□ frequency at 30℃; ■ flow rate at 30℃; O frequency at 60℃; ● flow rate at 60℃

4.3.4 Milk Flow Pattern through the PEF System

For flow rates of 240, 120, 60 and 30 mL s^{-1} at 30°C the *Re* numbers within the tubing was 698, 349, 174 and 87 which increased to 1694, 847, 424 and 212 at 60°C, respectively. Due to the fact that the gap between electrodes (2.9 mm) is narrower than the diameter of tubing (4 mm),. the maximum *Re* number achieved within the chamber with a flow rate of 240 mL min^{-1} and at 60°C was 2946 (Table 4-8). Therefore, the flow within the chamber became transitional which contributed to a more even treatment of the product. Alkhafaji and Farid (2008) also reported a similar observation. Table 4-8 shows the physical characteristics of milk used for calculation of *Re* numbers at various flow rates at both 30 and 60°C. Typical calculation of *Re* number is given in Appendix 5.

Table 4-8 Reynolds number and other flow physical attributes of milk flow through the PEF system

T (°C)	F_r[1] (mL min^{-1})	R_t[2] (s)	*P*[3] (g mL^{-1})	μ[4] (P.s)	*Re*[5] (tubing)	*Re* (chamber)
30	240	25	1.03	0.001881	698	1213
30	120	50	1.03	0.001881	349	607
30	60	100	1.03	0.001881	174	303
30	30	200	1.03	0.001881	87	152
60	240	25	1.02	0.000767	1694	2946
60	120	50	1.02	0.000767	847	1473
60	60	100	1.02	0.000767	424	737
60	30	200	1.02	0.000767	212	368

[1]Flow rate; [2]residence time; [3]fluid density; [4]fluid viscosity dynamic; [5]Reynolds number; the internal diameter of tubes and treatment chamber were 4 and 2.3 mm, respectively. For PEF specifications see section 3.2.2.

4.4 DISCUSSION

The activity levels of various milk enzymes have been determined by different researchers mostly in raw or pasteurised whole milk. Alkaline phosphatase activity level has been reported frequently in pasteurised milk as this enzyme is an indicator of pasteurisation adequacy (Rampling *et al*., 2004; Ludikhuyze *et al*., 2000). Total lipase activity level in skim milk has been reported to be 0.102 mU mL^{-1} (Iverius and Ostlund-Lindqvist, 1976). Xanthine oxidase initial activity in raw whole milk has been reported to be 85 to 110 mU mL^{-1} and in skim milk 78 to 90 mU mL^{-1} while plasmin levels in fresh milk have been reported as 0.089 to 0.12 AMC U mL^{-1} (Hayes and Kelly, 2003; Girotti *et al*., 1999; Cerbulis and Farrell, 1976). Alkaline phosphatase and XO are both MFGM-bound enzymes and skimming milk can decrease their levels. Plasmin and lipase, on the other hand are associated with the casein micelles and skimming the milk does not affect their levels (Fox and Mc Sweeney, 1998). The levels of plasmin and total lipase found in skim milk used in this study were close to values reported in the literature. However, seasonality and lactation period can change level of enzymes in milk such as AlP and plasmin (Politis and Ng Kwai Hang, 1989; Kelly and Fox, 2006).

The findings of this study on the effects of PEF on enzyme inactivation were consistent with those reported in literature i.e. PEF can partially inactivate enzymes. However, the different degrees of enzyme inactivation reported in these studies could be attributed to the variety in experimental parameters as well as the PEF equipment type and configurations. The mechanism of enzyme inactivation by PEF is not fully understood; however it is believed that electrical pulses can change the secondary and tertiary structures of the enzymes (Zhong *et al.,* 2007;

Castro *et al.,* 2001). The effects of electric fields on proteins include the association or dissociation of functional groups, movements of charged chains, and changes in alignment of helices. The factors that mainly influence PEF enzymatic inactivation are: 1) electric parameters (e.g., electric-field intensity, total treatment time, and pulse width); 2) enzymatic structures (e.g., secondary and tertiary structures); 3) PEF treatment temperatures; and 4) treatment media (Min *et al*., 2007).

The enzymes studied in the current project including total lipase were raw milk enzymes while in most studies pasteurised or sterilised milk or SMUF have been spiked by the isolated enzymes. The method used for determining lipase activity measured total lipase in milk which included native and microbial lipases.

The differences in the reported amounts of plasmin and AIP in both parts of the current study was due to seasonal changes and stage of lactation which are reported to increase the plasmin level up to 2.3 times (Politis and Ng Kwai Hang, 1989; Kelly and Fox, 2006).

4.4.1 Combined Effects of Field Intensity and Heat on Milk Enzyme Inactivation

The partial conversion of electric pulses to heat causes an increase in milk temperature which if high enough can contribute to thermal inactivation of enzymes. Whether PEF or induced heat or both are responsible for inactivation of enzymes is still unclear (Yang *et al*., 2004; Van Loey *et al*., 2002). All enzymes were partially inactivated by PEF treatment while induced heat (35, 45 and 55°C)

had an additive effect on enzyme inactivation, and a dominant effect at temperatures ≥ 65°C.

Alkaline phosphatase activity level at temperatures <55°C when the PEF was switched off (E=0) did not change; however, by increasing the temperature to the range of 55 to 75°C a significant reduction (p<0.05) was observed in the level of AlP activity. The same trend was also observed for total lipase and XO under the same conditions while plasmin activity level did not show any significant difference even at 65°C (Table 4-3). The inactivation of these enzymes in the non-PEF control milks could be attributed to the cold shock resulted from the large difference between the temperatures of inlet and outlet milk (Table 4-1). The milk enters the water bath at a temperature <15ºC and leaves the 4th treatment chamber at the maximum temperature followed by sudden chilling in the ice bath located after the 4th treatment chamber. Therefore, the heat shock could result in a further enzyme inactivation (Pinto *et al*., 1991).

When field intensities of 29 or 35 kV cm^{-1} (the maximum achievable field intensities) were applied at 35°C, all four enzymes were significantly inactivated (p<0.05). However, there was no significant difference (p>0.05) in enzymes inactivation level between the two field intensities, indicating that the 6 kV cm^{-1} difference in the filed intensity did not have a substantial effect on enzyme inactivation (Fig. 4-1, Table 4-3).

When the temperature was raised to 45°C, a significant difference (p<0.05) was observed in the AlP and XO activity levels between the two field intensities; 35 kV

cm^{-1} causing higher inactivation, while total lipase and plasmin activity levels did not show a significant difference. However, the activity levels of all four enzymes in PEF-treated milk samples were significantly different (p<0.05) from the 45℃ control samples when PEF was switched off. Structure of the enzyme, conductivity of the medium, treatment temperature or energy input determine the enzyms' sensitivity to PEF treatment. It is reported that the larger and more complex an enzyme molecule, the more susceptible it is to heat (Yang *et al.*, 2004). The data shown in Table 4-3 indicate that the sensitivity of the enzymes to PEF is also related to their thermostability and size as the molecular weight of plasmin (81 Da) is much less than AlP (170-190 kDa), lipase (40-400 kDa) and XO (300 kDa) (Fox and McSweeney, 1998) which makes it more resistant to heat and PEF treatment.

At 55℃, PEF effectively decreased the activity levels of all enzymes while there was no significant difference (p>0.05) between the two field intensities in inactivation levels. However, at 65℃ and above, a sudden drop in enzymes activity levels was observed so that it was difficult to differentiate between the heat or PEF effect. The factorial statistics presented in Table 4-4 demonstrated that once the PEF treatment temperature reached 65℃ and beyond, the enzyme inactivation was mostly dominated by heat rather than field intensity. However, the PEF effects cannot be ignored because there was a significant difference (p<0.05) between the activity levels of enzymes in PEF treated milks and the control (non-PEF-treated) samples at 65 and 75℃. Compared to other 3 enzymes plasmin showed resistance to both heat and PEF effect. Alkaline phosphatase was inactivated >90% at temperature 65℃ at both field intensities which makes it a suitable indicator for PEF treatment efficiency under the tested experimental

conditions; however, further pathogenic microbiology tests are needed to confirm inactivation of pathogens in conjunction with AlP.

Figure 4-2 shows the correlation between the natural log of relative enzymes activity at 29 or 35 kV cm^{-1} and different treatment temperatures. The proportionality constant (k_T) for temperature represents the slope of the inactivation curve, with a larger k_T value indicating a greater inactivation efficacy with the increase in the PEF treatment temperature. The proportionality constant for all enzymes and all temperatures significantly increased (p<0.05) from control to PEF treated samples which indicates the additive effects of PEF treatment and heat on enzyme inactivation. The k_T of AlP, total lipase, XO and plasmin, varied significantly with the highest k_T for AlP and lowest for plasmin. The slope of the curve for AlP and XO had a sudden fall from 65°C onwards while XO and plasmin had a milder slope which indicates the dominant effect of heat on AlP and total lipase while XO and plasmin were more resistant to both heat and PEF treatment. However, although the enzyme inactivation was dominated by heat at 65 to 75°C the role of PEF treatment was also evident on enzyme inactivation since the inactivation curves of all enzymes had a steeper slope than those of non-PEF-treated control samples (PEF off, E=0).

The inactivation curves in all temperatures were positioned close together (Fig. 4-2) which showed that the difference in field intensities used (29 or 35 kV cm^{-1}) did not result in a significant difference (p>0.05) in enzyme inactivation. The linear relationship between enzyme inactivation, field intensity and treatment temperature showed a regression coefficient of $R^2 \geq 0.71$ which indicates that the

first-order inactivation model was adequate for describing the enzyme inactivation in raw skim milk within the experimental conditions tested (Zar, 1999; Min *et al.*, 2003).

Based on the results of the first set of trials, PEF treatment temperatures exceeding 60°C would result in the dominance of heat effect on the enzyme inactivation. Therefore, for the second set of trials, 60°C was selected as the maximum treatment temperature.

4.4.2 Combined Effects of Treatment Time and Temperature on Milk Enzyme Inactivation

The relationship between the treatment time and the activity levels of enzymes is shown in Figure 4-3. The maximum levels of AlP activity after changing either the pulse frequency or the flow rate were close (1.07 & 1.16 U mL^{-1}) at 30°C which indicates that the means of achieving the given treatment time (whether by changing the pulse frequency or the flow rate) had no significant (p>0.05) effect on the enzyme activity level; however, at 60°C the difference was significant (p<0.05); flow rate being more efficient in inactivating AlP (Table 4-6). This could be due to longer exposure time of AlP to heat at a lower flow rate as AlP is rather thermolabile and its inactivation is reported to start at 60 and by the time the temperature reaches 86ºC, it is completely inactivated (Eckner, 1992). The difference between the activity levels of AlP at 30 and 60°C was statistically significant (p<0.05) which indicates the additive effects of heat and PEF treatment on enzyme inactivation (Fig. 4-3). Castro *et al.* (2001) reported that up to 65% of the added AlP was inactivated in SMUF and raw milk with a total treatment time of

460-800 µs at 18.8 - 22 kV cm^{-1} and a final temperature of <44°C. In the present study, the maximum inactivation level of native AlP achieved in milk was 75% at a treatment time of 38.5 µs (flow rate of 30 mL min^{-1} at 60°C). The treatment times in the study by Castro *et al.* (2001) was 12 to 21 times greater than 38.4 µs used in the present study while their achieved level of inactivation was 12% less than this study. Obviously, the treatment temperature (44°C in their study vs. 60°C in our study) as well as difference in field intensities played an important role in AlP inactivation process.

Total lipase inactivation trend was very similar to AlP inactivation. At 30°C and a treatment time of 38.4 µs, maximum inactivation level achieved by changing pulse frequency or flow rate was 42%. However, when the temperature was increased to 60°C (at 400 Hz and 30 mL min^{-1}), the maximum inactivation level was increased to 66% due to combined effect of PEF and heat (Fig. 4-3). Bendicho *et al.* (2002b) achieved a much lower level of inactivation on extracellular lipase from *Pseudomonas fluorescens* suspended in SMUF. They subjected the samples to up to 80 pulses of 37.3 kV cm^{-1}, at 3.5 Hz, and <35°C with a flow rate of 360 mL min^{-1} and achieved only 13% inactivation which was attributed to the low pulse frequency and high flow rate as well as the lipase type which was of microbial origin. The lipase from microbial source is more resistant to heat than the native lipase (Shamsuzzaman and Modler, 1987). The microbial lipases are 20–60 kDa proteins (Geraldine *et al.*, 2008) but the native milk lipases are lipoproteins with a molecular mass of 100 kDa (Kinnunen *et al.*, 1976) while Fox and McSweeney (1998) report a range of up to 400 kDa. It is believed that their comparative larger size makes them more sensitive to heat or PEF treatments.

Xanthine oxidase was significantly more vulnerable (p<0.05) to PEF treatment times achieved by flow rates rather than pulse frequency particularly at 60°C. This indicated that XO similar to AlP and total lipase was more susceptible to heat than PEF treatment. However, a significantly higher inactivation level (p<0.05) was achieved by combined effects of heat and PEF treatments.

Studies reported on XO inactivation in bovine milk are mostly limited to the effects of heat. For instance, Girrotti *et al*. (1999) determined the activity of XO in various milks (raw, skim, pasteurised, under-pasteurised, various heat treatments) from different breeds of cows. They found that at temperatures <60°C there was no "substantial" inactivation; however, heating the whole milk to 62°C for a "few seconds" inactivated XO by only 10 U L^{-1} (original 85 mU mL^{-1}). In the present study, the activity levels of XO at 60°C (PEF switched off, E=0) at flow rates of 240, 120, 60 and 30 mL min^{-1} (residence times of 25, 50, 100 and 200 s, respectively) were 79, 78, 66 and 59 mU L^{-1}, while the initial activity was 84±2.7 mU L^{-1}. The relatively higher inactivation level of XO in the present study could be attributed to the absence of MFGM in skim milk used that is claimed to have a protective effect on XO (Lewis *et al*., 1993).

Compared to other three enzymes, plasmin showed a higher resistance to both PEF and heat treatments. The initial activity level of plasmin at 30°C (PEF switched off, E=0) was 0.12 AMC U mL^{-1}, which was decreased to 0.093 AMC U mL^{-1} (23% lower) at 60°C. Maximum treatment time of 38.4 μs achieved by changing either flow rate or pulse frequency at 30 or 60°C did not result in significant inactivation levels (p>0.05) in plasmin (23 vs. 28% at 30°C; 43 vs. 47%

at 60°C) which indicated that the effect of flow rate and pulse frequency on plasmin inactivation was not significantly different ($p>0.05$). However, there was a significant difference ($p<0.05$) between the plasmin inactivation levels at 60°C before and after PEF treatment which indicates that to achieve a higher inactivation of plasmin, PEF treatment should be combined with heat effects (Table 4-6).

The level of plasmin inactivation in the present study was very different from that reported by Vega-Mercado *et al.* (1995), who achieved up to 90% inactivation of plasmin (from bovine plasma) spiked in SMUF by PEF treatments at 15, 30 or 45 kV cm^{-1} with 10, 20, 30, 40 and 50 pulses of 2 µs at 0.1 Hz and <20°C. Besides the number of pulses and field intensity used, they attributed the high level of inactivation to the absence of casein micelles in the SMUF, which is reported to increase the heat stability of plasmin. According to Grufferty and Fox (1988), the stability of plasmin is lower in non-micellar systems (such as SMUF) than in casein dispersions (such as milk) because plasmin is associated with casein micelles which have a protective effect on plasmin against any external forces (Fox and McSweeney, 1998). Besides, the plasmin system in milk includes the plasminogen and plasmin inhibitors which control plasmin activity in milk while in SMUF only the plasmin enzyme was present.

This observation can explain why the maximum inactivation of plasmin achieved by PEF treatment of milk in the present study was half that achieved by Vega Mercado *et al.* (1995). Sharp and Honig (1990) and Robertson and Astumian

(1990) attributed the PEF inactivation mechanism of plasmin to changes in charge and configuration of plasmin due to its electrostatic nature as a protein.

Heat treatment of skim milk by pasteurisation at 72ºC for 15 s (HTST) and 63ºC for 30 min (LTLT) was undertaken to compare enzyme inactivation results to those achieved by PEF treatment. Alkaline phosphatase was totally inactivated by both pasteurisation methods, while total lipase was inactivated by 88 and 90% with HTST and LTLT, respectively. Milk lipase is a heat-labile enzyme and pasteurisation of milk at 72°C for 15 s can result in a total inactivation (Farkye *et al.,* 1995; Deeth, 2006); however, compared to AlP it is more resistant to PEF effects. On the other hand, XO is fairly heat stable, being destroyed only at 80ºC for 10 s (Shahani *et al*., 1973; Walstra and Jenness, 1984; Lewis *et al*., 1993). The inactivation level of XO in pasteurised skim milk (HTST or LTLT) in this study was about 75 and 72%, respectively. In a study by Girotti *et al.* (1999), XO was inactivated by 50% at 74°C for 5 s in whole milk. The difference in the level of inactivation could be attributed to the fact that in whole milk XO is bound to MFGM which has a protective effect and shields the enzyme against heat and other physical treatments, while in skim milk that protective effect does not exist. Besides, in the method of pasteurisation in the present study, the come-up time for milk sample to reach 72ºC was 90 s which is longer than the standard industrial method. Xanthine oxidase was once suggested as an indicator of pasteurisation adequacy to replace AlP, however, owing to difficulties in measuring XO activity, the idea was abandoned (Fox and Kelly, 2006; Shahani *et al*., 1973).

When milk sample was pasteurised at 63°C for 30 min, the plasmin showed 14% activation which is reportedly due to the inactivation of its inhibitors at this temperature (Borda *et al.*, 2004), however, pasteurisation at 72°C for 15 s decreased plasmin activity level to 42%. Korycka-Dahl *et al.* (1982) achieved a 10% inactivation of plasmin in full-cream milk after HTST pasteurisation while Alichanidis *et al.* (1986) reported that plasmin was not affected by pasteurisation conditions. The relatively high inactivation level of plasmin achieved in the present study (similar to XO) could be attributed to the relatively long come-up-time of 90 s for the milk sample to reach 72°C.

Ahern and Klibanov (1985) related the thermal inactivation of enzymes to the conformational and chemical changes within the enzyme upon heating. They demonstrated that once the enzyme is exposed to heat, the catalytic activity is lost which depending on the temperature used could be an irreversible process.

Treatment times achieved by changing either the pulse frequency or the flow rate were the primary variables tested in this study for the inactivation of milk enzymes. As described earlier, when the flow rate was changed from 30 to 60, 120 and 240 mL min^{-1}, the pulse frequency was kept constant at 200 Hz and when the pulse frequency increased from 100 to 200, 300 and 400 Hz, the flow rate was kept constant at 60 mL min^{-1}. At all treatment temperatures tested, the linear relationship between enzyme inactivation and the treatment times showed a regression coefficient of $R^2 \geq 0.82$ (Table 4-7), which indicates that the first-order inactivation model is adequate for describing the inactivation of native AlP, XO, total lipase and plasmin in raw skim milk within the experimental conditions used.

Treatment time and total specific pulsing energy at 30 and 60°C followed a similar trend as enzyme inactivation is dependent on both (Fig. 4-3). Extending the treatment time has been reported to increase the enzyme inactivation. Min *et al.* (2003) achieved a higher level of inactivation in lipoxygenase in tomato juice by extending the treatment time from 20 to 70 µs at a maximum field intensity of 35 kV cm^{-1}.

Zhong *et al.* (2007) also found a strong linear relationship between the inactivation of POD and PPO (in 0.1 M acetate buffer at pH 5.6) and the treatment time with regression coefficients of 0.932 and 0.926, respectively. They found that increasing treatment time from 124 to 1740 µs (which was achieved by recirculating the buffer through the PEF system at temperature <40°C) resulted in 23 to 77% inactivation of both enzymes. The field intensity was 25 kV cm^{-1} with a pulse frequency of 10 Hz and flow rate of 52.5 mL min^{-1}.

The enzyme inactivation levels depend on the concentration of enzyme (Castro *et al.,* 2001) and the enzyme characteristics (Vega-Mercado *et al.,* 1995; Ho *et al.,* 1997). The field intensity, number of pulses and treatment temperature are also other factors determining the enzyme inactivation by PEF treatment (Grahl and Märkl, 1996; Van Loey *et al.*, 2002). This explains the differences in inactivation levels of various enzymes achieved in different studies.

Table 4-7 shows the R^2 and inactivation constant *k* for each enzyme. The slope of the trend lines at various pulse frequencies indicated that AlP, total lipase, XO and plasmin were more susceptible to changes in flow rates than pulse frequencies.

Compared to XO and plasmin, AlP and total lipase were more susceptible to changing flow rate than pulse frequency due to longer exposure time of milk to heat at lower flow rates. Plasmin and XO were less affected by PEF treatments at different flow rates and pulse frequencies at 30°C compared to 60°C. They had also a higher inactivation at various flow rates at 60°C compared to the inactivation levels achieved by changing pulse frequencies at the same temperature, which could be attributed to longer exposure time of enzymes to heat.

During processing of liquids in a continuous PEF system, the fluid flows through a series of high voltage treatment zones. The viscosity of a fluid, which is highly influenced by temperature, affects the flow of the fluid through the PEF chamber. Reynolds (*Re*) number indicates the flow's behaviour whether laminar or turbulent, and is independent of field intensity. It defines the flow dynamic and the relationship between the physical characteristics of fluid including its viscosity, temperature, and density. The higher the *Re* number, the more turbulent is the flow, thus resulting in an even exposure of enzymes or microorganisms to the heat and field intensity (Ruhlman *et al*., 2001; Schrive *et al*., 2006; Alkhafaji and Farid, 2008). For values above 4000 the flow is considered turbulent and between 2100 and 4000 the flow pattern is transitional (Earle, 1983). Flow pattern is important to be considered in PEF treatment since a turbulent flow provides a uniform PEF effect (Ruhlman *et al.,* 2001). Ideally, liquid flow through PEF treatment zone should be in turbulent pattern (*Re* number of 4000 or above) to limit deposits formation on the surface of the electrodes. Under the laminar flow regime, due to formation of boundary layers the enzyme inactivation may not be uniform. However, in the treatment chambers a transitional flow was observed which can

limit the thickness of the boundary layers thus resulting in an even distribution of the field intensity.

In the present study, skim milk flow rate through the chamber was set to 240, 120, 60 and 30 mL min^{-1} (Table 4-8). The maximum *Re* numbers of skim milk flow through the treatment chambers was 2946 at 60ºC at a flow rate of 240 mL min^{-1}. However, this transitional flow regime did not result in a higher enzyme inactivation due to reduction in treatment time. According to Alkhafaji and Farid (2008), the higher inactivation is achieved through keeping whole processing conditions constant. Increasing the *Re* number would be to the cost of reducing the treatment time. Therefore, a balance needs to be struck between these conflicting process parameters.

4.5 CONCLUSIONS

The results of the first set of trials confirmed the combined impact of heat and PEF treatment on enzyme inactivation up to 65°C. However, at 65°C and above, the PEF effects on enzyme inactivation was overtaken by heat effect; therefore, the PEF treatment temperature should never be allowed to exceed 60°C if the main aim of treatment is to predominantly achieve nonthermal PEF effects. Higher temperature above 65°C can be used to achieve more inactivation in heat-resistant enzymes such as plasmin. Pulsed electric field effects were clearly observed in inactivating enzymes at 35, 45 and 55°C since there was a significant difference between the enzymes in PEF-treated and control samples. The kinetic study showed that the first order kinetic equation was adequate to describe the relationship between the field intensity and treatment temperature with enzyme inactivation.

The effects of treatment time achieved through adjusting pulse frequency or flow rate on native milk enzymes indicated that the enzyme inactivation was more pronounced by increasing the treatment time; however, changing the flow rate at both temperatures was found to be more effective than changing the pulse frequency. Therefore, adjusting the flow rate is recommended when a higher level of enzyme inactivation is pursued. Based on the experimental conditions, the most susceptible enzyme to PEF and heat treatments was AlP followed by total lipase, XO and plasmin while AlP was found to be a suitable indicator of PEF treatment adequacy at 65ºC. Using a kinetic model it was demonstrated that the enzyme inactivation, treatment time and total specific pulsing energy were linearly correlated. The flow pattern in this study was transitional at the maximum flow rate as shown by calculated *Re* number; therefore, to achieve a higher *Re* number and more even distribution of heat in the PEF system, a higher flow rate is needed.

CHAPTER 5

EFFECTS OF PULSED ELECTRIC FIELD ON THE RHEOLOGICAL AND TEXTURAL PROPERTIES OF RENNET-INDUCED GELS OF BOVINE MILK

ABSTRACT

This study was conducted using two different PEF units, one at Food Science Australia (FSA) and the other at Berlin University of Technology (BUT). In the set of trials performed at FSA, raw skim milk was treated at 38 and 35 kV cm^{-1} with monopolar pulses at final temperatures of 30 or 60°C, respectively with a flow rate of 60 mL min^{-1}. Rennet was added to the milk samples and incubated at 32°C for 60 min. Elastic or storage modulus (*G'*) and viscous or loss modulus (*G''*) of gels were determined using a rheometer, while the gel firmness was measured by a Texture Analyser. Gels made from non-PEF treated 30°C control skim milk showed the highest *G', G''* and firmness followed by the gels of 60°C control. The PEF treatment of milk at both field intensities and temperatures resulted in a lower *G', G''* and firmness compared to gels made from control milk. However, in comparison, the HTST and LTLT pasteurisation processes resulted in much more pronounced effects on rheological and textural properties of the gels. High heat-treated (97°C for 10 min) skim milk formed no gel. Casein micelle size was not affected by PEF treatment but high-heat treatment of milk resulted in a significant increase ($p<0.05$) in micelle size. The rennet coagulation time (RCT) increased as a result of PEF treatment; however, compared to pasteurisation process it was shorter.

In the set of trials conducted at BUT on both raw skim and whole milk, the field intensities of 40, 45 and 50 kV cm^{-1} with monopolar pulses and final temperatures of 30 or 60°C, with a flow rate of 60 mL min^{-1} were selected. The fat globule size did not change after PEF treatment or LTLT pasteurisation while high-heat treatment and HTST pasteurisation increased the size significantly (p<0.05). The *G', G"* and firmness of gels made from PEF-treated milk decreased while the RCT increased. Similar to the results obtained in the first set of trials, the extent of these changes was much less pronounced than those caused by heat treatments. Scanning electron microscopy of treated gels indicated that PEF or heat treatment increases the size of pores in the gel network resulting in a lower *G', G"* and firmness.

5.1 INTRODUCTION

Milk gelation is a critical step in cheese and yoghurt production processes. In milk gels microstructure and rheological properties as well as the overall visual appearance are important since they contribute to the sensory properties of these products. Milk gels are viscoelastic and their rheological properties can be characterised using both the viscous and elastic moduli. Dynamic rheology testing (applied oscillatory strain or stress) provides useful information on gels and their formation process. The elastic or storage modulus (*G'*) is a measure of the energy stored per oscillation cycle and the viscous or loss modulus (*G''*) describes dissipation of energy into heat when a material is deformed (Lucey, 2003).

Rennet coagulation of milk is an irreversible phase change resulting in a semi-solid gel whose porous structure is formed by a protein network confining the whey (Zoon *et al*., 1988 a & b). The coagulation stage has an important effect on physical properties of the gel, particularly on its porosity and permeability (Lagoueyte *et al*., 1994).

Milk is coagulated through cleaving of κ-casein by rennet enzyme and subsequent aggregation of casein micelles (Zoon *et al*., 1988a; Walstra, 1990). Heating milk at above 70°C even for normal pasteurisation purpose hinders rennet action on κ-casein through denaturing whey proteins to varying degrees. The denatured whey proteins interact with κ-casein and adversely affects coagulation process and gel strength (Dalgleish, 1984 and1990; Parris *et al*., 1991; Jelen, 1992; Singh and Latham, 1993; Qi *et al*., 1995; Jelen and Rattray, 1995; Law *et al*., 1998; Beaulieu *et al*., 1999).

The effects of PEF on microorganisms and enzymes in liquid foods i.e. milk or juices are well reported (Barbosa-Canovas and Sepulveda, 2005; Calderon-Miranda *et al*., 1999a; Craven *et al*., 2008). However, there are only a small number of reports on the effects of PEF on protein components of foods, mostly on egg albumin, and very few on milk proteins.

The aim of this study was to investigate the effects of various field intensities (35 to 50 kV cm^{-1}) on the rheological and textural properties of rennet-induced gels of bovine milk through using different PEF systems compared to heat treatments. In a previous report (Floury *et al.* 2006b), it was reported that applying field intensities below 40 kV cm^{-1} can hardly affect the milk protein or fat.

The PEF trials were conducted at two different locations, Food Science Australia (FSA) (671 Sneydes Road, Werribee VIC 3030**,** Australia) and Berlin University of Technology, Department of Food Biotechnology and Process Engineering (BUT) (Königin, Luise, Strasse 22, 14195 Berlin, Germany). The rheological, textural and microscopic evaluations on PEF-treated samples were carried out at RMIT University (RMIT University, La Trobe Street Melbourne VIC 3001, Australia, Food Science) and German Institute of Food Technologies (DIL) (Professor-von-Klitzing Strasse 7 DE-49610 Quakenbrück, Germany). The reason to use the PEF system at BUT, was that the maximum achievable field intensity with PEF system at FSA was 38 kV cm^{-1} while the PEF system at BUT was capable of achieving a higher field intensity of up to 50 kV cm^{-1}. Therefore, it was decided to investigate the impact of various field intensities higher than 40 kV cm^{-1} on gelation process of milk.

5.2 MATERIALS AND METHODS

5.2.1 Raw Skim and Whole Milk Properties

The fresh raw cold-skimmed milk (harvested in September 2007) used for FSA study had an electric conductivity (σ) of 4.51 mS cm^{-1} at 30℃ and 5.53 mS cm^{-1} at 60℃, and a pH of 6.71 at 15℃ (see section 3.2.1 in Chapter 3 for technical specifications of pH meter, conductivity meter and source of milk).

For BUT study, two lots of fresh whole milk (harvested in March 2008, fat content = 4.56%) were supplied by Department of Food Biotechnology and Process Engineering of BUT. The pH of whole milk was measured using a digital pH meter (CG 811, Schott Geräte GmbH, Hofheim, Germany) was 6.61 at 15ºC. One lot of milk was preheated in a water bath to 35℃ and skimmed using a thermo-controlled laboratory centrifuge (Sorvall RC5B Refrigerated Superspeed Centrifuge, Sorvall GmbH, Bad Homburg, Germany) at 2560 × g for 10 min. The fat layer on top was removed using a spoon followed by filtering the milk through a strainer (mesh size=10 µm).

The conductivity of the skim and whole milk measured by a digital conductivity meter (Konduktometer CG 858, Schott Geräte GmbH, Hofheim, Germany) was 4.61 and 4.71 mS cm^{-1} at 30ºC and 5.29 and 5.38 mS cm^{-1} at 60ºC, respectively. The whole milk lot was investigated for the fat globule particle size while the skimmed lot was used for rheological, textural and microscopic studies.

5.2.2 PEF Treatments

The PEF equipment at FSA, configuration and specifications, sanitisation protocol, the start-up of the system and handling of treated samples as well as monitoring the temperature, flow rate and electric voltage input during the process have been described in Chapter 3, section 3.2.2.

The PEF treatments at FSA were conducted using monopolar pulses with a pulse frequency of 200 Hz and a pulse width of 2 µs. The flow rate used for all PEF treatments was 60 mL min^{-1}, which resulted in a total treatment time of 19.2 µs. An input voltage of 9.5 kV was applied to the treatment chamber resulting in a field intensity of 38 kV cm^{-1} at an outlet temperature of 30°C (after the 4th treatment chamber), and 35 kV cm^{-1} at 60°C due to increase in the electrical conductivity of milk. The outlet temperature after the 4th treatment chamber was taken as the highest PEF treatment temperature. The total specific pulsing energy input for all PEF treatments was calculated as 154 kJ L^{-1} depending on conductivity of the milk used.

The inlet temperatures were set to 15°C for the 30°C trials, and to 45°C for the 60°C trials. The non-PEF treated control samples were subjected to the same temperature regimes without the application of electrical pulses by passing the skim milk at a flow rate of 60 mL min^{-1} through the PEF treatment chambers with water bath temperatures adjusted at 35 or 65°C to deliver 30°C and 60°C, respectively at the outlet of the forth treatment chamber. Based on the flow rate and volume of tubing between the outlet of the 4th treatment chamber and the ice water bath which was used for immediate cooling of the milk, the residence time of

milk in the highest temperature was approximately 5 s; however, the total exposure time of milk to a range of temperatures below the target temperature was 100 s. The temperature, flow rate and electric voltage input were monitored continually during the process as described in Chapter 3, section 3.2.2. The PEF trials were conducted twice and from each trial three samples were collected for reproducibility purpose and were duplicated for rheological and textural analyses.

The PEF system at BUT was equipped with a pulse modulator of 7 kW (ScandiNova System AB, Uppsala, Sweden) which converted the 3-phase line voltage to a regulated DC voltage. The maximum achievable pulse frequency of the system was 400 Hz with a pulse width of 3 to 8 µs; however, the maximum pulse frequency applied in this study was only 43 Hz since the higher frequencies would result in raising the milk temperature to over 60ºC. The electrodes were made of stainless steel with polyoxymethylene insulators. The treatment chamber was a cofield type with two cells with a total volume of 220 µL. Total residence time of milk inside treatment chamber was 230 µs. The line temperature was monitored by a thermometer connected to an optic fibre (FT 1110, Chiyoda, Tokyo, Japan). The product handling system comprised a peristatic pump (Watson Marlow 323 Du, Wilmington, USA) with 6 mm silicone tube. Two water baths (Peter Huber GMBH, Offenburg, Germany) filled with silicon oil were used before and after treatment chamber to control the inlet and outlet temperatures (Fig. 5-1).

The milk (both whole and skim) was treated with square monopolar pulses of 3 µs, at field intensities of 40, 45 or 50 kV cm^{-1}, and final outlet temperatures of 30 and 60ºC with a flow rate of 60 mL min^{-1}. Duplicate samples were collected in 250 mL

plastic bottles and transferred overnight to DIL for rheology, texture, microscopy and fat globule size measurements. Table 5-1 shows the experimental conditions for PEF treatment of milk at BUT.

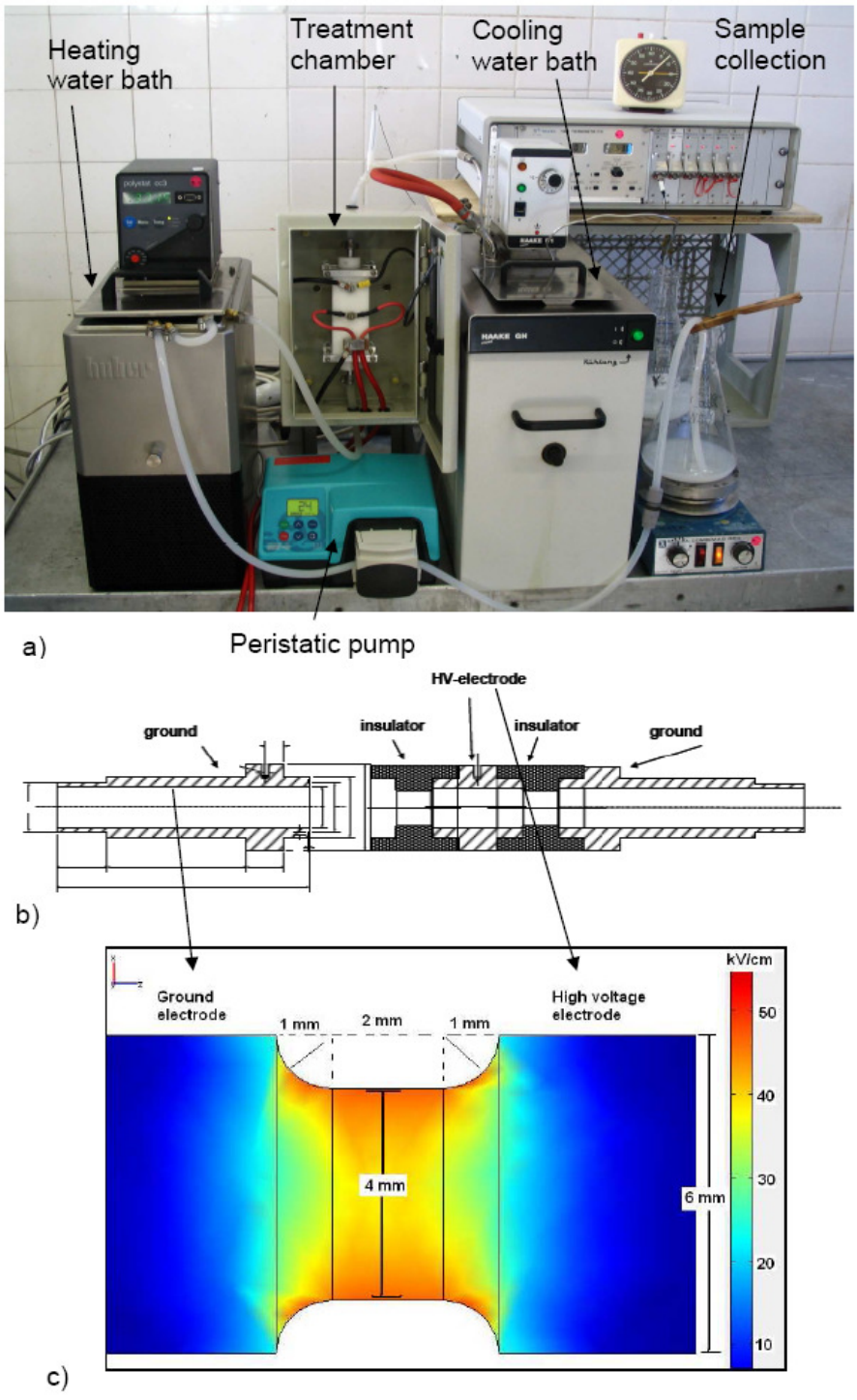


Figure 5-1 (a) The PEF unit at Berlin University of Technology, Germany; (b & c) treatment chamber

Table 5-1 Treatment conditions of milk treated with PEF unit at Berlin University of Technology

Treatment conditions	Skim milk							Whole milk						
Field intensity (kV cm^{-1})	50	45	40	50	45	40	off	50	45	40	50	45	40	off
Pulse frequency (Hz)	28	34	41	16	18	23	0	16	17	22	28	34	43	0
Treatment time (μs)	19	23	27	11	12	15	0	10.5	11	13.5	18	23	29	0
Number of pulses	6	8	9	4	4	5	0	4	4	5	6	8	9	0
Inlet T (ºC)	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Outlet T (ºC)	60	60	60	30	30	30	60	30	30	30	60	60	60	60
Water bath T (ºC)	6	6	6	6	6	6	67	6	6	6	6	6	6	67
Total energy (kJ L^{-1})	203	203	203	94	94	94	0	87	87	87	193	193	193	0
Peak voltage (kV)	30	27	24	30	27	24	0	30	27	24	30	27	24	0
Peak current (A)	94	86	76	76	68	61	0	70	62	56	86	80	71	0

5.2.3 Casein Micelles Size Measurement by Dynamic Light Scattering

The average diameter of casein micelles (Z Ave) was measured by photon correlation spectroscopy using a Malvern Zetasizer Nano ZS (model ZEN 3600, Malvern Instruments Ltd, Malvern, UK) at FSA equipped with a He-Ne 633 nm laser scanner with a scattering angle of 90° and refractive index of 1.57. The Dispersion Technology Software version 4.20 was used for operation of the instrument and data acquisition. The skim milk samples were diluted 40 times with Milli Q water and placed in disposal cuvettes (DTS0012, Malvern) and the measurement was conducted at 25℃ with sample equilibration time of 2 minutes. The results were expressed as intensity of distribution in volume versus particle size, and the average particle size was calculated.

5.2.4 Fat Particle Size Measurement by Dynamic Light Scattering

A Malvern Mastersizer (Zetasizer 3000, Malvern Instruments, Worcestershire, UK) was used to measure the particle size distribution of fat globules in milk samples. The scattering angle was 90º, the wavelength 633 nm, and the temperature 25ºC. The reflective index for fat particles was set at 1.59. The measurement was performed by diluting 100 µL of each milk sample with 3 mL of Milli Q water in disposal cuvettes. The results were expressed as intensity of distribution versus particle size. This measurement was conducted at DIL on the milk treated with PEF unit of BUT.

5.2.5 Rheological Analysis

The viscoelastic properties of gels prepared at FSA and DIL were determined by low amplitude dynamic oscillation measurement of elastic or storage modulus (*G'*) and viscous or loss modulus (G″) (Lucey *et al*., 2000) using a rheometer (RheoStress RS-50, Haake Mess-Technik GmbH, Co., Karlsruhe, Germany). The rheometer consisted of a jacketed stainless steel cylinder with an internal diameter of 22 mm and height of 58 mm connected to a circulating temperature-controlled water bath set to 32°C, and a cylindrical probe (Z20 DIN, 20 mm Ø). Aliquots (20 mL) of treated and untreated milk samples were transferred into the cylinder pre-warmed to 32°C. Ten microlitre of Naturen microbial double strength liquid rennet 280 (Chr. Hansen Pty Ltd, Brandwood, SA, Australia) diluted with 400 µL Milli Q water was added to each sample and mixed in. The probe was lowered and submerged in the milk to a depth of 1 mm from the bottom of the cylinder. The surface of the milk was covered with a thin layer of paraffin oil to prevent evaporation during coagulation. The frequency and strain of the probe were set at

0.1 Hz and 0.01 Pa, respectively, and the data was collected for a period of 60 min after adding rennet into the milk, mixing and covering milk surface with paraffin (Guinee *et al*., 1997). Rennet coagulation time was taken as the time for the *G'* of gels to reach 1 Pa (Lucey *et al*., 2000; Guillaume *et al*., 2004).

5.2.6 Texture Analysis

Aliquots of 40 mL of treated and untreated milk samples were transferred into plastic containers (50 mm d x 40 mm h), sealed and equilibrated in a water bath at 32℃ for 10 min. Similar to rheology sample preparation, 10 µL of double strength rennet diluted with 400 µL Milli Q water was added to each container, followed by stirring and subsequent incubation at 32^{o}C for 60 min (Guinee *et al*., 1997).

At the completion of coagulation time (after 60 min at 32^{o}C), the firmness of the resultant gels was determined within the containers with a TA-XT2 Texture Analyser (Stable Micro System, Godalming, London, UK) using the 'Force in Compression' mode. A cylindrical probe (P20) was lowered into the sample at a speed of 1.7 mm s^{-1} and a post-compression speed of 10 mm s^{-1}. The penetration depth was set to 40% of the gel height and the force value was set at 0.1 N. The penetration force was plotted against the compression time, and the firmness of the gel was defined as the maximum force prior to breakage of the gel. The entire measuring cycle took 8 seconds and the breaking point of all gels was between 4 and 5 seconds. All measurements were performed in duplicates.

5.2.7 Scanning Electron Microscopy of Gel Structure

A Scanning Electron Microscope (SEM, Jeol JSM 6460 LV, 1-2, Musashino 3-chome Akishima,Tokyo 196-8558, Japan) was used to observe the structure of milk gels. Random samples were taken from the gels and immediately frozen in liquid nitrogen. The frozen gels were then inserted into the cryo-preparation system (Emitech K 1250 29b Kripes Road, East Granby, Connecticut USA 06026-9669), broken and sublimated. Finally, the surface of the dried gel was sputtered with a thin layer of gold (20 nm) and the prepared samples were transferred into the SEM unit pre-set to approximately -180℃ (Lagoueyte *et al*., 1995).

5.2.8 Heat Treatment of Milk

For description of heat treatment of milk including HTST, LTLT and high-heat treatment of milk refer to section 3.2.3 in Chapter 3.

5.2.9 Statistical Analyses

Statistical analyses were performed by applying one-way ANOVA to determine the significance of the 95% confidence interval and correlation coefficient using Minitab software (Version 14, Minitab Inc., State College, PA, USA). The means and standard deviations were the results of 3 duplicates each obtained in two separate sets of experiments.

5.3 RESULTS

5.3.1 Casein Micelle Size of Skim Milk

Figure 5-2 shows the size distribution of casein micelles in PEF and non-PEF treated skim milk samples. The average diameter of casein micelles calculated by Malvern Zetasizer in the 30 and 60°C controls was 168 and 165 nm, respectively. The treatment at 38 kV cm^{-1} (30°C) and 35 kV cm^{-1} (60°C) did not result in a significant change ($p>0.05$) in casein micelle size (163 nm, 164 nm) compared with the respective non-PEF controls. Pasteurisation of milk (LTLT and HTST) had no significant effect ($p<0.05$) on the casein micelle size (167 nm, 169 nm). High-heat treatment of milk at 97°C for 10 min increased the casein micelle size to 187 nm. The percentage of size distribution difference from the minimum (163 nm) to maximum (187 nm) size was 1.8%.

5.3.2 Fat Globule Size of Whole Milk

Figure 5-3 shows the average size of the fat globules measured by Malvern Zetasizer. No significant difference ($p>0.05$) was observed between the average fat globules size in PEF-treated and control samples (30 and 60°C) regardless of the field intensities and temperatures applied. However, high-heat treatment and HTST pasteurisation of milk caused a marginal drop in the fat globules size. The results were expressed as the intensity of distribution versus particle size.

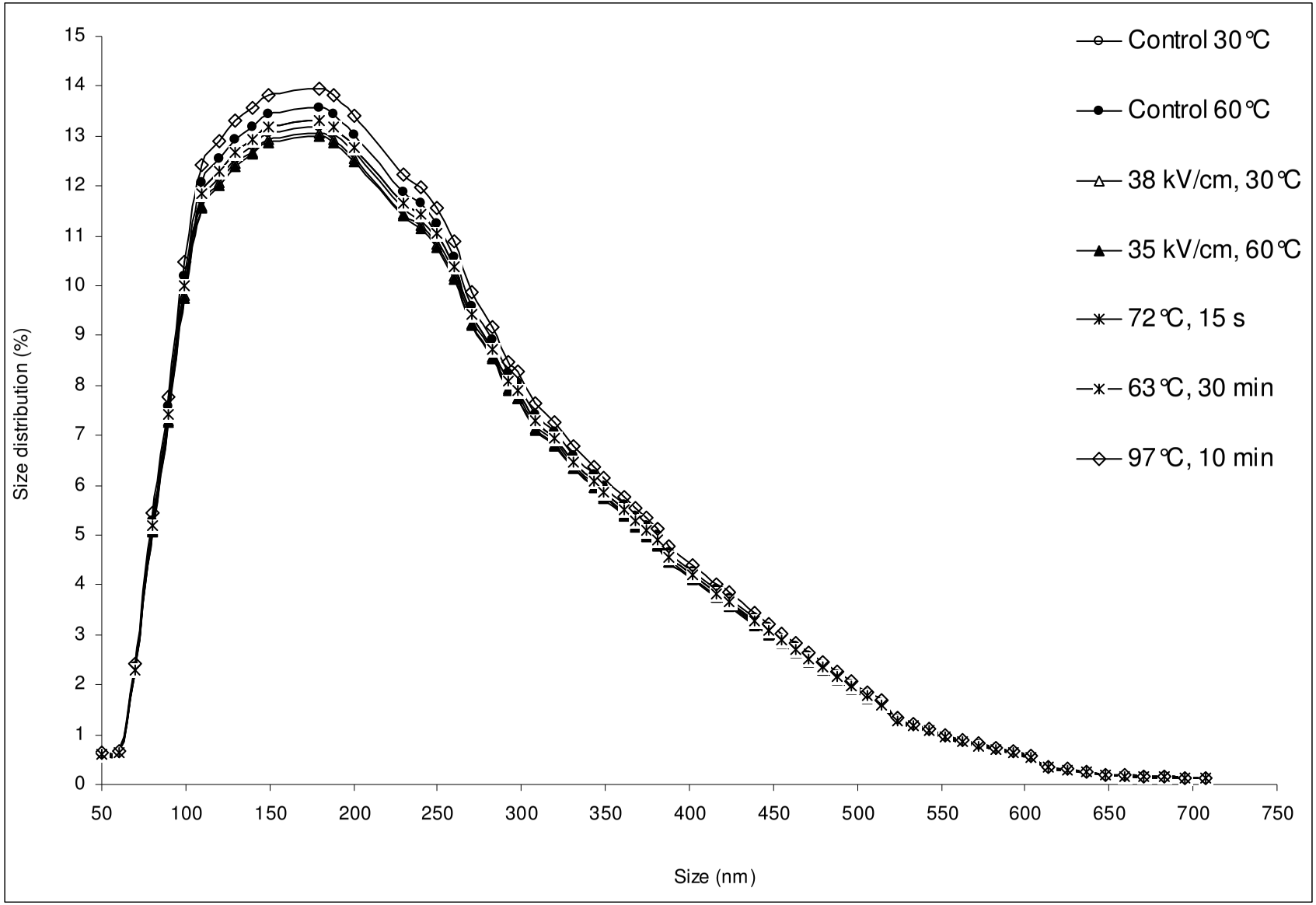


Figure 5-2 Size distribution of casein micelles measured by Zetasizer.

The standard deviation of particle size for duplicate samples of different treatments was from ±5 to ±8 nm.

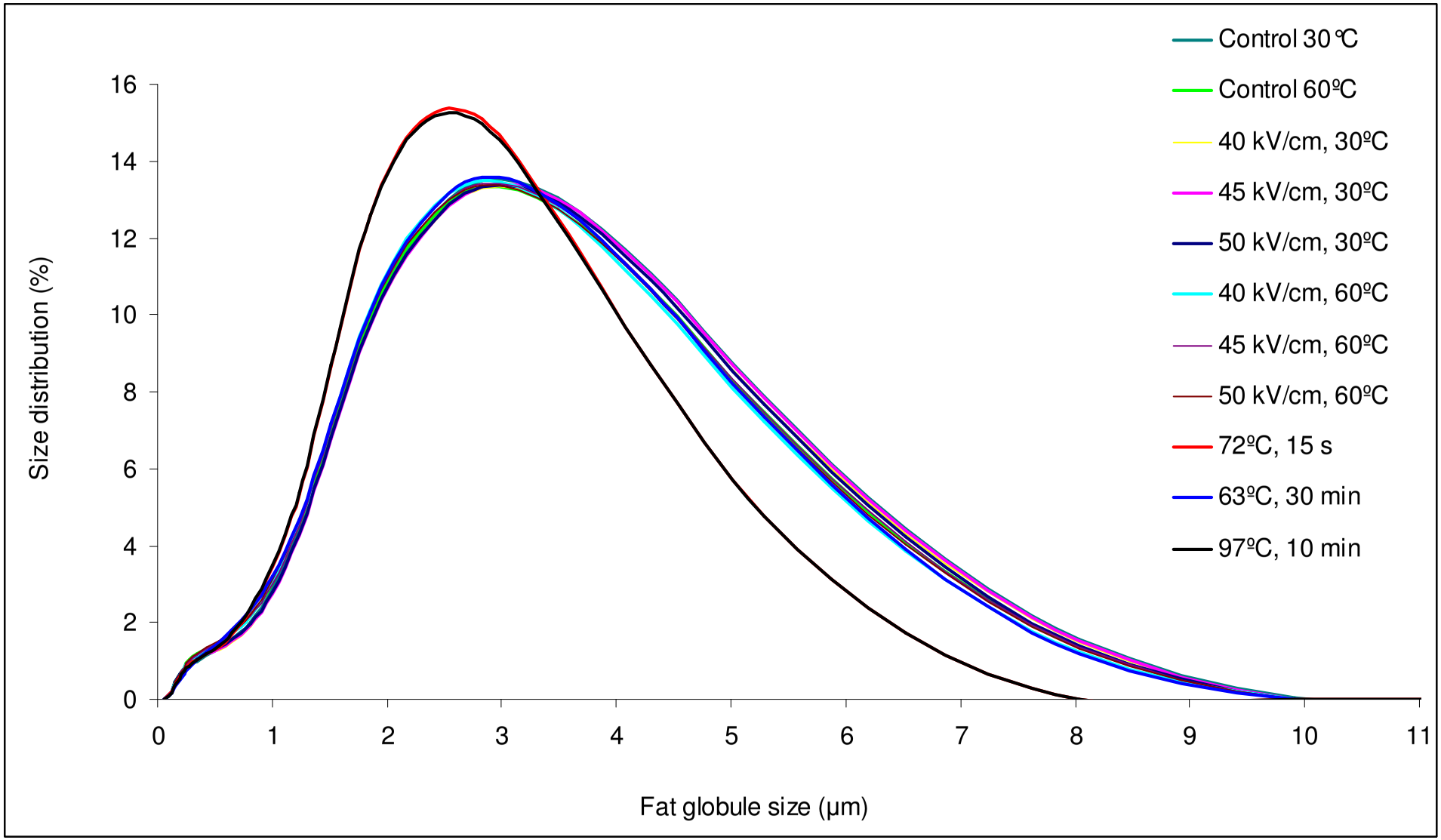


Figure 5-3 Average size of fat globule particle measured by Zetasizer.

The standard deviation of duplicate samples for different treatments was from ±5 to ±8 µm.

5.3.3 Rheological Properties of Skim Milk Gels

The elastic or storage modulus (*G'*) and viscous or loss modulus (*G''*) of gels made from all milk samples as a function of coagulation time are shown in Figure 5-4 and 5-5. The gels made from the 30 and 60°C control milk had the highest ($p<0.05$) *G'* and *G''* values compared to other samples. The PEF treatment of milk at 38, 40, 45 and 50 kV cm^{-1} and 30°C resulted in a significant decrease ($p<0.05$) in *G'* and *G''*. A further decrease in *G'* and *G''* of gels was observed at field intensities of 35, 40, 45 and 50 kV cm^{-1} and 60°C. The rheological properties of the gels made from the PEF-treated milks showed a decreasing trend regardless of using two different PEF systems correlated with increasing field intensity and temperature since heat (60°C) had an additive effect on PEF treatment and resulted in further decrease in *G'* and *G''* of gels.

The LTLT pasteurisation of milk resulted in the significantly lowest ($p<0.05$) *G'* and *G''* while the HTST method resulted in a milder change to *G'* and *G''* (Table 5-2 a & b).

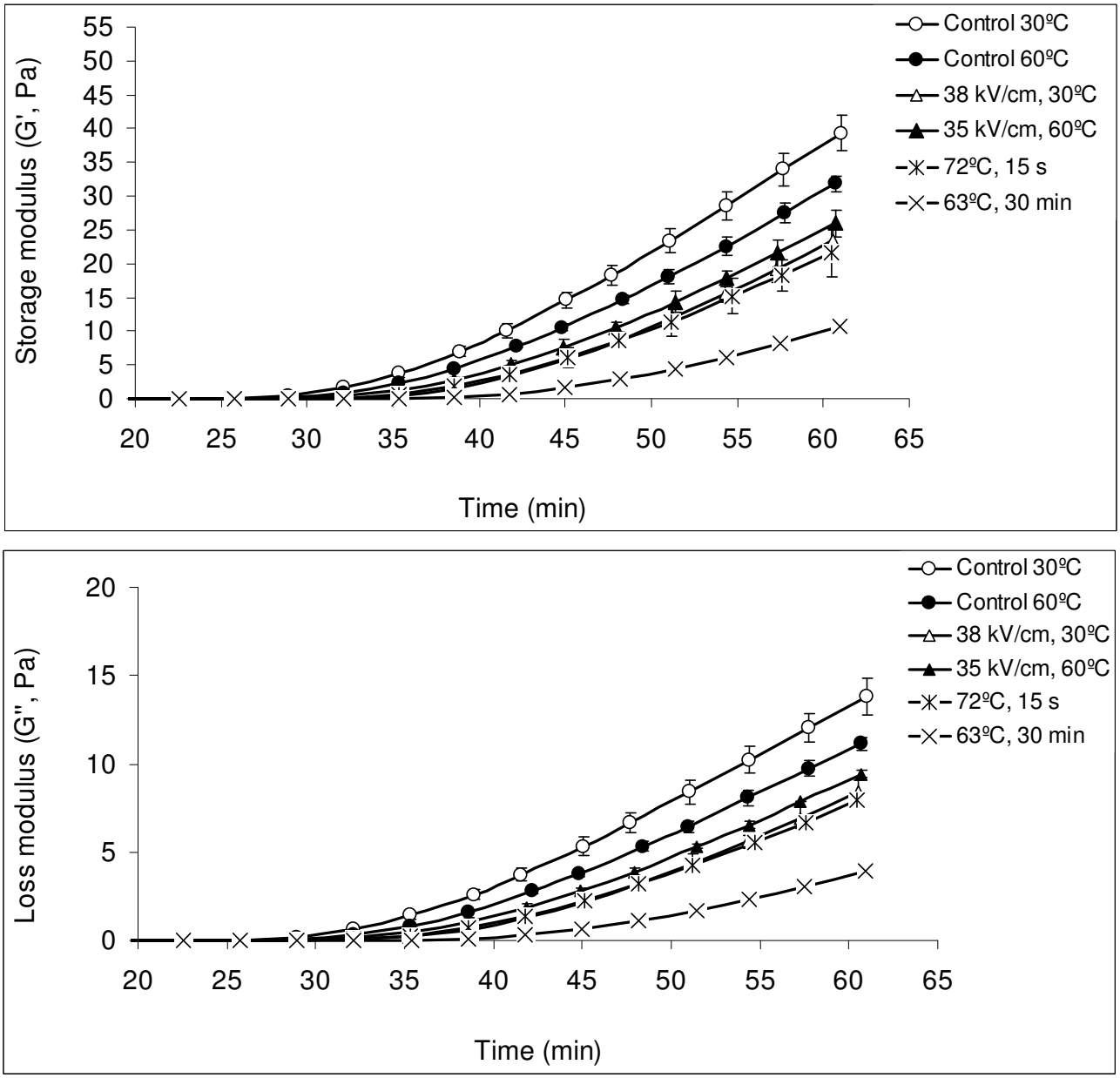


Figure 5-4 Storage modulus (*G'*) and loss modulus (*G''*) of gels of thermal and PEF-treated milk samples at 30℃ and 60℃ made at FSA.

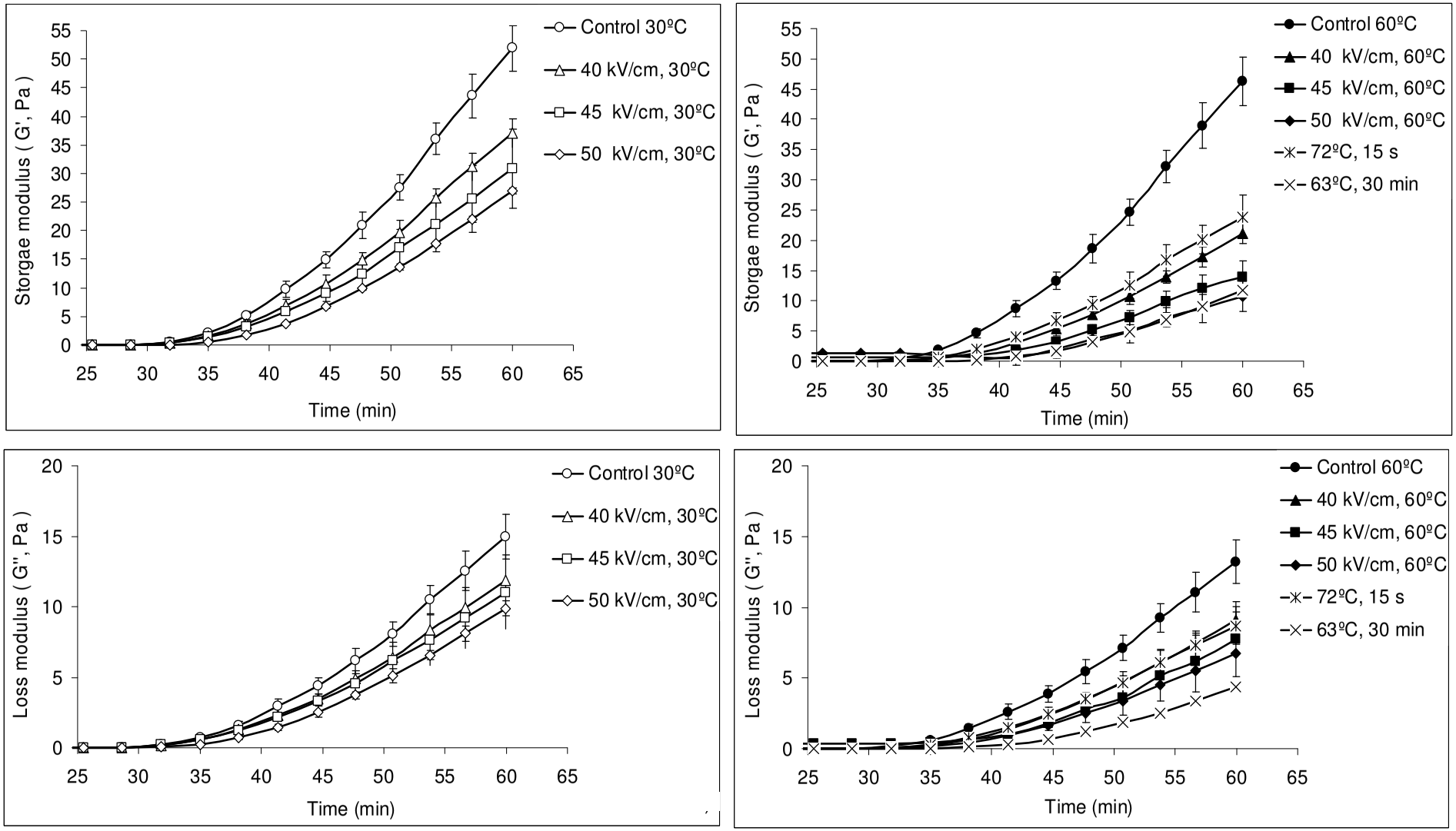


Figure 5-5 Storage modulus (*G'*) and loss modulus (*G''*) of gels made from thermal and PEF-treated milk samples at 30℃ and 60℃ at BUT.

5.3.4 Rennet Coagulation Time

The time taken for the *G'* of the gel to reach 1 Pa during the rennet coagulation process is presented in Table 5-2 (a & b). The RCT of the 30°C control milk was the shortest (32.2 min) while the RCT of 60°C control milk was slightly longer ($p>0.05$) (32.3 min). The PEF treatment of milk at 38 kV cm^{-1} and 30°C increased the RCT significantly ($p<0.05$) to 35.4 min and that at 35 kV cm^{-1} and 60°C further increased the RCT ($p<0.05$) to 38.8 min. The LTLT pasteurisation of skim milk resulted in the longest RCT (45 min) while the HTST treatment resulted in a more moderate increase in RCT (38.6 min).

The RCTs of milk treated at field intensities of 40, 45 and 50 kV cm^{-1} indicated an increasing trend at both 30 and 60°C; the RCT of milk samples treated at 50 kV cm^{-1} being significantly longer ($p<0.05$) than other milk samples. The RCTs of PEF-treated milk at 60°C were significantly longer ($p<0.05$) than RCTs of PEF-treated milk samples at 30°C indicating the combined effect of heat and PEF on RCT. The longest RCT belonged to LTLT (48.3 min) and HTST (43.4 min) pasteurisation of milk which was significantly different ($p<0.05$) from the control and PEF-treated milk samples at 30°C.

The RCTs of milk treated with PEF units at FSA and BUT showed an increasing trend from the lowest field intensity (35 kV cm^{-1}) to the highest (50 kV cm^{-1}) while temperature had an additive effect on extending RCT. Pasteurisation of milk conducted at FSA and DIL both resulted in the longest RCT compared to other treated samples. High-heat treated milk did not form any gel.

Table 5-2 Rheological and textural properties and rennet coagulation time of gels made at FSA and BUT from PEF-treated and control milk samples

a)	PEF treatment at FSA				Heat treatment		
	30 ℃		60 ℃				
Attributes	Control	38 kV cm^{-1}	Control	35 kV cm^{-1}	72 ℃/15 s	63 ℃/30 min	97 ℃/10 min
Avg. micelle size (nm)	168 ± 8^{a}	163 ± 5^{a}	165 ± 7^{a}	164 ± 6^{a}	169 ± 7^{a}	167 ± 6^{a}	187 ± 5^{b}
RCT (min)	$32.2^{a}\pm2$	$35.4^{a}\pm2$	$32.3^{a}\pm2$	$38.8^{b}\pm2$	$38.6^{b}\pm2$	$45^{c}\pm2$	No gel
G′ (Pa)	40 ± 3^{a}	24 ± 3^{c}	32 ± 3^{b}	26 ± 3^{c}	22 ± 1^{c}	11 ± 1^{d}	No gel
G″ (Pa)	14 ± 2^{a}	8 ± 1^{c}	11 ± 2^{b}	10 ± 1^{b}	8 ± 1^{c}	4 ± 1^{d}	No gel
Firmness (g)	69 ± 4^{a}	55 ± 2^{b}	65 ± 2^{a}	58 ± 3^{b}	40 ± 4^{c}	36 ± 1^{c}	No gel

b)	PEF treatment at BUT								Heat treatment	
	30ºC				60ºC					
Attributes	Control	40 kV cm^{-1}	45 kV cm^{-1}	50 kV cm^{-1}	Control	40 kV cm^{-1}	45 kV cm^{-1}	50 kV cm^{-1}	72ºC/15 s	63 ºC/30 min
RCT (min)	$35.4^{a}\pm2$	$36.7^{a}\pm2$	$38.6^{ab}\pm2$	$41.8^{b}\pm3$	$35.5^{a}\pm0.31$	$40.4^{b}\pm0.24$	$42.2^{c}\pm0.26$	$43.3^{c}\pm0.3$	$43.4^{c}\pm0.2$	$48.2^{d}\pm0.1$
G′ (pa)	$52^{a}\pm3$	$37^{b}\pm3$	$31^{c}\pm3$	$27^{c}\pm3$	$46^{a}\pm3$	$21^{d}\pm2$	$14^{e}\pm2$	$11^{c}\pm1$	$24^{c}\pm1$	$12^{e}\pm1$
G″ (pa)	$14^{a}\pm2$	$12^{a}\pm1$	$11^{a}\pm1$	$10^{b}\pm1$	$13^{a}\pm2$	$9^{c}\pm1$	$8^{c}\pm0.5$	$7^{c}\pm0.2$	$9^{c}\pm0.51$	$4^{d}\pm1$
Firmness (g)	$99^{a}\pm4$	$83^{b}\pm2$	$80^{b}\pm3$	$77^{b}\pm6$	$94^{a}\pm2$	$71^{b}\pm4$	$66^{c}\pm6$	$61^{c}\pm5$	$42^{d}\pm4$	$36^{e}\pm3$

The casein micelle size was not measured for the milk treated at BUT.

Different letters in each row in both tables depict a significant difference ($p<0.05$).

5.3.5 Textural Properties of Milk Gels

Figure 5-6 (a) shows the penetration force versus compression time of rennet-induced gels made from PEF and non-PEF treated milks at FSA. The 30℃ control milk formed the firmest gel (69 g) followed by the gel from 60℃ control (65 g). Treatment of milk at 38 kV cm^{-1} (30℃) and 35 kV cm^{-1} (60℃) resulted in a significantly lower (p<0.05) gel strength than control milks. However, the strength of gels from PEF treatments at both temperatures and field intensities were not significantly different (p>0.05). Compared to gels made from HTST and LTLT pasteurised milk, the gels made from PEF-treated milk were significantly (p<0.05) firmer.

Figure 5-6 (b & c) shows the strength of gels made from PEF and non-PEF treated milks with PEF unit at BUT. Similar to above results, the 30℃ control milk formed the firmest gel (99 g) followed by the gel from 60℃ control (94 g). Treatment of milk samples at 40, 45 and 50 kV cm^{-1} at 30 and 60℃ resulted in a significantly (p<0.05) softer gel with lower elasticity and viscosity (G', G'') than gels made from control milks. However, the gels made from PEF-treated milks were significantly (p<0.05) firmer than the gels made from HTST and LTLT pasteurised milk.

In both experiments above increasing the field intensity and temperature resulted in a softer gel; however, compared to gels from pasteurised milks, the gels from PEF-treated milks were firmer. The difference between the maximum gel strength in FSA and BUT trials could be due to the different breed of cow, milk protein content and milking season. High-heat treated skim milk formed no gel.

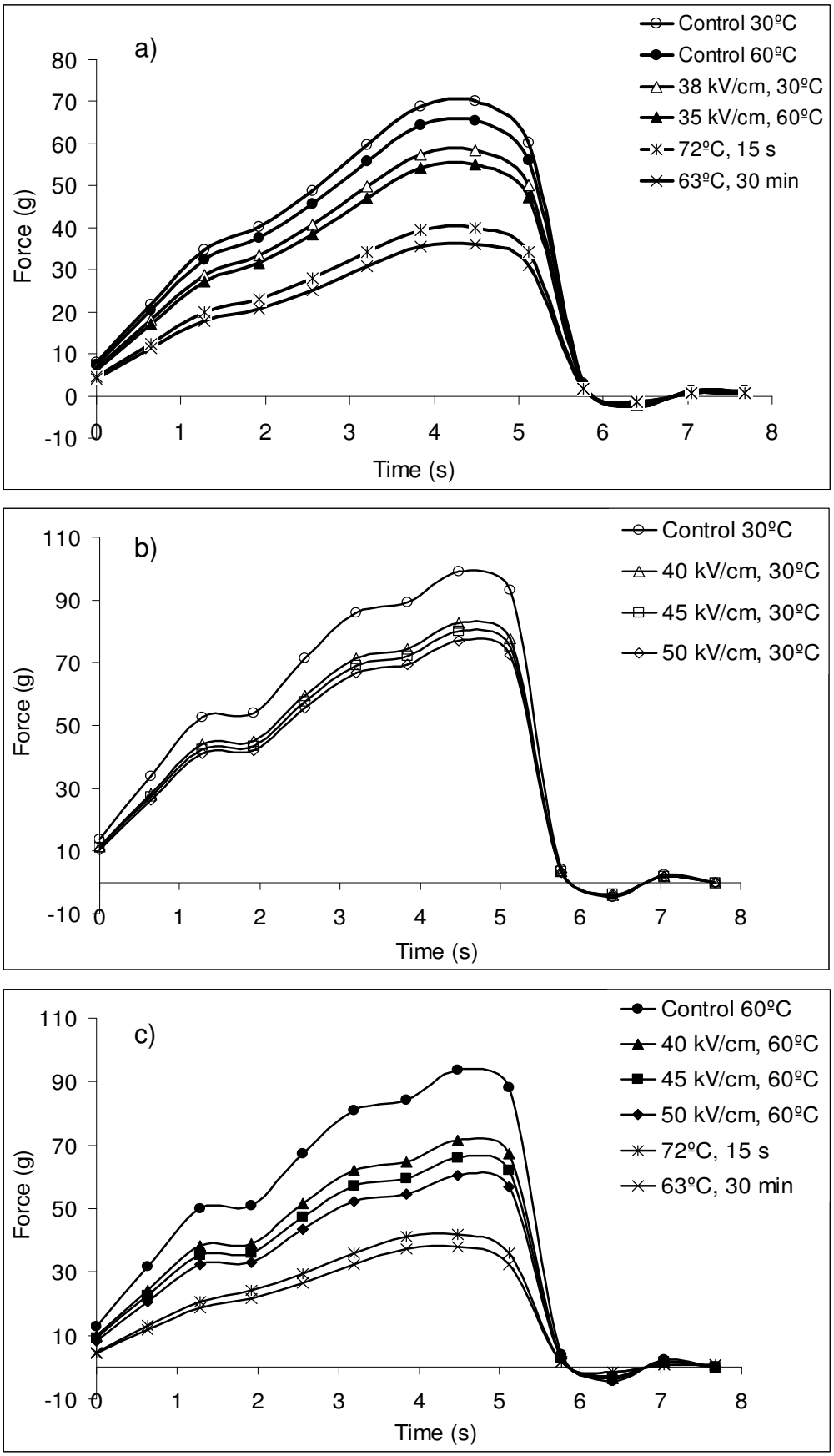


Figure 5-6 Firmness of gels made from PEF-treated and untreated skim milk samples.

The standard deviations of duplicate samples for different treatments were ±5 to ±9.

(a) PEF trials at FSA and (b & c) PEF trials at BUT.

5.3.6 Scanning Electron Microscopy of Milk Gels

The SEM micrographs of milk gels made from treated skim milks are presented in Figure 5-7. The samples were taken randomly from the gels and observed in the microscope with a magnification of ×4,000 with a scale bar of 5 µm. The gels matrix made from untreated 30°C and 60°C control skim milk (Fig. 5-7 a & b) had a similar structure in terms of pores' size indicating that holding milk for a few seconds at 60°C did not adversely affect the gel structure. The pores in the matrix of gels from PEF-treated milk became larger as the field intensity and temperature increased. The gels made from pasteurised milk samples showed the largest pore size compared to all other samples. The electron microscopy was conducted at DIL on the gels made from PEF-treated milk with PEF unit at BUT.

5.4 DISCUSSION

The casein micelle size in the PEF-treated and pasteurised samples was measured only at FSA in September 2007 and did not show any significant change (p>0.05) (165-168 nm) while high-heat treatment of milk resulted in a significant increase in the casein micelle size (185 nm). However, the percentage of the size distribution difference was only 1.8%. The findings of this study are inconsistent with Floury *et al*. (2006b) who reported a decrease in the casein micelle size from 215 nm to 185 nm following the PEF treatment at 45-55 kV cm^{-1} (T < 50 °C) with a total treatment time of 2.1 - 3.5. This inconsistency could be attributed to the greater difference in the field intensities used in both studies (45-55 kV cm^{-1} vs. 35 and 38 kV cm^{-1}). The size of casein micelles measured in the current study was below the range measured by Floury *et al.* (2006b) and Daviau *et al.* (2000).

The composition and casein micelle size varies significantly due to different geographical location, lactation period and genetics of cows (Fox, 1982; Auldist *et al*., 2002). The shape of casein micelles as observed by electron microscopy is spherical, ranging in size from 50-500 nm in diameter (average about 120 nm) and a molecular mass from 106-109 Da (Fox and McSweeney, 1998).

The mechanism of PEF impact on casein micelles is still unclear although Floury *et al*. (2006b) suggested an apparent charge modification of micelles after exposure to intense electrical field resulting in a reduction in hydrodynamic volume of casein micelles. The increased casein micelle size after heat treatment of milk observed in the current study is reported to be due to aggregation of heat-denatured whey proteins at the surface of casein micelles via the β-lg/κ-casein complex formation (Singh and Latham, 1993; Fox, 1982; Beaulieu *et al*., 1999; Mohammad and Fox, 1987; Dalgleish *et al*., 1987).

In relations to the impact of PEF on fat globule size, no significant difference (p>0.05) was found between the fat globule size of control, PEF or LTLT-treated milk samples (Fig. 5-3). However, a marginal reduction in fat globule size was observed in HTST and high-heat treated milk samples with a drop from *ca.* 3.2 µm to 2.7 that increase the percentage distribution of this size by 2%. Fat globules in milk are surrounded by a thin layer of surface active membrane material consisting of a complex mixture of proteins, glycoproteins, triglycerides, cholesterol and enzymes. Exposing milk to heat above 70ºC causes the denaturation of the cryoglobulins resulting in size reduction of fat globules (Fox and McSweeney, 1998; Mather, 2000; Je Lee and Sherbon, 2002).

The effects of PEF treatment on rheological properties of the gels were more obvious compared to the impact of PEF treatment on fat globule and casein micelle size. The elasticity (*G´*) and viscosity (*G´´*) of gels made from milk treated at field intensities of 40, 45 and 50 kV cm^{-1} were significantly lower (p<0.05) than those made from milks treated at 35 and 38 kV cm^{-1} regardless of the heat applied, indicating the effect of field intensity alone on the structure of milk gel (Fig. 5-4 & 5-5). In view of the observed similarities in physical attributes of gels made from PEF and heat-treated milks, it is postulated that PEF treatment may cause similar changes in milk proteins as the heat treatment. Therefore, the formation of less elastic and less viscous gels was attributed to the possible aggregation of the denatured whey proteins with κ-casein that hinders the coagulating action of the rennet (Shalabi and Wheelock, 1976; Van Hooydonk *et al*., 1987). Raising temperature during PEF treatment to 60°C had an additive effect on further lowering the *G´* and *G´´* of milk gel.

Perez and Pilosof (2004) reported aggregation and denaturation of β-lactoglobulin after PEF treatment at 12.5 kV cm^{-1}, using 3-10 exponential decaying pulses of 2-2.3 ms, as shown by differential scanning calorimetry and SDS-PAGE analyses. However, a study by a different group showed that PEF treatment of skim milk at 29 kV cm^{-1} using 200 exponentially decaying pulses did not cause marked unfolding and aggregation of β-lactoglobulin (Barsotti *et al*., 2002).

In contrast, Sepulveda *et al.* (2000) and Floury *et al.* (2006) reported that the rennet induced gels of PEF-treated skim milk were firmer than those of the non-PEF controls. Although Sepulveda *et al.* (2000) did not offer a hypothesis for their

observation, Floury *et al.* (2006) suggested that the reduction in casein micelle size by PEF treatment may play a role.

The *G´* and *G´´* of gels made from PEF-treated milk at 35 and 38 kV cm^{-1} (at both temperatures) were not significantly different ($p>0.05$) from *G´* and *G´´* of gels made from HTST pasteurised milk (Fig. 5-4). However, the gels made from milks treated at 40, 45 and 50 kV cm^{-1} and 60°C were significantly less elastic ($p>0.05$) than gels made from HTST pasteurised milks (Table 5-2 a & b). Inversely, gels made from PEF-treated milk at 30°C and 40, 45 and 50 kV cm^{-1} had significantly ($p<0.05$) higher *G´* and *G´´* compared to HTST pasteurisation. Low temperature long time pasteurisation of milk resulted in the weakest gel compared to all other gels. Long exposure of milk to heat had an adverse effect on gel structure due to denaturation of whey proteins and their aggregation with casein micelle which hinders the coagulation process resulting in a soft gel (Dalgleish, 1990; Omar, 1985).

Regardless of the difference in the protein contents of the two lots of milk used at FSA and BUT trials (3.15 and 3.32%, respectively) and differences in the PEF systems configurations and field intensities used, the viscoelastic and textural properties of gels made from PEF-treated milks demonstrated an inverse relationship with increased field intensity. The milk used for the PEF trials at FSA formed a gel with lower rheological and textural properties compared to the gels made from the milk used at BUT trials which could be attributed to differences in the milk seasonality, its protein and total solids content and breed of the cows (Fox and McSweeney, 1998).

The RCT of milks treated at field intensities of 35 and 38 kV cm^{-1} (30 and 60°C) was significantly shorter (p<0.05) than those treated at 40, 45 and 50 kV cm^{-1} at both temperatures. Raising PEF treatment temperature of milk to 60°C resulted in a significant increase (p<0.05) of RCT in both sets of PEF trials (Fig. 5-4 and 5-5). However, compared to LTLT pasteurisation, PEF treatment resulted in a significantly (p>0.05) shorter RCT while the RCT of HTST milk was not significantly different (p>0.05) from the 60ºC trials. The long exposure time of milk to heat denatures the whey proteins and aggregates them with casein micelle resulting hindered coagulation process (Renault *et al*., 2000). This explains why the gel from LTLT-pasteurised milk had the longest RCT while 60°C control gel had an RCT close to that of 30°C control. The results showed that the RCT was affected by PEF treatment and/or heat. In cheese making, long RCT results in an increase in the water holding capacity of the curd (affected by denatured whey proteins) and producing a cheese with a higher moisture content which may not be suitable for producing quality cheddar or similar cheeses (Fox, 2004; Floury *et al*., 2006b; Johnson *et al*., 2001). In this study, the protein content of milk used at FSA trials was 5% less than that used at BUT trials which may have contributed to the changes observed in RCT.

The increase in the RCT of skim milk after PEF treatment obtained in the current study is in contrast to the findings of Floury *et al.* (2006) who reported that the RCT was reduced as a result of the PEF treatment. The definition of RCT used by Floury *et al.* (2006) was different from that of RCT used in the current study, and was based on a more primitive formagraphic method described by Kopelman and Cogan (1976) and McMahon and Brown (1982). Despite the difference in

definition, the reduced RCT reported by Floury *et al.* (2006) was consistent with the reduction in casein micelle size and enhanced gel firmness reported.

The results of texture analyser were consistent with the rheology findings. In both sets of trials at FSA and BUT, there was a significant difference (p<0.05) between the firmness of gels made from 30 and 60ºC control milks and the gels made from PEF-treated milks. The gel firmness showed an inverse relationship with increasing field intensity (35, 38, 40, 45 and 50 kV cm^{-1}) and treatment temperature (30 and 60ºC). According to Floury *et al.* (2006b) only PEF treatments above 40 kV cm^{-1} could modify the casein micelles behaviour. However, based on the results of the current study there were still measurable changes in textural and rheological properties of gels made from milks treated at field intensities below 40 kV cm^{-1} which were intensified by increasing the field intensities to 40, 45 and 50 kV cm^{-1}. The decrease in firmness of gels made from PEF or heat-treated milks is (similar to rheological changes) due to hindering the coagulation process since the denatured whey proteins aggregate with casein micelles and hamper the action of rennet enzyme on cleaving κ-casein (Fox and McSweeney, 1998) which could result in a less firm gel compared to the gels made from control 30 and 60ºC milks. Although the PEF treatment lowered the gel firmness (due to the combined effects of heat and PEF treatment), the gels were still firmer than those made from pasteurised milks which indicated that the PEF impact on gel structure was less pronounced compared with that of pasteurisation. Low temperature long time treatment of milk resulted in the least firm gel due to longer exposure time of milk to heat.

Scanning electron microscopy of gels provided clear micrographs for observation of changes to gel matrix before and after thermal and PEF treatments (Fig. 5-7). The gels made from 30 and 60ºC control milks had a dense structure (Fig. 5-7 a & b). By increasing the field intensity from 40 to 45 and 50 kV cm^{-1} and temperature from 30 to 60ºC the pore size became larger. The pores are in fact the sublimated gel matrix which were once filled with trapped whey during gel formation. Large pores are the result of weaker interactions between the casein micelles making up the gel network resulting in a lower *G'*, *G"* and firmness and a gel that is easier to fracture. They also indicate that heat (or as in this study PEF treatment alone or combined with heat) has considerably rearranged the gel matrix (Lucey *et al*., 2001) compared to gels made from control milks since the size of the pores in the former gels have become much larger. The size of the pores indicates the water holding capacity of the gel matrix which increases by the extent of whey protein denaturation. Compared to PEF effects, the impact of heat on gel structure was much more pronounced as the micrographs indicated much larger pores in the structure of gels made from HTST and LTLT-pasteurised milks. Figure 5-7 (I & J) shows that the effects of higher field intensities (>45 kV cm^{-1}) at 60ºC on gel matrix is similar to thermal pasteurisation. Based on the results of the textural analyses PEF treatment could result in less damage to gel structure thus resulting in a firmer gel compared to gels made from pasteurised milks.

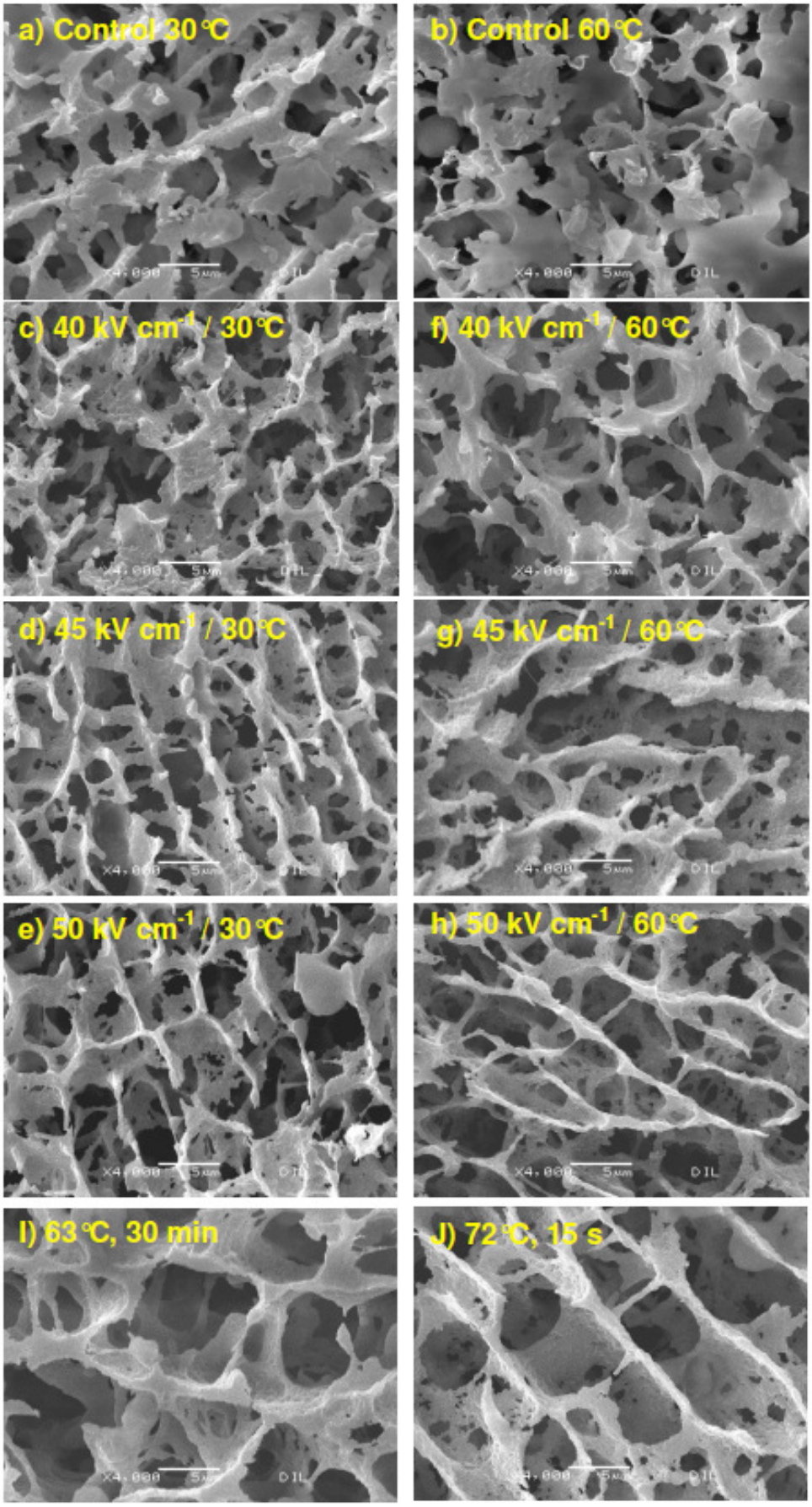


Figure 5-7 SEM micrographs of rennet-induced milk gels made from PEF-treated and untreated skim milk.

5.5 CONCLUSIONS

This study revealed that PEF treatment of milk at ≥ 35 kV cm^{-1} and 30 or 60ºC did not affect casein micelle size nor fat globule size while high-heat treatment of milk increased the casein micelle size and decreased the fat globule size significantly. The effect of HTST pasteurisation on fat globule size was similar to high-heat treatment. The extent of rheological and textural changes caused by PEF treatments of milk and increasing RCT was much less pronounced than those caused by LTLT and HTST pasteurisation. Therefore, based on the results of this study PEF treatment of milk if applied below field intensity of 50 kV cm^{-1} and 60ºC can supersede thermal pasteurisation in terms of rendering better rheological and textural properties in rennet-induced milk gels.

CHAPTER 6

GENERAL DISCUSSION

The effects of PEF on microbial and enzymatic inactivation has been extensively studied over the last two decades and numerous research groups in different countries have tried to make the PEF treatment more applicable to a wide range of products such as fruit juices, egg pulp, milk, water and even semi-solid foods like sausages meat. However, the only industrial application of PEF emerged in 2005 when Genesis Juice Cooperative (325, West Third Suite B, Eugene Oregon, 97401 USA) started to use PEF treatment for cold pasteurisation of fruit juices such as apple, apple-strawberry, carrot, carrot-celery-beet, herbal tonic, strawberry lemonade and ginger lemonade. The company has recently abandoned using PEF technology for unknown reasons and presently is employing high pressure technology to “cold-pasteurise” its natural fruit juices.

Pulsed electric field is potentially an alternative to thermal pasteurisation and is capable of providing microbiologically-safe products with longer shelf-life, fresh-like quality and nutritional properties. However, to date a number of contradictory results regarding the inactivation of enzymes and microorganisms have been reported which arise from the lack of a standard procedure for treating various products. The versatility in the number and type of PEF units and treatment chambers which are often custom-designed by the research groups have added to the variability of the reported results. The relatively high cost of the installations including pulse generators and discharge switches, control of heat generation

within the system, and concerns with the survival of spores are among the hurdles in the way of adopting PEF technology as a processing method of choice for milk and dairy products. As a guide, the cost of a bi-polar 400 L h^{-1} pilot PEF system is approximately US$ 250,000, while the commercial PEF system with higher capabilities and process volume is US$ 500,000 (Gaudreau *et al*., 2008). Nonetheless, research is ongoing to make the PEF treatment industrially and commercially viable.

There are a number of technical hurdles to overcome in achieving successful application of PEF technology at industrial scale such as designing treatment chambers with maximum throughput and minimum ohmic heating (which can adversely affect heat-sensitive products) as well as building highly specific pulse generators and switches capable of handling high voltages, and developing corrosion-resistant electrodes (Fox *et al*., 2007).

Considering these challenges, more research is necessary to make PEF applicable in dairy industry and to achieve a higher rate of enzymatic and microbial inactivation in milk more research. Currently, PEF systems used for treatment of food products are limited to the laboratory or pilot scale systems.

Pulsed electric field has a number of shortcomings which must be taken into account in future research. The following list gives a few of these hurdles:

1. The presence of air bubbles within the treatment chambers may lead to non-uniform treatment as well as operational and safety problems. Milk for

instance could have up to 7% dissolved air which could be released in the form of micron-size bubbles and disrupts the electric field.

2. Change in product conductivity at various temperatures can increase the electric current flow through the product resulting in increased total specific pulsing energy. It may also lead to creation of two different field intensities for the same input voltage.
3. The particle size of the food containing particulate matters could cause system malfunction. The maximum particle size in the liquid must be smaller than the gap between the electrodes in the treatment chamber in order to maintain a proper flow of the product, and there should be no clumping of particles together (Barbosa-Canovas, 2000).
4. Control of temperature in every stage of treatment is essential in a successful PEF operation for the following reasons:
    a. Rise of temperature during PEF operation above a certain temperature (e.g. 60ºC) can lead to microbial and enzymatic inactivation by thermal effect rather than PEF effect and defies the purpose of nonthermal processing
    b. Sudden rise in treatment temperature can result in coagulation of proteins due to their sensitivity to heat and deposit formation on the electrodes surface leading to obstruction of treatment chamber (Zhang *et al.*, 1995).
5. High costs of electrodes since they are usually made from expensive inert metals (such as platinum) resistant to chemical and electrolytic corrosion.
6. Designing treatment chambers with optimum throughput, minimal ohmic heating and high-voltage discharge switches is costly (Fox *et al.*, 2007).

Despite these shortcomings, PEF has been shown to be effective in eliminating pathogens and spoilage organisms and capable of producing safe fresh-like foods. Thus it seems to be a promising alternative for traditional thermal methods in cold pasteurisation of the liquid foods (Barbosa-Canovas *et al*., 1998).

6.1 CONCLUSIONS

Based on the findings of this project it could be concluded that:

Applying field intensities of 25 to 37 kV cm^{-1} at a final temperature of 30°C resulted in 24-42% inactivation of AlP and <1 log reduction in TPC and *Pseudomonas* count, while the *Enterobacteriaceae* count was reduced by at least 2.1 logs below the detection limit of 1 CFU mL^{-1}. Pulsed electric field was shown to be more effective when temperature was raised to 60°C since AlP was inactivated by 29-67% while *Pseudomonas* and *Enterobacteriaceae* counts were reduced by at least 5.9 and 2.1 logs CFU mL^{-1}, respectively. Total plate count reduction was due to temperature effect rather than the effect of PEF. Fitting AlP inactivation data to kinetic model suggested that the slope of the AlP inactivation line at 60°C was more than twice of that at 30°C indicating an additive effect between the field intensity and heat in the inactivation of both AlP and the microflora in raw skim milk. As the AlP inactivation was not complete under the experimental conditions of this project, it was considered to be an unsuitable indicator for PEF treatment adequacy of milk. However, optimisation of PEF treatment conditions might achieve reasonably high inactivation level of AlP.

Enzyme inactivation could be increased by using different combinations of heat, field intensity and treatment time. However, at temperatures ≥65°C heat becomes the dominant inactivating factor rather than PEF. It was also demonstrated that the

flow rate was an important processing parameter in the enzyme inactivation and the most effective way of controlling the treatment time for maximum enzyme inactivation could be achieved by controlling the flow rate. It was also shown that AlP was the most sensitive enzyme to combined heat and PEF treatments followed by lipase, XO and plasmin, and that even AlP could not be inactivated completely in a moderate PEF treatment temperature of 60℃. However, at both field intensities and 65℃, AlP was inactivated >90% which could make it as a suitable indicator for PEF treatment at the given experimental conditions.

The study on rheological and textural properties of rennet-induced milk gels and fat globule size indicated that PEF treatment compared to LTLT or HTST pasteurisation had less adverse effects on the physical properties of the gel. Although PEF treatment reduced viscoelastic properties and firmness of rennet-induced gels and increased the RCT, the extent of rheological and textural changes was much less pronounced than those caused by LTLT and similar to HTST pasteurisation. Pulsed electric field did not result in any change in casein micelle or fat globule size while high-heat treatment increased the casein micelle size and decreased the fat globule size. Scanning electron microscopy of gels made from PEF-treated milks showed a more stable structure with a higher viscosity, elasticity and firmness compared to gels made from pasteurised milk.

6.2 RECOMMENDATIONS

This study generated interesting findings on the application of PEF technology to milk. However, to facilitate the realisation of the full potential of PEF treatment of milk the following issues need additional research:

1. Alkaline phosphatase was found to be an unsuitable indicator for adequacy of PEF-treatment at 60ºC but a suitable indicator at 65ºC. However, further tests on milk pathogenic microorganisms are needed to confirm inactivation of pathogens in conjunction with AlP. None of the other enzymes investigated in this study are considered suitable indicator because they were even more resistant to combined heat and PEF treatments than AlP. Further research is needed to either optimise PEF treatment conditions or to find a biological or chemical indicator as an index of PEF treatment efficiency.

2. To improve the bactericidal efficiency of PEF treatment, apart from application of higher field intensities (above 40 kV cm^{-1}), investigation on other parameters such as total specific pulsing energy, pulse frequency or flow rate may improve the microbial or enzymatic inactivation.

3. Temperature rise during PEF treatment is an important issue to be considered. The temperature in the electrode zone needs to be measured by using sensitive thermocouples to give an estimation of heat generation in the PEF treatment zone.

4. Further studies are needed on effects of PEF on microbial protease or lipase since there are only few studies conducted on these enzymes and even in the current study the focus was on the native enzymes in milk.

5. Further investigations on the effects of field intensities of 40, 45 and 50 kV cm^{-1} on casein micelle size and whey proteins denaturation can better elucidate the impact of PEF treatment on these proteins.

6. Pulsed electric field could be employed to pre-treat milk prior to ultra-high treatment to achieve a higher inactivation of plasmin in milk since this enzyme could result in proteolysis of UHT milk after a few months.

7. Gel electrophoresis to be conducted to investigate the protein structure of milk gel after PEF or heat treatment.

8. It is relevant to measure the zeta potential in casein micelles before and after PEF treatment of milk to investigate the effect of field intensity on the hydrodynamic status and aggregation of the micelles.

9. Since plasmin is generated by plasminogen, it is of great importance to measure the activity of plasminogen after PEF treatment of milk.

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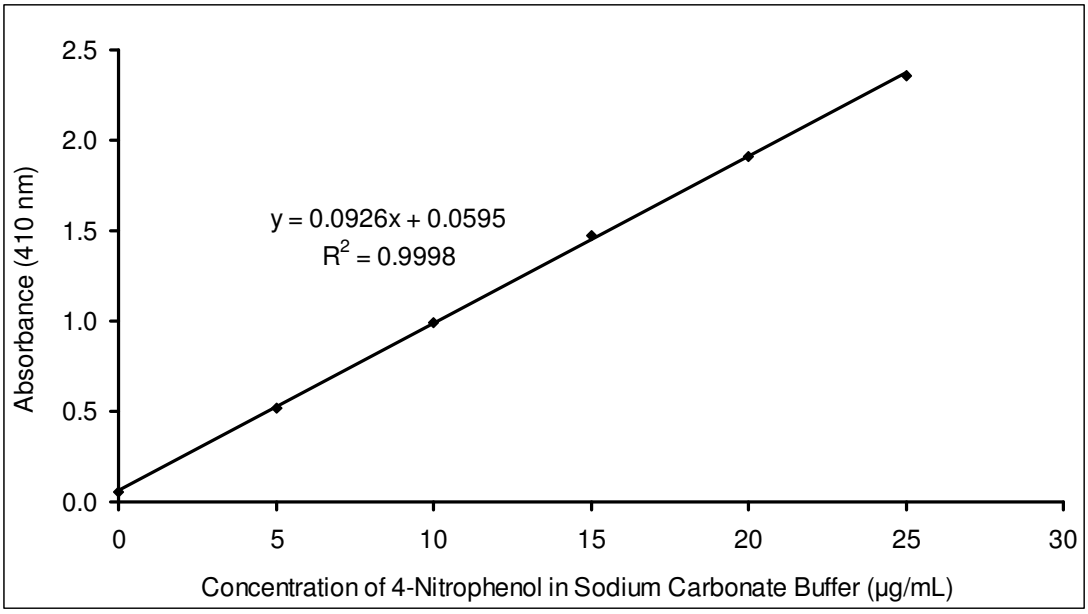
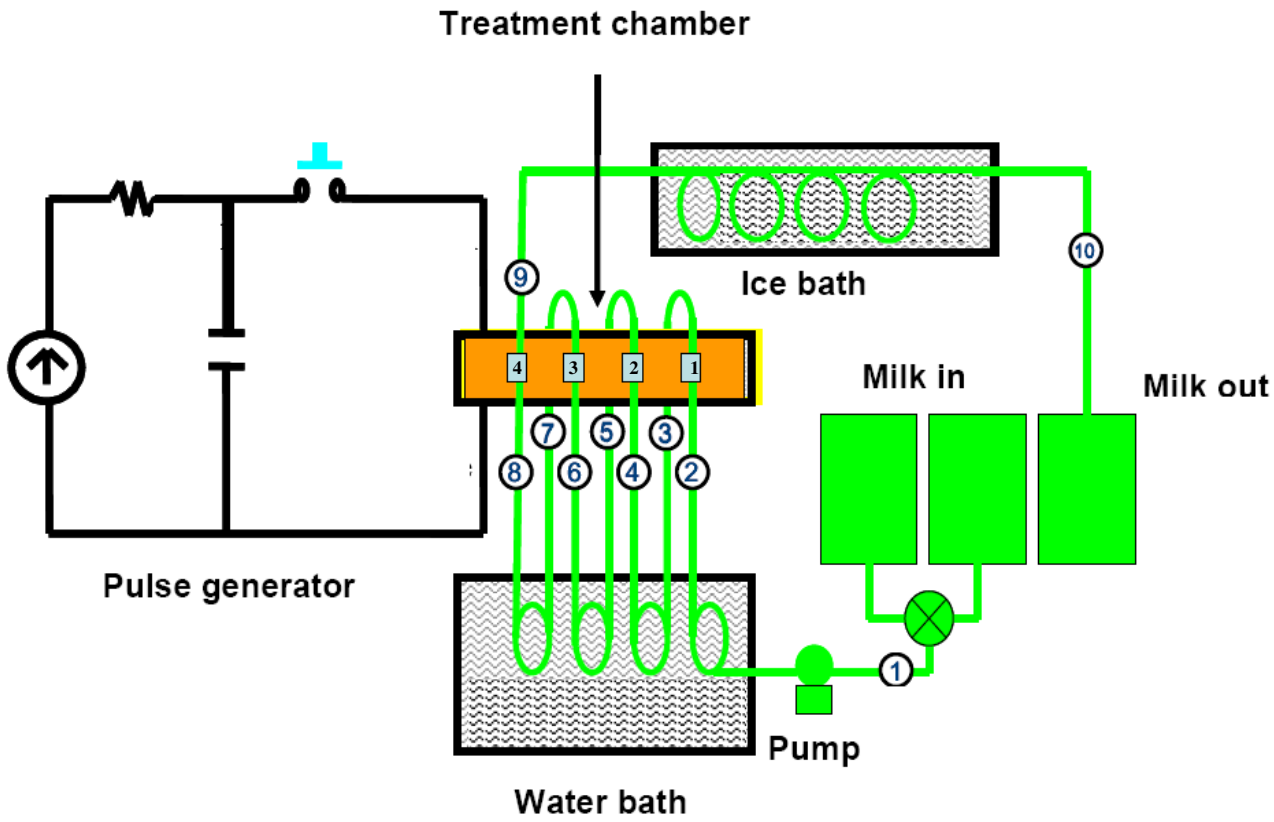
APPENDICES

Appendix 1. Standard Curve for Alkaline Phosphatase

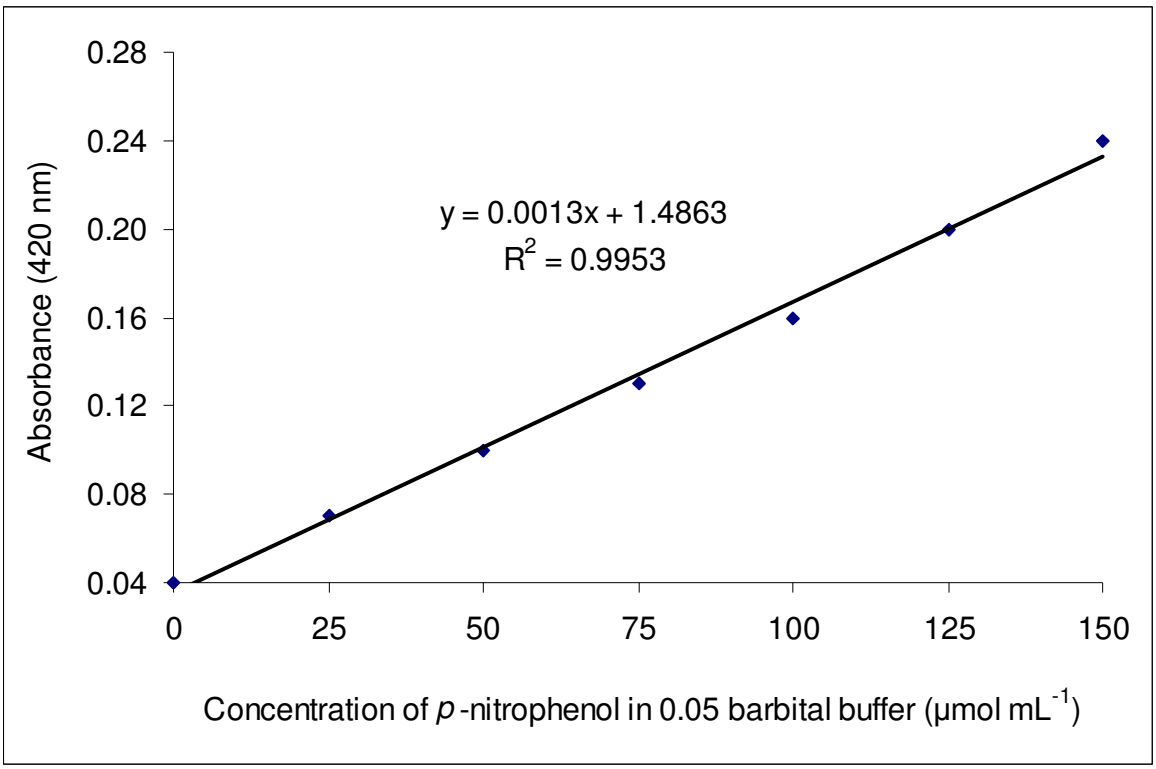


Appendix 2. Schematic diagram of the PEF unit at Food Science Australia, with Numbers 1 to 10 representing the 10 in-line thermocouples referred to as T1 to T10. From T1 to T9 the exposure time of milk to heat was 100 s before it enters the ice bath.

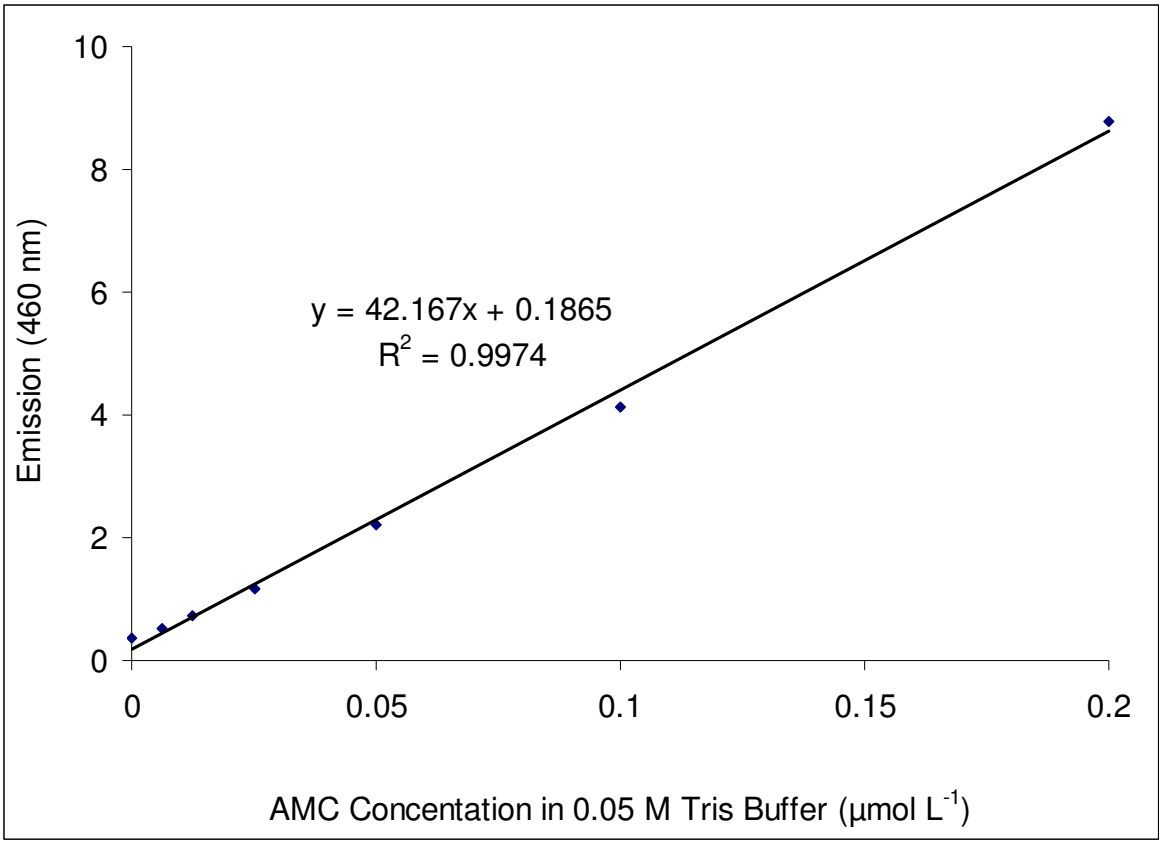


Source: Sui *et al.*, 2008

Appendix 3. Standard Curve for Total Lipase



Appendix 4. Standard Curve for Plasmin



Appendix 5. Calculation of Reynolds Number

Reynolds number for the tubing and treatment chamber was calculated from the following formula:

$$Re = \frac{\rho V D}{\mu}$$

Where ρ is the fluid density (g mL^{-1}); V is fluid velocity (cm s^{-1}); D is the chamber or tubes diameter (cm) and μ is the fluid viscosity dynamic (Pa·s). The viscosity of skim milk at 30 and 60°C has been calculated by Ruhlman *et al.* (2001). The constant parameters were as follows:

- D (tubing) and treatment chamber = 0.4 and 0.23 cm, respectively.
- μ for skim milk at 30 and 60ºC = 0.01881 and 0.00767 Pa.s, respectively.
- ρ for skim milk at 30 and 60ºC = 1.03 and 1.02 g mL^{-1}, respectively.
- Area of cross section of tubing and chamber = 0.1256 and 0.0415 and cm^2, respectively ($A = л \times r^2$).

The fluid velocity (V) was calculated from the following equation by Ruhlman *et al.* (2001):

$$V = \frac{Flow\ rate\ (mL\ s^{-1})}{Chamber\ area\ (cm^2)}$$

Therefore, for flow rates of 30, 60, 120 and 240 mL min^{-1} through tubing the V was calculated as 3.98, 7.96, 15.92 and 31.84 cm s^{-1}, and for treatment chamber as 12.041, 24.081, 48.162 and 96.324 cm s^{-1}, respectively.

As an example, the Re number at 30 mL min^{-1} for treatment chamber was calculated as: $Re = 1.03 \times 3.98 \times 0.23 / 0.01881 = 152$; and for tubing $Re = 1.03 \times 3.98 \times 0.4 / 0.01881 = 87$.

By changing the flow rate and μ, the Re number was calculated for each flow rate and temperature.