APPLICATION OF PULSED ELECTRIC FIELD TREATED MILK ON CHEESE PROCESSING: COAGULATION PROPERTIES AND FLAVOR DEVELOPMENT

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ABSTRACT

Raw milk cheeses have unique flavor and texture not obtainable in cheeses from pasteurized milk. However, cheeses made from pasteurized milk are widespread for public health reasons. Pulsed electric field (PEF) treatment as a non-thermal pasteurization method has shown its advantage over conventional heat processing. Understanding the effect of PEF on cheese making is crucial. This study firstly determined the effect of fat content in milk on the microbial inactivation by PEF treatment. Fat content showed different behavior on microbial inactivation by PEF at different temperature levels. At 5 to 35°C, milk fat content did not affect the microbial inactivation by PEF. However, from 45 to 55°C, microbial reduction in whole milk was lower than that in skim and 2% fat milk. Secondly, this study investigated the effects of PEF parameters and temperature on the inactivation of pathogenic microorganisms (*E. coli* O157:H7 and *Salmonella enteritidis*) in whole milk. PEF treatment indicated effective inactivation of *E. coli* O157:H7 and *S. enteritidis* in whole milk. The maximum reduction of *E. coli* O157:H7 and *S. enteritidis* was 4.1 and 5.2 logs, respectively at 30 kV/cm and 50°C. The inactivation kinetics for both bacteria was primarily exponential, except in some cases with some tailing. *E. coli* O157:H7 in whole milk was more resistant to heat-PEF treatment compared to *S. enteritidis*. Further on, this study determined the effects of PEF and temperature on the rennet coagulation properties (curd firmness, CF, and rennet coagulation time, RCT) of raw milk using the rheological approach. PEF treated milk showed better rennetability compared to thermally pasteurized milk in terms of CF and RCT. Finally, this work investigated the effects of PEF and temperature on the ripening properties (proteolysis) of raw milk cheese curd using the RP-HPLC technique and Cd-Ninhydrin method. Peptide and free amino acid analysis showed that PEF treated milk could give similar flavor to cheddar cheese as raw milk, superior to that of pasteurized milk. These indicated that PEF treatment could have a chance to supplement or replace traditional pasteurization process with minimum impact on cheese quality.

RÉSUMÉ

Les fromages au lait cru ont une saveur et une texture uniques que l'on ne retrouvent pas dans les fromages de lait pasteurisé. Toutefois, les fromages au lait pasteurisé sont plus répandus pour des raisons de santé publique. Les champs électriques pulsés (CEP) sont un traitement non thermique de pasteurisation qui a démontré son avantage sur les méthode classiques de traitement thermique. Comprendre l'effet des CEP sur le fromage est crucial. Cette étude a d'abord déterminé l'effet de la teneur en matière grasse dans le lait sur l'inactivation microbienne par traitement CEP. La teneur en matière grasse a montré un comportement différent sur l'inactivation microbienne par CEP à différents niveaux de température. De 5 à 35°C, la teneur en matière grasse du lait n'a pas d'incidence sur l'inactivation microbienne par CEP. Toutefois, de 45 à 55°C, la réduction microbienne dans le lait entier a été plus faible que dans le lait écrémé à 2% de matière grasse. Deuxièmement, cette étude a étudié les effets des paramètres du CEP et de la température sur l'inactivation de microorganismes pathogènes (*E. coli* O157:H7 et *Salmonella enteritidis*) dans le lait entier. Les CEP peuvent être des traitements efficaces d'inactivation de *E. coli* O157: H7 et *S. enteritidis* dans le lait entier. La réduction maximale de *E. coli* O157: H7 et *S. enteritidis* a été de 4.1 et 5.2 logs, respectivement à 30 kV / cm et 50°C. La cinétique d'inactivation des deux bactéries a été principalement exponentielle, sauf dans certains cas avec certains résidus. *E. coli* O157: H7 dans le lait entier est plus résistant à la chaleur et au traitement CEP comparé à *S. enteritidis*. En outre, cette étude a déterminé les effets de la CEP et de la température sur les propriétés de coagulation (fermeté du caillé, FC, temps de coagulation avec présure, TCP) du lait cru à l'aide de la méthode de détermination des caractéristiques rhéologiques. Le lait traité par CEP a montré un meilleur emprésurage thermique par rapport au lait pasteurisé. Enfin, ce travail a enquêté sur les effets de la CEP et de la température sur la maturation des propriétés (protéolyse) de fromage au lait cru à l'aide de la technique d'analyse RP-HPLC et de la méthode Cd-ninhydrine. Les peptides et acides aminés libres ont révélé que, le lait traité au CEP pouvait donner une saveur de fromage cheddar de lait cru, supérieure à celle du lait pasteurisé. Ces CEP ont indiqué que le traitement pourrait avoir

une chance de compléter ou de remplacer les processus de pasteurisation avec un minimum d'impact sur la qualité du fromage.

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CONTRIBUTION OF AUTHORS

The role and contribution made by different authors are as follows: The principal author is Li Juan Yu. She is the Ph.D. candidate who planned and executed all the experiments, data analysis and wrote the manuscript for scientific publications. Dr. Michael Ngadi is the thesis supervisor, who guided the candidate in the planning and execution of experiments and analysis of data during the course of the entire program. He corrected, edited and reviewed all the manuscripts sent for publication. Dr. Vijaya Raghavan contributed in planning and execution some aspects of the project. Dr. James Smith also contributed in planning and execution some aspects of the project. He allowed the candidate to use his laboratory and contributed in editing manuscripts for scientific conferences.

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CHAPTER I: GENERAL INTRODUCTION

Cheese is a popular dairy product. The world production of cheese is roughly 15 x 10^6 tons per year (about 35% of total milk production) and has increased at an average annual rate of about 4% over 30 years (Fox et al. 2000). Canada produces more than 450 different varieties of fine cheeses. In 2005, Canadian cheese production increased to 379, 286 tons, 24% higher compared to 1994 (CFIA 2007).

Raw milk cheeses possess unique flavor and texture characteristics not obtainable in cheeses from pasteurized milk. However, raw milk cheeses have been involved in the majority of cheese related illness outbreaks (IFST 1998). *Salmonella* spp. and *Escherichia coli* O157:H7 are among the most important pathogens found in contaminated cheese products. *Escherichia coli* O157:H7 has been implicated in sporadic outbreaks of hemorrhagic colitis and hemolytic-uremic syndrome in humans. *Salmonella enteritidis* may contaminate milk and cheese products and cause salmonellosis in humans (Fox et al. 2000).

The control of these microorganisms is the most important issue in the handling and manufacturing of milk and cheese products. In most countries, raw milk must be pasteurized as required by strict regulations. Conventional heat pasteurization could be accomplished by two schemes: low temperature long time (LTLT 63 – 65°C, 30 min) and high temperature short time pasteurization (HTST 72°C, 15 s) (Fox et al. 2000). However, apart from microbial inactivation, heat treatment can adversely affect the flavor, taste and nutrients of the product. Therefore, applying non-thermal pasteurization, such as pulsed electric field (PEF) in cheese making is attracting attention.

PEF involves the application of high voltage pulses at relatively low temperature to a food placed between two electrodes for very short time (normally less than 1 second). Inactivation of microorganisms exposed to PEF results from electromechanical destabilization of the cell membrane (Zimmermann 1986; Castro et al. 1993) due to the high voltage pulses, which destroy unwanted bacterial cells without significant generation of heat (Barbosa-Cánovas et al. 1999). Successful application of PEF depends on many factors such as electric field intensity, treatment temperature, target microorganisms and food systems.

A number of studies on milk pasteurization by PEF have been carried out (Dunn and Pearlman 1987; Zhang et al. 1995b; Grahl and Markl 1996; Martin et al. 1997; Calderon-Miranda et al. 1999; Dutreux et al. 2000; Fernandez-Molina 2001; Evrendilek and Zhang 2005). These studies have demonstrated that higher microbial inactivation could be obtained with the combination of PEF treatment and mild heat. However, there are still some challenging works in this area.

For example, the influence of milk ingredients, such as milk fats on microbial inactivation by PEF needs to be clarified. Some investigators observed a protective effect of milk fats (Grahl and Markl 1996; Picart et al. 2002) on milk bacterial inactivation by PEF, whereas others did not report the effect of milk fat (Reina et al. 1998; Dutreux et al. 2000; Manas et al. 2001; Sobrino-Lopez et al. 2006) in milk and buffer systems.

Also, whole milk is commonly used for cheese making; therefore, the microbial inactivation study by PEF in whole milk is important. However, most research works in this area are focused on skim milk or reconstituted milk. So the

inactivation of both *Salmonella enteritidis* and *Escherichia coli* O157:H7 in whole milk by PEF in combination with mild heat is necessary.

Further on, the information of PEF effect on cheese making is very limited. Cheeses from PEF pasteurized milk have been investigated by Dunn (1996) and Sepulveda-Ahumada et al. (2000). Dunn (1996) reported that milk treated with PEF suffered less flavor degradation. The author proposed the possibility of manufacturing dairy products such as cheese, butter and ice cream using PEF treated milk. Sepulveda-Ahumada et al. (2000) evaluated the quality of cheese produced from PEF treated milk in terms of sensory and texture evaluation. The authors claimed that using milk pasteurized by PEF to make cheese appeared to be a feasible option to improve the product quality in terms of texture and sensory aspects.

Cheese processing is a complex process involving many steps. The rennet coagulation of milk is the first and most important step in cheese making process. Curd Firmness (CF) and Rennet Coagulation Time (RCT) are among the primary coagulation properties that influence cheese quality, yield and economic returns (Fox et al. 2000). Knowledge of PEF effects on these factors is extremely necessary for understanding the structure changes in cheese made from PEF treated milk.

Cheese ripening is another crucial step in cheese processing. It involves a complex series of biochemical events. Major biochemical changes occurring in cheese ripening include proteolysis, glycolysis and lipolysis. Proteolysis is considered the most important issue in terms of flavor development. Information of PEF effects on cheese proteolysis is needed in order to understand the flavor development profile of cheese made from PEF treated milk.

The hypothesis of this study was that cheese made from PEF treatment milk had similar flavor development profile as raw milk. Based on the hypothesis, the overall objective of this study was to evaluate the potential of producing consumer-safe cheddar cheese with raw milk like flavor characteristics through PEF processing. The specific objectives were as follows:

1. To verify the effect of fat content in milk on the microbial inactivation by PEF treatment;
2. To investigate the effects of temperature, electric field intensity and treatment time on *E. coli* O157:H7 and *Salmonella enteritidis* in whole milk by kinetic approach;
3. To determine the rennet coagulation properties of PEF treated milk using rheological measurement approach and compare the results with those from raw milk and thermally pasteurized milk;
4. To evaluate the proteolysis process of cheddar-type cheese curd made from PEF treated milk and compare the results with those from raw milk and thermally pasteurized milk.

CHAPTER II: LITERATURE REVIEW

2.1 Pulsed electric field (PEF) and its application in the food industry

2.1.1 Introduction of PEF

PEF processing involves a short burst of high voltage application to a food placed between two electrodes (Qin et al. 1995a). When high electric voltage is applied, a large flux of electric current flows through food materials, which may act as electrical conductors due to the presence of electrical charge carriers such as large concentration of ions (Barbosa-Cánovas et al. 1999).

In general, a PEF system consists of a high-voltage power source, an energy storage capacitor bank, a charging current limiting resistor, a switch to discharge energy from the capacitor across the food and a treatment chamber. The bank of capacitors is charged by a direct current power source obtained from amplified and rectified regular alternative current main source. An electrical switch is used to discharge energy (instantaneously in millionth of a second) stored in the capacitor storage bank across the food held in the treatment chamber. Apart from those major components, some adjunct parts are also necessary. In case of continuous system a pump is used to convey the food through the treatment chamber. A chamber cooling system may be used to diminish the ohmic heating effect and control food temperature during treatment. High-voltage and high-current probes are used to measure the voltage and current delivered to the chamber (Barbosa-Cánovas et al. 1999; Floury et al. 2006; Amiali et al. 2006a).

The type of electrical field waveform applied is one of the important descriptive characteristics of a pulsed electric field treatment system. The

exponentially decaying or square waves are among the most common waveforms used. To generate an exponentially decaying voltage wave, a DC power supply charges the bank of capacitors that are connected in series with a charging resistor. When a trigger signal is applied, the charge stored in the capacitor flows through the food in the treatment chamber. Exponential waveforms are easier to generate from the generator point of view. Generation of square waveform generally requires a pulse-forming network (PFN) consisting of an array of capacitors and inductors. It is more challenging to design a square waveform system compared to an exponential waveform system. However, square waveforms may be more lethal and energy efficient than exponentially decaying pulses since square pulses have longer peak voltage duration compared to exponential pulses (Zhang et al. 1995a; Evrendilek and Zhang 2005; Amiali et al. 2006a). In order to produce effective square waveform using a PFN, the resistance of the food must be matched with the impedance of the PFN. Therefore, it is important to determine the resistance of the food in order to treat the food properly.

The discharging switch also plays a critical role in the efficiency of the PEF system. The type of switch used will determine how fast it can perform and how much current and voltage it can withstand. In increasing order of service life, suitable switches for PEF systems include: ignitrons, spark gaps, trigatrons, thyratrons, and semiconductors. Solid-state semiconductor switches are considered by the experts as the future of high power switching (Bartos, 2000). They present better performance and are easier to handle, require fewer components, allow faster switching times and are more economically sound (Gongora-Nieto et al. 2002).

2.1.2 Application of PEF in food industry

There is a growing interest in the application of PEF in food processing (Barbosa-Cánovas et al. 1999; Dutreux et al. 2000; Fleischman et al. 2004; Floury et al. 2006; Huang et al. 2006; Sobrino-Lopez et al. 2006). Generally, applications of PEF in food processing have been directed to two main categories: microbial inactivation and preservation of liquid foods, and enhancement of mass transfer and texture in solids and liquids.

Large portion of works on PEF have been focused on reducing microbial load in liquid or semi-solid foods in order to extend their shelf life and ensure their safety. The products that have been mostly studied include milk (Dunn and Pearlman 1987; Grahl and Markl 1996; Sensoy et al. 1997; Reina et al. 1998; Dutreux et al. 2000; Fleischman et al. 2004, Evrendilek and Zhang 2005); apple juice (Vega-Mercado et al. 1997); orange juice (Zhang et al. 1997) and liquid egg (Jeantet et al. 1999 and 2004; Hermawan et al. 2004, Amiali et al. 2006b). These studies and others have reported successful PEF-inactivation of pathogenic and food spoilage microorganisms as well as selected enzymes, resulting in better retention of flavors and nutrients and fresher taste compared to heat pasteurized products (Barbosa-Cánovas et al. 1999; Ho and Mittal, 2000; Van Loey et al. 2001; Barsotti et al. 2002; Bendicho et al. 2002; Espachs-Barroso et al. 2003; Sepulveda et al. 2005a; Sobrino-Lopez et al. 2006).

Another area that is showing a great potential is applying PEF on plant tissues as a pre-treatment to enhance subsequent processes such as juice extraction (Bazhal and Vorobiev 2000; Eshtiaghi and Knorr 2002) and dehydration (Angersbach and

Knorr 1997; Rastogi et al. 1999; Ade-Omowaye et al. 2000; Taiwo et al. 2002, Lebovka et al. 2007).

2.2 Mechanism of microbial inactivation by PEF

PEF treatments cause electroporation (generation of pores) of the cell membrane, leading consequently to microbial destruction and inactivation (Tsong 1991; Knorr et al. 1994; Ho and Mittal, 1996; Pothakamury et al. 1996; García et al. 2007). Although it is still unclear whether the pore formation occurs in the lipid or the protein matrices, it is believed that electric fields induce structural changes in the membranes of microbial cells based on the transmembrane potential, electromechanical compression and the osmotic imbalance theories (Zimmermann 1986; Barbosa-Cánovas et al. 1999; Gongora-Nieto et al. 2002, Ohshima and Sato 2004).

2.2.1 Transmembrane potential

The membrane in a biological cell acts as an insulator to the cytoplasm, whose electrical conductivity is six to eight orders of magnitude greater than that of the membrane (Chen and Lee 1994). The cell membrane can be regarded as a capacitor filled with a low dielectric constant material. When a certain electric field is applied to the cell suspension, the ions inside the cell move along the field until the free charges are accumulated at both membrane surfaces. This accumulation of charges increases the electromechanical stress or transmembrane potential (V_t), to a value that is much greater than the applied electric field (Zimmermann 1986). The V_t gives rise to a pressure that causes the membrane thickness to decrease. A further increase in the electric field intensity reaching a critical transmembrane potential (V_c) leads to a

reversible membrane breakdown (pore formation). When the size and number of the pore become larger compared to the membrane surface, irreversible breakdown occurs (Zimmermann 1986; Chen and Lee 1994; Sepulveda et al. 2005a).

2.2.2 Electromechanical compression

Naturally, the charges on the capacitor plates of the biological cell membrane attract each other. This causes a thinning of the membrane provided that the membrane is compressible (Ho and Mittal 1996). The membrane thickness attained at a given membrane potential is determined by the equilibrium between the electric compression forces and the resulting electric restoring forces. With increasing membrane potential, a critical membrane thickness is reached at which the electric compressive forces change more rapidly than the generated electric restoring forces. The membrane becomes unstable and pores occur. The emerging pores fill up the internal and external solution, both of which are highly conducting. The resulting increase in the electrical permeability of the membrane leads to a rapid discharge of the membrane capacitor. An increase in the intensity of the external field will lead to membrane breakdown at the poles of the cells. The required field strength for this transmembrane breakdown is in the range of 1 to 20kV/cm depending on cell radius. The breakdown voltage itself is of the order of 1V depending on temperature, field duration, among others. At higher field strength, the breakdown voltage is reached for other membrane sites (Coster and Zimmermann 1975; Ohshima and Sato 2004).

2.2.3. Osmotic imbalance

It is also believed that the cause of membrane rupture maybe due to the osmotic imbalance generated by the leakage of ions and small molecules induced by

the PEF treatment (Kinosita and Tsong 1977). Due to the osmotic pressure of the cytoplasmic content, the cell begins to swell. When the volume of the cell reaches 155% of its normal volume, rupture of the cell membrane and lysis of the cell occur (Tsong 1990).

Vega-Mercado (1996) further confirmed the osmotic imbalance theory. The authors investigated pH, ionic strength effect and PEF combined effect on *E. coli* inactivation and found that the inactivation of microorganisms is caused mainly by an increase in their membrane permeability due to mechanical compression and poration. Reductions of more than 2.2 logs were observed when both pH and electric field are modified: pH from 6.8 to 5.7 and electric field from 20 to 55 kV/cm. Similar results are obtained when the ionic strength is reduced from 168 mM to 28 mM. The authors concluded that the electric field and ionic strength are more likely related to the poration rate and physical damage of the cell membranes, while pH is more likely related to changes in the cytoplasmic conditions due to the osmotic imbalance caused by the poration.

2.3 Factors affecting PEF inactivation of microorganisms

Major factors that affect PEF inactivation of microorganisms include the process factors (electric field intensity, pulse type, treatment time, and treatment temperature), product factors (pH, ionic strength, electrical conductivity and constituents of foods) and microbial factors (type, concentration and growth stage of microorganisms).

2.3.1 Process factors

2.3.1.1 Electric field intensity

Electric field intensity (*E*) is one of the main factors that influence the microbial inactivation (Dunn 1996). It is defined as electric potential difference (*V*) between two given points in space divided by the distance (*d*).

$$E = \frac{V}{d} \tag{2.1}$$

To achieve microbial inactivation, the applied electric field needs to be greater than the critical electric field for a particular microorganism (Castro et al. 1993). The electric field should be evenly distributed in the treatment chamber in order to achieve an efficient treatment. An electric field intensity of 16 kV/cm or greater is usually sufficient to reduce the viability of Gram negative bacteria by 4 to 5 log cycles and Gram positive bacteria by 3 to 4 log cycles (Pothakamury et al. 1995a). In general, the electric field intensity required to inactivate microorganisms in foods is in the range of 12-45 kV/cm. However, some studies have reported that electric field intensity of up to 90 kV/cm could be applied to food under a continuous treatment conditions (Zhang et al. 1994a; Dunn 1996; Liang et al. 2002). The fact that microbial inactivation increases with increasing applied electric field strength is consistent with the electroporation theory, in which the induced potential difference across the cell membrane is proportional to the applied electric field.

The most famous model showing the relationship between the survival ratio ($S=N/N_0$) of microorganisms and the electric field intensity was proposed by Hülsheger et al. (1981) and listed below:

$$\ln(S) = -b_E(E - E_C) \tag{2.2}$$

where b_E (cm/kV) is the regression coefficient and E_c is the critical electric field intensity obtained by the extrapolated value of E for 100% survival.

Grahl and Markl (1996) found that the critical value of electric field intensity (E_c) was a function of cell size; the bigger the size of a cell, the lower the E_c. They attributed this phenomenon to the transmembrane potential experienced by the cell, which is proportional to the cell size. Also, Hülsheger et al. (1983) found that E_c for gram-negative bacteria was lower than that of gram positive bacteria, which may be explained by the smaller resistance of the former.

2.3.1.2 Treatment time and frequency

PEF treatment time is calculated by multiplying the pulse number by the pulse duration. An increase in any of these variables increases microbial inactivation (Sale and Hamilton 1967).

Schoenbach et al. (1997) proposed that pulse width between 1 and 5 μs produced the best results for microbial inactivation. Martin-Belloso et al. (1997) found that pulse width influenced microbial reduction by affecting the E_c. Longer widths decreased E_c, therefore resulting in higher inactivation. However, an increase in pulse duration may also result in an undesirable food temperature increase and promote electrolytic reactions and electrodeposition at the electrode surfaces (Zhang et al. 1995a).

Normally, the inactivation of microorganisms increases with an increase in the pulse number, up to a certain number (Hülsheger et al. 1983). Grahl and Markl (1996) reported that the log reduction of *E. coli* in UHT milk increased from 1 to 4 with the pulse number increasing from 5 to 20 at less than 45°C and 22.4 kV/cm of electric

field intensity. Zhang et al. (1994b) also reported that the log reduction of *E. coli* in skim milk increased from 1 to 4 with the pulse number increasing from 16 to 64 at 15°C and 40 kV/cm. Liu et al. (1997) found that microbial inactivation was usually achieved during the first several pulses, additional pulses display a lesser lethality. Zhang et al. (1994a) also noticed that the inactivation of *Saccharomyces cerevisiae* by PEF in apple juice reached saturation up to 10 pulses at an electric field of 25kV/cm.

Different models relating the survival ratio of microorganisms to treatment time have been proposed. Hülsheger et al. (1981; 1983) proposed the following two models:

$$\ln(S) = -b_t \times \ln\left[\frac{t}{t_c}\right] \tag{2.3}$$

where b_t is the regression coefficient, t is the treatment time (μs), t_c is the extrapolated value of t for 100 % survival.

$$\log(S) = \frac{E_c - E}{k} \times \log\left[\frac{t}{t_c}\right] \tag{2.4}$$

where, t_c is the maximum treatment time (μs) that results in an S value of 1, k is a first-order kinetic constant (kV/cm).

Later, Martin-Belloso et al. (1997) presented a first order kinetic model:

$$S = e^{-kt} \tag{2.5}$$

where, S is the survival ratio, k is the specific death rate of the population (μs^{-1}), t is the treatment time (μs).

However, further investigations with data on microbial inactivation using PEF suggested that this model might be inadequate to describe PEF inactivation mostly due to the saturation of inactivation during PEF treatment. Therefore, other models were

proposed to describe non-linear kinetics. For example, Smelt et al. (2002) and Amiali et al. (2004) have reported rapid inactivation of microorganisms within early pulses and subsequent tailing phenomena. A two phase kinetic model has been proposed by Amiali et al. (2004) which sufficiently fit their experimental data of whole egg and egg yolk.

$$S = S_e + (1 - S_e)e^{-kt} \tag{2.6}$$

where S is survival fraction, S_e is the tailing survival fraction, k is the kinetic rate constant and t is the treatment time (μs).

Apart from electric field intensity and treatment time, pulse frequency is also an important factor. Elez-Martinez and Martin-Belloso (2007) evaluated the effects of PEF processing conditions on vitamin C and antioxidant activity of orange juice. The treatments were performed at 25 kV/cm and 400 μs with square bipolar pulses of 4 μs and pulse frequency from 50 to 450 Hz. The retention of vitamin C in orange juice and gazpacho increased with a decrease of pulse frequency.

2.3.1.3 Pulse shape and polarity

Exponential decaying and square wave pulses are the two commonly used pulse shapes. Other waveforms such as bipolar, instant charge reversal or oscillatory pulses have been used depending on the circuit design.

An exponential decay voltage wave is a unidirectional voltage that rises rapidly to a maximum value and decays slowly to zero. Therefore, food is subjected to the peak voltage for a short period of time. Hence, exponential decay pulses have a long tail with a low electric field, during which excess heat is generated in the food without an antimicrobial effect (Zhang et al. 1995a). Oscillatory decay pulses are the least

efficient as they prevent the cell from being continuously exposed to high intensity electric field for an extended period of time, thus preventing the cell membrane from irreversible breakdown over a large area (Jeyamkondan et al. 1999).

The square waveform may be generated by using a pulse-forming network (PFN) consisting of an array of capacitors and inductors or by using long coaxial cable and solid-state switch devices. The disadvantage of using high voltage square waves lies in trying to match the load resistance of the food with the characteristic impedance of the transmission line. By matching the impedances, a higher energy transfer to the treatment chamber can be obtained. Zhang et al. (1994a) reported 60% more inactivation of *Saccharomyces cerevisiae* when using square pulses than exponentially decaying pulses.

Bipolar pulses are more lethal than mono-polar pulses (square or exponential decay) because bi-polar pulses cause the alternating changes in the movement of charged molecules which lead to extra stress in the cell membrane and enhance its electric breakdown (Qin et al. 1994; Barbosa-Cánovas et al. 1999; Evrendilek and Zhang 2005). Bipolar pulses also offer the advantages of minimum energy utilization, reduced deposition of solids on the electrode surface, and decreased food electrolysis. These advantages were tested by Qin et al. (1994) on *B. subtilis* and Evrendilek and Zhang (2005) on *E. coli* O157: H7 in skim milk.

Ho et al. (1995) proposed instant reversal pulses where the charge is partially positive at first and partially negative immediately thereafter. The inactivation effect of an instant reversal pulse is believed to be due to a significant alternating stress on microbial cell which causes structural fatigue. Amiali et al. (2006b) used instant

reversal square wave pulses and found that this kind of waveforms to be more efficient than others in terms of egg product pasteurization, since it combines instant reversal charge and square waveform pulses.

2.3.1.4 Treatment temperature

Treatment temperature is a very important parameter regarding microbial inactivation by PEF. On one hand, PEF treatment increases the product temperature; therefore, a proper cooling device is necessary to maintain the temperature below levels that affect nutritional, sensory or functional properties of products. On the other hand, application of PEF at mild temperature enhances the microbial inactivation.

Dunn and Pearlman (1987) found that a combination of PEF and heat was more efficient than conventional heat treatment alone. A higher level of inactivation was obtained using a combination of 55°C temperature and PEF to treat milk. Dunn (1996) obtained a 6 log reduction of *L. innocua* inoculated in milk after few seconds at 55°C accompanied with PEF.

Zhang et al (1995b) reported that increasing treatment temperature from 7 to 20°C significantly increased PEF inactivation of *E. coli* in simulated milk ultra-filtrate (SMUF). However, additional increase in temperature from 20 to 33°C did not result in any further increase in PEF inactivation.

Pothakamury et al. (1996) subjected SMUF inoculated with *E. coli* to PEF treatment (36kV/cm, 40 pulses) and found that microbial reduction increased from 4 to 5-log when temperature increased from 7 to 20°C.

Sensoy et al. (1997) treated *Salmonella dublin* inoculated in skim milk by PEF (25kV/cm, 100 pulses) and observed that when temperature increased from 25 to 50°C, the microbial reduction increased by 1 log.

Reina et al. *(*1998) reported a higher inactivation rate of *L. monocytogenes* in milk with a temperature increase from 25 to 50°C. At 30°C and 30 kV/cm, a 3.5 log reduction of *L. monocytogenes* was obtained after 600 μs of treatment, whereas at 50°C more than 4 log reductions were obtained.

Sepulveda et al. (2005a) found a marked increase of PEF inactivation at 55°C on *L. innocua* suspended in a buffer. The electric field intensity and number of pulses were applied in the range of 31-40kV/cm and 5-35 pulses. These authors found synergy between thermal and PEF treatments. The authors thought the marked increase of PEF inactivation effectiveness at 55°C may be due to the occurrence of phase transition on the cell membrane of *L. innocua* at this temperature, since it is possible that a thinning of the bacterial membrane would render bacterial cells more susceptible to disruption by electric fields (Jayaram et al. 1992).

Ravishankar et al. (2002) investigated *E. coli* O157:H7 at electric field strength of 15 to 30 kV/cm, pulse number of 1 to 20 and temperature up to 65°C using a static chamber and gellan gum gel as a suspension medium. The authors found thermal energy began taking effect at 55°C. At this temperature, a 1 log reduction was attributable to thermal energy. Above this temperature, all reductions were attributable entirely to thermal energy. The authors suggested no synergy between thermal and PEF energy.

Bazhal et al. (2006) investigated the combined effect of heat treatment with PEF on the inactivation of *E. coli* O157:H7 in liquid whole egg. The electric field strength was varied from 9 to 15 kV/cm and the treatment temperatures were 50, 55 or 60°C. At 60°C, a 2 log reduction of *E. coli* O157:H7 was obtained using thermal treatment alone, while a combination of heat and PEF resulted in a 4 log reduction. These results indicated a synergy between temperature and electric field.

An increase in the rate of inactivation with temperature is attributed to reduced trans-membrane breakdown potentials at higher temperatures (Zimmermann 1986). Stanley (1991) proposed that phospholipid molecules in cell membrane undergo temperature-related transitions, changing from a firm gel-like structure to a less ordered liquid crystal phase at higher temperature, thus reducing the mechanical resistance of the cell membrane. Another deduction proposed by Schwan (1957) was that the higher lethal effect of PEF combined with heat might be due to the increase in electrical conductivity of the medium, making it similar to electrolytic conduction (Barbosa-Cánovas et al. 1999; Sepulveda et al. 2005a).

2.3.1.5 Operation mode

In general, continuous processes reached higher inactivation rates than those in batch mode. Martin et al. (1997) reported that to achieve a 2 log reduction of *E. coli* inoculated in milk by a batch mode, 64 pulses and 35 kV/cm is needed, while for a continuous mode, 25 pulses and 25 kV/cm is enough.

2.3.2 Product factors

2.3.2.1 PH and ionic strength

Vega-Mercado (1996) studied the effect of pH and ionic strength of the medium (SMUF) during PEF treatment. The author reported that the lower the pH and ionic strength, the higher the inactivation rate. When the ionic strength decreased from 168 to 28mM, the inactivation ratio increased from not detectable to 2.5 log cycles. Also, when the pH reduced from 6.8 to 5.7, the inactivation ratio increased from 1.5 to 2.2 log cycles. The PEF treatment and ionic strength were responsible for electroporation and compression of the cell membrane, whereas the pH of the medium affected the cytoplasm when the electroporation was complete.

Alvarez et al. (2000) studied the influence of pH of treatment medium on the inactivation of *Salmonella senftenberg* by PEF treatment. The authors found that at the same electrical conductivity, inactivation of *S. senftenberg* was greater at neutral (7.0) rather than for acidic pH (3.8).

2.3.2.2 Electrical conductivity

The electrical conductivity of a medium (σ, s/m), which is defined as the ability to conduct electric current, is an important variable in PEF treatment.

$$\sigma = \frac{d}{RA} = \frac{1}{\rho} \tag{2.7}$$

where σ (s/m), R (Ω), A (m^2), d (m) and ρ are the electrical conductivity of medium, the resistance of the medium, the electrode surface area, the gap between electrodes and the resistivity, respectively.

The electrical conductivity of a medium depends on treatment temperature as defined by

$$\sigma = \alpha T + \beta \quad (2.8)$$

where α and β are constants depending on the composition and concentration of the medium.

At constant temperature conditions, foods with high electrical conductivities (low resistivity) exhibit smaller electric fields across the treatment chamber and therefore are difficult to be treated with PEF process. An increase in electrical conductivity results from increasing the ionic strength of a liquid while an increase in the ionic strength of a food results in a decrease in the inactivation rate. Furthermore, an increase in the difference between the electrical conductivity of a medium and microbial cytoplasm weakens the membrane structure due to an increased flow of ionic substance across the membrane (Jayaram et al. 1992).

Alvarez et al. (2000) studied the influence of conductivity of treatment medium on the inactivation of Salmonella *senftenberg* by PEF treatment. The authors found that at constant input voltage, electric field strength obtained in the treatment chamber depended on medium conductivity. At the same electric field strength, conductivity did not influence S. *senftenberg* inactivation.

2.3.2.3 Protective factor

Food components such as fat and protein may exhibit protective effects against PEF inactivation of microorganisms. These effects may be related to the capacity of some substances to shield microorganisms from applied field, or the ability of some chemical compounds to stabilize or prevent ion migration.

Martin et al. (1997) found that inactivation of *E. coli* in milk was more limited than in a buffer solution, because of the presence of milk proteins. There is currently no agreement on the possible influence of fat content on PEF inactivation.

Grahl and Markl (1996) subjected different media (milk with 1.5 and 3.5% fat, solutions of sodium-alginate) inoculated with *E. coli* and other microorganisms to PEF. The treatment conditions are 5-15kV/cm, 1-22Hz, and the temperatures did not exceed 45-50^{o}C. The authors noticed that the fat particles of milk seemed to protect the bacteria against electric pulses. Picart et al. (2002) also claimed that whole milk with a higher fat content (3.6%) appeared to reduce *Listeria innocua* inactivation compared to skim milk at temperatures between 25 and 45^{o}C, a pulse repeat frequency of 1.1Hz and electric intensity of 29kV/cm.

However, this protective effect is not always the case. Reina et al. (1998) compared the effect of PEF treatment under 25^{o}C at 30 kV/cm and frequency of 1700Hz in milk with different fat content. The authors inoculated *L. monocytogenes* into skim milk, 2% fat milk, and whole milk, and evaluated the effects of the fat content on the inactivation rates, no differences were observed among the results. Manas et al. (2001) used 33% emulsified fat cream to test fat effect on the inactivation of *E. coli* by PEF treatment. The treatment was conducted under 34kV/cm with a pulse frequency of 1.1Hz and temperatures less than 30^{o}C. The result was that the emulsified lipids do not appear to protect against microbial inactivation by electric pulses. Sobrino-Lopez et al. (2006) also claimed that fat content of the milk did not modify the resistance of *Staph. aureus* to a PEF treatment. Three types of milk (whole, 1.5% and skim) were treated under 25^{o}C, 30-35kV/cm and frequency of 100Hz.

The contradicting results indicated that further study is needed to better understand these phenomena.

2.3.3 Microbial factors

2.3.3.1 Type and size of microorganism

In general, gram-positive bacteria are more resistant to PEF than gram-negative ones (Hülsheger et al. 1983). Yeasts are more sensitive to electric fields than bacteria due to their larger size (Sale and Hamilton 1967; Qin et al. 1995a).

2.3.3.2 Concentration of microorganisms

The number of microorganisms in foods may or may not have an effect on their inactivation by PEF, depending on the specific conditions of the treatment process. Barbosa-Cánovas et al. (1999) reported that inactivation of *E. coli* in SMUF was not affected when the concentration of microorganisms varied from 10^3 to 10^8 cfu/mL after being subjected to 70 kV/cm, 16 pulses, and a pulse width of 2 μs. The authors also reported that increasing the number of *S. cerevisiae* in apple juice resulted in slightly lower microbial inactivation, which could possibly be explained by the cluster formation of microbial cells or concealed microorganisms in low electric field regions.

2.3.3.3 Growth stage of microorganisms

In general, logarithmic phase cells are more sensitive to stress than lag and stationary phase cells. Hülsheger et al. (1983) concluded that cells from logarithmic growth phase were more sensitive to PEF than from the stationary growth phase. Microbial growth in logarithmic phase is characterized by a high proportion of cells

undergoing division, during which the cell membrane is more sensitive to the applied electric field.

Pothakamury et al. (1996) confirmed this conclusion with *E. coli* cells. The authors harvested *E. coli* after 0.5 to 6.5 hours of bacterial growth. They observed that the stationary phase was reached after 4 hours. The treatment conditions were 4 pulses of 2 μs each at an electric field intensity of 36 kV/cm. The cells in logarithmic phase were more sensitive and their viability reduced by more than 2 log reductions, whereas cells in the stationary phase were reduced by only 1.4 logs and cells in the lag phase were reduced by less than 0.5 logs.

Alvarez et al. (2000) studied the influence of cell age on PEF treatment effect by treating *S. senftenberg* at different growth stages with 200 pulses of 2 μs each at an electric field intensity of 19 kV/cm. The authors found that in the stationary phase and the beginning of the logarithmic phase, cells were more resistant to PEF than cells in the logarithmic phase. However, the influence of cell age was not that much since the maximum difference in the number of survivors between the most and the least resistant cells was only a 1.5 log cycles.

2.4 PEF inactivation of microorganisms in milk

Milk is one of the most popular and commercialized liquid food products. Canadian milk production reached 7.6 million tons in 2008 (Statistics Canada 2009), indicating the enormous size of the market. As a highly nutritious medium of almost neutral pH, milk can be easily contaminated by many bacteria, including spoilage and pathogenic bacteria. Numerous outbreaks of food poisoning have been traced to milk. *Escherichia coli* O157:H7 is one of the most important pathogens found in un-

pasteurized milk products. It has been implicated in sporadic outbreaks of hemorrhagic colitis and hemolytic-uremic syndrome in humans. A typical example of *E. coli* O157:H7 outbreak in Canada was associated with goat's milk from a co-operative farm on Vancouver Island, British Columbia. Five children were infected and two of them were hospitalized and got hemolytic-uremic syndrome (Public Health Agency of Canada 2002). *Salmonella* spp. are also dangerous food-borne bacterial pathogen. They may contaminate milk and cheese products and cause salmonellosis in humans (Fox et al. 2000). A typical outbreak occurred in California in 1997 where 31 persons were infected by *S. typhimurium* from eating fresh Mexican-style cheese and raw milk (Cody et al. 1999). Other equally important bacterial pathogens in milk include *Listeria monocytogenes, Staphylococcus aureus*, and *Campylobacter jejuni.*

The control of these microorganisms is the most important function in the handling and manufacture of milk and dairy products. In most countries, raw milk must be pasteurized as required by strict regulations. The sale of raw milk has been strictly prohibited under the Food and Drug Regulations of Canada since 1991. Because of these health concerns, Food and Drug Regulations of Canada require that all milk available for sale in Canada be pasteurized, which means that the raw milk has been subjected to heat to eliminate disease-causing bacteria that may be present (Health Canada 2006).

Conventional heat pasteurization could be accomplished by two schemes: low temperature long time (LTLT 63 – 65°C, 30 min) and high temperature short time pasteurization (HTST 72°C, 15 s) (Fox et al. 2000). However, apart from bacterial inactivation, heat treatment can adversely affect flavor, taste and nutritional quality of

milk. Therefore, there is a need to develop alternative processing technologies based on non-thermal techniques.

PEF as a non-thermal pasteurization method has the potential to prevent these kinds of outbreaks without a sacrifice on the quality. A great number of researches on PEF have focused on the inactivation of different microorganisms in milk due to the importance of the dairy industry (Table 2.1).

Table 2.1 Inactivation of selected microorganisms in milk by PEF[a]

Source	Milk type[b]	Microorganisms	Max. log reduction	Treatment vessel[c]	Process conditions[d]
Dunn and Pearlman (1987)	Pasteurized milk	*E. coli*	3	B, parallel plate	43°C, 43 kV/cm, 23 pulses
Grahl and Markl (1996)	UHT milk (1.5% fat)	*E. coli*	4	B, 25 mL, d = 0.5cm	< 45°C, 22.4 kV/cm, 20 pulses
Zhang et al. (1994b)	Skim milk	*E. coli*	3	B, d = 0.95cm, v = 25.7 mL	< 25°C, 25kV/cm, 20 pulses, exponential
Zhang et al. (1995b)	Modified SMUF	*E. coli*	9	B, parallel plate, 14 mL, 0.51 cm	7, 20, 33°C, 70 kV/cm, 2µs, 80 pulses
Qin et al. (1994)	SMUF	*E. coli*	1.5	B, 80J/pulse, parallel plate	<30°C, 40 kV/cm, 8 pulses, oscillatory decay
Qin et al. (1994)	SMUF	*E. coli*	3	B, 60J/pulse, parallel plate	<30°C, 40 kV/cm, 4 pulses, bipolar

Source	Milk type[b]	Microorganisms	Max. log reduction	Treatment vessel[c]	Process conditions[d]
Qin et al. (1994)	Skim milk	*E. coli*	2.5	B, parallel plate, 14mL	<30°C, 50 kV/cm, 62 pulses, 2µs, square wave
Qin et al. (1994)	Skim milk	*E. coli*	3.5	C, parallel plate	<30°C, 50 kV/cm, 48 pulses, 2µs, square wave
Qin et al. (1995a)	SMUF	*E. coli*	3.6	C, parallel plate, 8 cm^3, d = 0.51cm	<30°C, 50 kV/cm, 62 pulses, 2µs, square wave
Qin et al. (1995a)	SMUF	*E. coli*	7	C, coaxial, 29mL, d = 0.6cm	<30°C, 25kV/cm, 300pulses, 2µs, square wave
Qin et al. (1998)	SMUF	*E. coli*	6	C, coaxial, 29mL, d=0.6cm	<40°C, 60kV/cm, 50 pulses, 2µs, Exponential decay
Pothakamury et al. (1995a)	SMUF	*E. coli*	4	B, parallel plate, 12.5 mL, d = 0.5 cm	<30°C; 16 kV/cm; 200-300µs, Exponential decay
Pothakamury et al. (1996)	SMUF	*E. coli*	4, 5	B, parallel plate	7, 20°C; 36 kV/cm, 60pulses

Source	Milk type[b]	Microorganisms	Max. log reduction	Treatment vessel[c]	Process conditions[d]
Vega-Mercado (1996)	Modified SMUF	*E. coli*	2.56	B, parallel plate, 12.5 mL, d = 0.5 cm	15°C, 20, 40, 55 kV/cm, 2µs, 8 pulses
Martin et al. (1997)	Skim milk diluted with water (1:2:3)	*E. coli*	Nearly 3	B, parallel plate, 13.8 mL, d = 0.51 cm	15°C, 45 kV/cm, 6 µs, 64 pulses
Martin et al. (1997)	Skim milk	*E. coli*	2	C, parallel plate, 45 mL/s, v = 8mL	15°C, 25 kV/cm; 1.8 µs
Dutreux et al. (2000)	Fat-free milk	*E. coli*	4.0	C, coaxial, 29mL, flow rate 0.5 L/min, d = 0.6 cm	Inlet 17°C, outlet 37°C, 41kV/cm, 2.5µs, up to 63 pulses
Manas et al. (2001)	Sterile dairy cream (33% fat)	*E. coli*	5.0	B, parallel electrode, cylindrical chamber, d = 0.5 cm, 5.7 mL	<30°C; 34 kV/cm; 7 µs, 1.1 Hz, 64 pulses, exponential decay
Evrendilek and Zhang (2005)	Skim milk	*E. coli* O157:H7	1.96	C, 1mL/s, cofield	24 kV/cm,2.8 µs, 700 Hz, bipolar pulses, square wave, t = 141 µs
Alkhafaji and Farid (2007)	SMUF	*E. coli*	6.6	C, Multi-pass treatment chambers	17°C, 43.4kV/cm, 1.7 µs, 518 pulses, bipolar square wave, , up to 200 Hz,

Source	Milk type[b]	Microorganisms	Max. log reduction	Treatment vessel[c]	Process conditions[d]
Dunn and Pearlman (1987)	Milk	*Salmonella dublin*	4	B, parallel plate	63°C, 36.7kV/cm, 36µs, 40 pulses
Sensoy et al. (1997)	Skim milk	*Salmonella dublin*	3.0	C, cofield	10 to 50°C, 15-40 kV/cm, 12-127 µs
Floury et al. (2006)	Skim milk	*Salmonella enteritidis*	1.0	C, coaxial, d = 2mm	<50°C, 35kV/ cm, 500 ns, 64 pulses square wave
Qin et al. (1994)	SMUF	*B. subtilis*	4.5	B. parallel plate, 100 µL, d = 0.1cm	16 kV/ cm, monopolar, 180 µs, 13 pulses
Qin et al. (1994)	SMUF	*B. subtilis*	5.5	B. parallel plate, 100 µL, d = 0.1cm	16 kV/ cm, bipolar, 180 µs, 13 pulses
Michalac et al. (2003)	Raw skim milk UHT skim milk	*B. subtilis*	3.0	Co-field, d = 0.19 cm, diameter = 0.23	52-22°C, 35 kV/ cm, bipolar square wave, 3 µs, 500 Hz, 64 pulses
Pothakamury et al. (1995b)	SMUF	*Lactobacillus delbrueckii* ATCC 11842	4-5	B, 1 mL, d = 0.1cm	<30°C, 16kV/ cm, 200-300 µs, 40 pulses exponential decay, t = 10,000 µs

Source	Milk type[b]	Microorganisms	Max. log reduction	Treatment vessel[c]	Process conditions[d]
Pothakamury et al. (1995b)	SMUF	*Bacillus subtilis* spores	4-5	B, parallel plate,1 mL, d = 0.1cm	<30°C, 16 kV/cm, 200-300 µs, 50 pulses, exponential decay, t = 12,500 µs
Pothakamury et al. (1995a)	SMUF	*S. aureus ATCC 6538*	3	B, parallel plate,1 mL, d = 0.1cm	<30°C, 16 kV/cm, 200-300 µs, 60 pulses, exponential decay
Evrendilek et al. (2004)	Skim milk	*S. aureus*	3.0	Co-field chamber, d = 0.6 cm, diameter = 2.3 mm	40°C, 250 Hz, 35 kV/cm, 3.7 µs, 250 pulses, bipolar square wave
Sobrino-Lopez et al. (2006)	Skim milk	*S. aureus*	4.5	C, cofield, d = 0.29, v = 0.012 cm^3	25°C, 100 Hz, 35 kV/cm, 8 µs, 150 pulses, bipolar square wave
Sobrino-Lopez et al. (2006)	Skim milk	*S. aureus*	6.3	C, cofield, d = 0.29, v = 0.012 cm^3	25°C, 75 Hz, 35 kV/cm, 6 µs, 200 pulses, bipolar square wave, pH 6.8, Lysozyme (300 IU/mL) and Nisin (1 IU/mL)
Reina et al. (1998)	Pasteurized milk (whole and skim)	*Listeria monocytogenes*	3.0-4.0	C, cofield flow, 0.07L/s, 20 mL,	10 to 50°C, 30 kV/cm,1.5 µs, 1,700 Hz , bipolar pulses, t = 600 µs

Source	Milk type[b]	Microorganisms	Max. log reduction	Treatment vessel[c]	Process conditions[d]
Calderon-Miranda et al. (1999)	Raw Skim milk	*Listeria innocua*	2.5	C, 29 mL, 0.5 L/min, d = 0.6 cm	22 to 34°C, 2 µs, 3.5Hz, 32 pulses, 50 kV/cm, Exponential decay
Fernandez-Molina (2001)	Pasteurized skim milk (0.2% milkfat)	*Listeria innocua*	2.6	C, coaxial, 29 mL, 0.5 L/min, d = 0.63 cm	15 to 28°C, 100 pulses, 50 kV/cm, 2 µs, 3.5 Hz, Exponential decay
Fernandez-Molina (2001)	Pasteurized skim milk (0.2% milk fat)	*Pseudomonas fluorescens*	2.7	C, coaxial, 29 mL, 0.5 L/min, d = 0.63 cm	15 to 28°C, 30 pulses, 50 kV/cm, 2 µs, 4.0 Hz, Exponential decay
Michalac et al. (2003)	Raw skim milk UHT skim milk	*Pseudomonas fluorescens*	2.5	Co-field, d = 0.19 cm	52-22°C, 35 kV/ cm, bipolar square wave, 3 µs, 500 Hz, 64 pulses
Shamsi et al. (2008)	Raw skim milk	*Pseudomonas*	5.9	Co-field chamber, flow rate: 60mL/min, d = 0.29 cm,	60°C, 200 Hz, 35 kV/cm, 2 µs, 19.6 µs, bipolar square wave
Dutreux et al. (2000)	Fat-free milk	*Listeria innocua*	3.9	C, coaxial, 29mL, flow rate: 0.5 L/min, d = 0.6 cm	Inlet 17°C, outlet 37°C, 41kV/cm, 2.5µs, up to 63 pulses

Source	Milk type[b]	Microorganisms	Max. log reduction	Treatment vessel[c]	Process conditions[d]
Picart et al. (2002)	Whole milk, skim milk, dairy cream	*Listeria innocua*	6	B, cylindrical vessel, 5 mm gap, v = 5.6 mL	21.5-42°C, 17-46 kV/cm, 545 pulses, 1.1 or 100Hz, 1.51µs, exponential decay
Fleischman et al. (2004)	Water and skim milk	*Listeria monocytogenes*	zero	B, cylindrical chamber, v = 20 mL	35°C, 3.25µs, 20kV/cm, 10 pulses
Fernandez-Molina et al. (2006)	Skim milk	*Listeria innocua*	3	C, coaxial, flow rate 8.33 mL/min, d = 0.69cm,	< 30°C, 38.9kV/cm, 3µs, 100 pulses

[a] Part of the information was adapted from Barbosa-Cánovas et al. 1999

[b] UHT: ultra high temperature sterilization; SMUF: Simulated milk ultra-filtrate

[c] B: batch; C: continuous, d: gap, v: volume

[d] Temperature, peak electric field, pulse width, number of pulse and shape, or total treatment time (t).

2.5 Cheese making

2.5.1 Introduction to cheese

Cheese is a group of fermented milk-based food products produced worldwide in a great diversity of flavors, textures and forms. There are at least 1000 named cheese varieties, which can be classified into four groups based on the method used to coagulate the milk: (1) rennet coagulated cheeses, which represents about 75% of total production and includes most major international cheese varieties; (2) acid coagulated cheeses (e.g., Cottage and Quark); (3) heat and acid coagulated cheeses (e.g., Ricotta); (4) concentrated /crystallized cheese such as "Mysost" produced in Norway (Fox et al. 2000).

Cheddar-style cheese is now one of the most important cheese varieties. It is described as a hard variety, rennet coagulated cheese, originated around the village of Cheddar, England. The production of cheddar cheese is worldwide.

Canada produces more than 450 different varieties of fine cheeses. In 2005, Canadian cheese production increased to 379, 286 tons, 24% higher compared to 1994. The amount of cheddar cheese occupied 42.9% (131,872 tons) of the total cheese production (Agriculture and Agri-Food Canada 2008).

2.5.2 Cheddar cheese processing

The production of Cheddar cheese consists of several steps before yielding the final product (Figure 2.1). Firstly, milk is pasteurized in order to destroy any harmful bacteria. The whole milk is standardized to a casein-fat ratio of 0.67-0.72:1.0 (Fox et al. 2000). Special starter cultures (*Lactococcus lactis ssp. cremoris* and *Lactococcus lactis ssp. lactis*) and calf rennet are then added to the warm milk (30°C). This process acidifies the milk and within a short time a coagulum is produced. The coagulum is cut and cooked

to 37-39°C for 30 min and held at this temperature for about 1 hr. This process starts the shrinking process, which, with the steady production of lactic acid from the starter culture, will change the cut coagulum into small grains called curds. At a chosen point, the curd grains are allowed to fall to the bottom of the cheese vat and the whey is drained off. The curd grains then mat together to form large slabs of curd. The cheese goes through a process of cheddaring at this point, which involves pilling the slabs of curd on top of one and another with regular turning, stacking and restacking. When the pH has declined from around 6.1 to 5.3, the curd is milled to form small-sized cheese curds, salted, and pressed into blocks or barrels. The blocks are then packaged and ripened for about 3 months to two years.

Among the complex system of cheese making, coagulation and ripening are the two major steps.

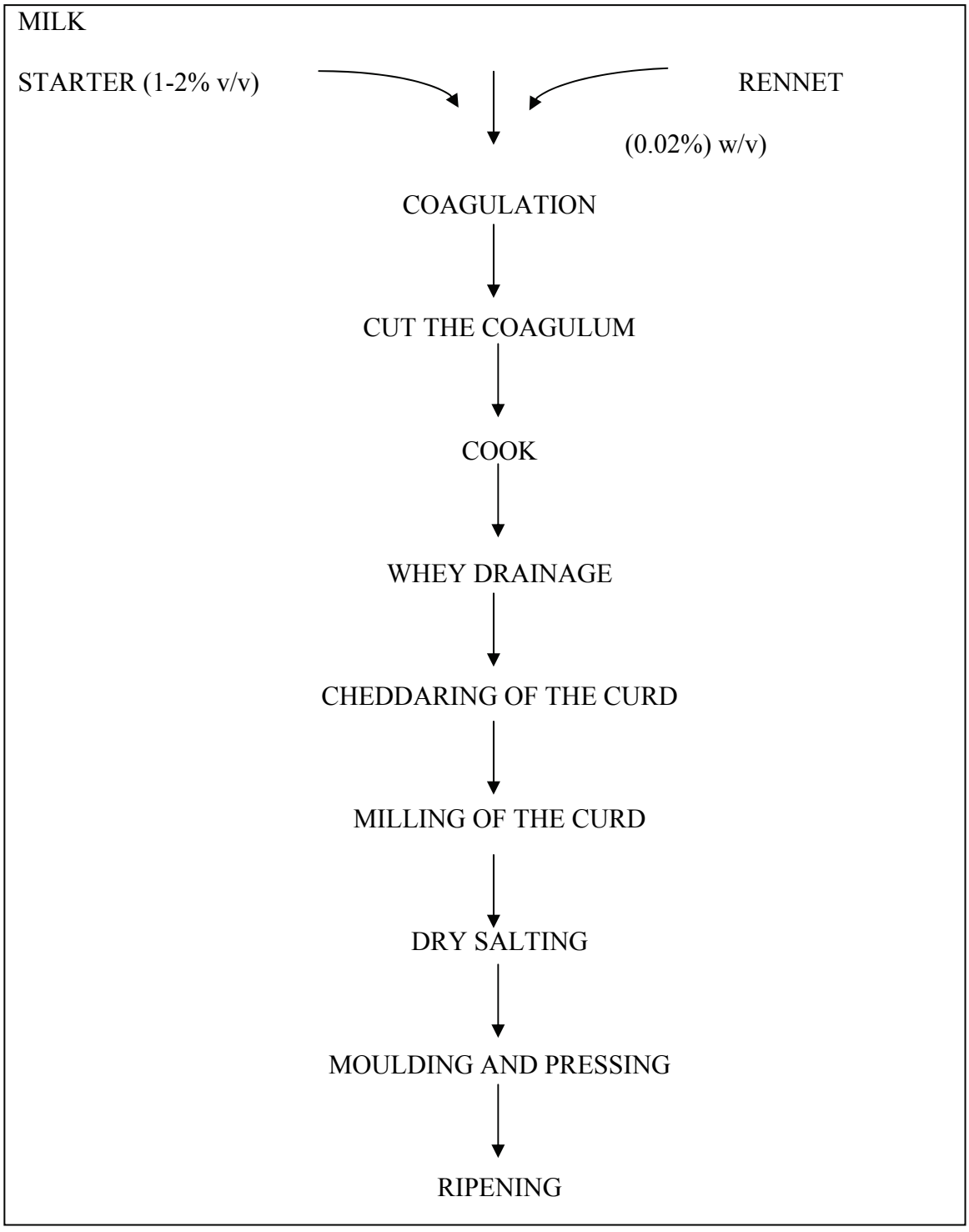

Fig. 2.1 General steps in Cheddar cheese making (Fox et al. 2000)

2.5.3 Milk coagulation mechanism

One of the important steps in cheese making is the rennet induced coagulation of milk. Rennet coagulation properties of milk could be used as indicators of processed and final cheese quality. The rennet coagulation of milk under still conditions involves the conversion of milk from a colloidal dispersion of stable micelles to a gel network of aggregated micelles, which forms a continuous phase, entrapping moisture and fat globules in its pores. The gel formation is accompanied by a number of physicochemical changes, including primary phase and secondary phase changes. The primary phase involves enzymatic hydrolysis of κ-casein whereas the secondary stage involves aggregation of the rennet-altered micelle (para-casein) into a three dimensional gel network as shown in Figure 2.2 and 2.3 (Fox et al. 2000). During the gel formation process, the viscosity and elasticity of the milk is increased, and the ratio of the viscous to elastic character of the milk is decreased. Numerous methods for detecting these changes have been developed such as the Formagraph, rotational viscometers, hot wires, ultrasounds and dynamic rheometers.

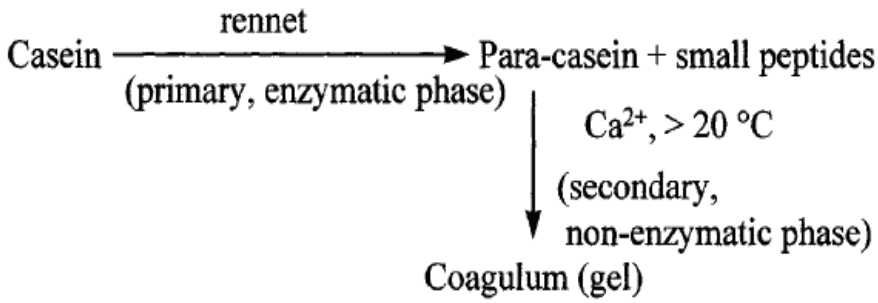

Figure 2.2 Summary of the rennet coagulation of milk (Fox et al. 2000)

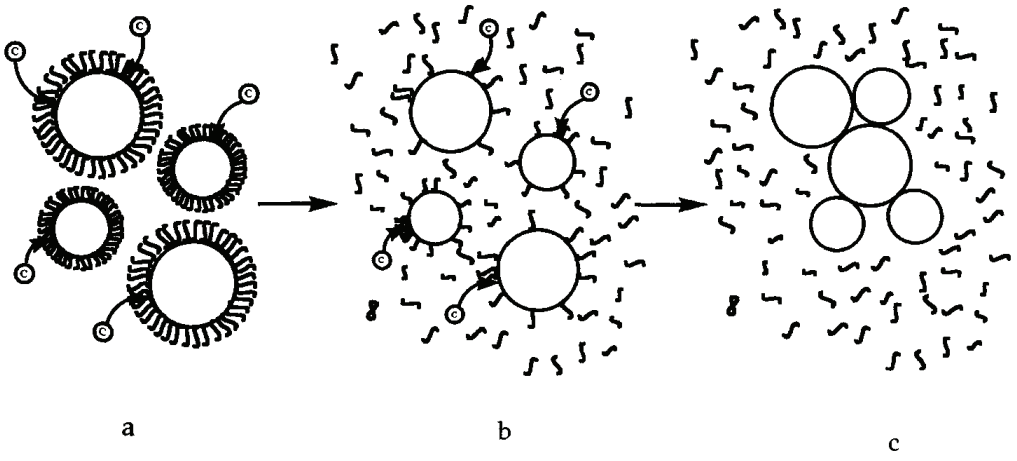

Figure 2.3 Schematic representation of the rennet coagulation of milk, (a) Casein micelles being attacked by the chymosin (C); (b) micelles partially denuded of κ-casein; (c) extensively denuded micelles in the process of aggregation (Fox et al. 2000).

Some earlier measuring methods for milk coagulation process, such as the Formagraph, were based on mechanical systems which sense the drag force on a slowly moving body suspended in the milk. Drag force techniques are not suitable for monitoring curd formation as they cause structural damage (Gervais and Vermeire 1983).

Compared to mechanically moving systems, vibrational systems are much less intrusive and could be adapted to commercial cheese vats. Sharma et al. (1989) used a torsional vibration viscometer in reconstituted skim milk and found that the instrument could detect an early stage of coagulation before visible clots are formed. Torsional vibration such as rheometers has the advantage of responding to viscous and elastic modulus and is able to follow the curd coagulation process.

Hot wire is commonly used in monitoring coagulation in cheese manufacturing. The principle of the technique is based on the temperature difference between a heated probe and the milk. Research has shown that hot wire probes are highly consistent in detecting the occurrence of a gel point but are at present incapable of monitoring the rate of curd firming (O'Callaghan et al. 1999).

Ultrasonic waves with wavelengths (i.e. frequencies as larger as 1 to 10MHz) similar to those of casein micelles, can be used to monitor milk coagulation. Cosgrove (2000) used ultrasounds to monitor milk coagulation. The author noticed that air bubbles in milk confounded some of the measurements. Further study is needed to bring this technology to a commercial application.

Coagulation of milk is accompanied by changes in optical properties, such as color, absorbance, and reflectance. This has led to the use of optical measurement systems in monitoring milk coagulation. However, such optical measurements are only an indirect measure of gel strength; they still need to be calibrated either by the cheese maker or by data from a rheometer (Dybowska and Fujio 1996).

Low-strain or stress rheometer have been used increasingly as research tools for the continuous measurement of the viscoelastic properties of renneted milks as a function of time to monitor coagulation. Dynamic rheometers give parameters that are rheologically precise and accurately quantify the dynamic rheological changes that occur during renneting without altering the process of gel formation (Bohlin et al. 1984). It thus accurately reflects the changes in curd firmness that occur upon renneting milk in the cheese vat under quiescent conditions. Many studies on the determination of milk coagulation properties chose dynamic rheometers as a practical approach (Samuelsson et al. 1997; Curcio et al. 2001; Lucey et al. 2001; Srinivasan and Lucey 2002).

Storage modulus, G' and Phase angle, δ are major rheological parameters. Various objective rennet coagulation parameters pertinent in cheese-making may be derived from the G'-time and δ-time curves. Curd Firmness (CF), normally defined as G' value after a fixed renneting time (e.g., 60 min), is the primary coagulation property that influences cheese quality, yield and economic returns (Fox et al. 2000). Rennet Coagulation Time (RCT) could be defined as the time at which phase angle (δ) fell to unity (O'Callaghan et al. 1999). RCT provides a very good index of the gelation potential of milk.

2.5.4 Cheese ripening and its measurement

Cheese ripening is a complex process driven by three basic reactions: proteolysis, lipolysis and glycolysis (Sousa et al. 2001; Collins et al. 2003). These reactions, responsible for the changes that occur in the curd during ripening, are carried out by rennet, indigenous milk enzymes, adventitious starter and non-starter bacteria. Factors such as maturation time, temperature, pH and water activity also affect the rate of ripening. In the flavor formation of matured hard cheeses such as Cheddar, proteolysis, in generating small peptides and amino acids, is the main biochemical flavor generating process (Law 2001).

Proteolysis is a process by which protein in cheese is broken down from casein to low molecular peptides and amino acids by various kinds of proteolytic enzymes. Proteolysis during cheese ripening plays an important role in forming the texture, body and flavor in all matured cheese. Proteolysis contributes to textural changes of the cheese matrix, due to breakdown of the protein network. It contributes directly to flavor and bitterness of cheese through the formation of peptides and free amino acids as well as liberation of substrates (amino acids) for secondary catabolic changes, catabolism of

aromatic amino acids and reactions of amino acids with other compounds (Fox et al. 2000).

There have been a number of analytical techniques used to monitor proteolysis in many cheese varieties. The most common index of proteolysis is to analyze the water soluble nitrogen (WSN) in an extract of cheese. Generally, an acid, trichloroacetic (TCA) or phosphotungstic (PTA) is added to the extract to precipitate protein and large peptides. Subsequently, the analysis and identification is performed on the remaining pH (water) soluble or insoluble fractions by Urea-polyacrylamide gel electrophoresis (urea-PAGE), capillary electrophoresis (CE) or reverse phase high performance liquid chromatography (RP-HPLC).

In recent years, the HPLC technique, using reversed phase (RP) columns, has been used widely to analyze peptides in cheese. HPLC is a powerful method for the study of protein degradation during cheese ripening because of its high resolution power, reproducibility and speed.

McGoldrick and Fox (1999) studied proteolysis in different varieties of cheeses (Cheddar and other varieties) by urea-PAGE and RP-HPLC. The authors reported that RP-HPLC analysis of the 70% ethanol-soluble or insoluble fractions of the cheese was more effective than urea-PAGE when classifying cheese according to variety.

Albenzio et al. (2001) compared the heat pasteurization effect on proteolysis characteristics of Canestrato Pugliese cheese. Cheese made from raw milk (R) and pasteurized milk (P) was analyzed by both urea-PAGE and the RP-HPLC. The authors found more complex profiles in cheeses produced by raw milks by both analytical methods.

Benfeldt and Sorensen (2001) investigated the effect of heat treatment on the proteolytic process in cheeses for a 16-week ripening period. The pH 4.6 soluble peptide profile by RP-HPLC varied significantly with increasing temperature during heat treatment of cheese milk.

Wallace and Fox (1997) monitored the effect of adding free amino acids to cheddar cheese curd on the proteolysis, flavor and texture development over a 6-month ripening period. Polyacrylamide gel electrophoresis indicated no differences in proteolysis between experimental and control cheeses. While RP-HPLC indicated minor qualitative differences between the cheeses.

2.5.5 Accelerating cheese ripening

Cheese ripening processing, even on a pilot scale, is expensive and time-consuming. Therefore, acceleration of ripening, especially of low-moisture, slow-ripening varieties like cheddar, is desirable. The methods used to accelerate ripening include elevating ripening temperature, adding exogenous enzymes, modifying starters and using cheese slurries made from cheese curd, distilled water and salts (Fox et al. 1996). The principal attractions of cheese slurries are the short ripening time, the low cost and the possibility of including numerous parameters in a single study which is not possible with actual cheese making. Cheese slurry systems that allow cheese to ripen at 30°C for 5 to 30 d have been used to rapidly evaluate flavor and proteolytic potential of starters and nonstarters (Kristoffersen et al. 1967; Farkye et al. 1995; Muehlenkamp-Ulate and Warthesen 1999; Coskun 2006; Kumar et al. 2007). Cheese slurries may be an efficient way to gather information; however, higher moisture and temperatures influence its chemical and enzymatic reactions.

Kristoffersen et al. (1967) prepared cheese curd slurries and incubated them at 30°C for five days. The authors found that the slurries could yield flavors similar to that of cheeses ripened for 3 months. The accelerated ripened cheese slurry approach closely proximates conditions in cheese and can be used to screen starter bacteria for cheese making (Farkye et al. 1995, Muehlenkamp and Warthesen 1999). In addition, this method can be used to study the contribution of various adjuncts and enzymes to cheese ripening and flavor development (Briggs et al. 2003; Coskun 2006; Kumar et al. 2007).

Briggs et al. (2003) used cheese slurries to evaluate the potential of accelerating cheese ripening via the addition of attenuated starter cultures by pulsed electric fields (PEF). The results suggested that a successful attenuation of the starter cultures is possible when the cells are treated with an electric field strength of 20 kV/cm and 100 pulses.

Coskun (2006) determined the effect of low pressure-homogenization of lactic acid bacteria (LAB) on the development of proteolysis in a cheese slurry medium. The cheese slurries were incubated with and without homogenized cultures at 30°C for up to 72 h. During incubation, the changes in trichloroacetic acid-soluble nitrogen (TCA-SN) and phosphotungstic acid-soluble nitrogen (PTA-SN) as well as pH were monitored. The results showed that pH development was slower in the slurries to which homogenized cultures were added. Higher TCA-SN and PTA-SN values were obtained from the slurries incubated with homogenized cultures.

Kumar et al. (2007) investigated the influence of exogenous enzymes and elevated ripening temperatures on biochemical characteristics of a cheese slurry during accelerated ripening**.** Flavor development in cheese was accelerated using exogenous proteolytic and lipolytic enzymes (1:1 ratio) and ripening at elevated temperatures (20, 30 and 37°C) up

to 12 days. Biochemical characteristics and sensory evaluation of the product were studied. The usage of exogenous enzymes, temperature of ripening, ripening period had significant influence on all biochemical characteristics. Such cheese slurry could be successfully incorporated in the preparation of processed cheese products to obtain appropriate flavor. At the same time, increased dosage of enzymes or use of elevated temperatures of ripening may lead to acid, bitter and rancid flavors.

2.5.6 Heat effect on milk and cheese quality

Traditionally, all cheeses were made from raw milk. The pasteurization of cheese milk became increasingly popular from the beginning of twentieth century, primarily for public health reasons. Under pasteurization conditions, several major known important changes in milk in addition to microbial reduction have been reported and these changes should be well understood and closely related to the final qualities of cheeses.

2.5.6.1 Whey protein denaturation and its interactions with casein

Heat treatment of milk during commercial processing results in a series of physico-chemical changes in the milk constituents. Significant changes in heated milk (> 60°C) include the denaturation of whey proteins, interactions between denatured whey proteins and the casein micelles and the transfer of soluble minerals to the colloidal state. Casein micelles are very stable at high temperatures although changes in zeta potential, size, hydration of micelles, as well as some association-dissociation reactions do occur at severe heating temperatures (Fox 1981; Singh and Kanawjia 1988; Singh and Creamer, 1992). Thermal denaturation and aggregation of whey proteins have been well studied. When heating milk above 65°C, whey proteins are denatured by the unfolding of their polypeptides, thus exposing the side chain groups originally buried within the native structure. The unfolded proteins then interact with casein micelles or simply aggregate

with themselves, involving thiol-disulphide interchange reactions, hydrophobic interactions and ionic linkages (Oldfield et al. 1998; Corredig and Dalgleish 1999; Oldfield et al. 2000; Vasbinder et al. 2003).

Denatured whey proteins have been shown to interact with the casein micelles. The interaction is largely between β-lactoglobulin and κ-casein and involves both disulphide and hydrophobic interactions (Smits and van Brouwershaven, 1980, Singh and Fox, 1987; Sandra and Dalgleish 2007).

2.5.6.2 Rennet coagulation properties of heated cheese milks

It is well established that the interaction of whey proteins with the casein micelles has both positive and negative implications in cheese manufacture. On one hand, denatured whey proteins could be incorporated into cheese curd, resulting in a higher yield from a given quantity of milk; on the other hand, the interactions of whey protein with casein micelles interfere with the rennet coagulation process, resulting in long coagulation times and weak curd structures (Singh and Waungana 2001).

The reduction in gel firmness is presumably caused by the disruption of the continuity of the gel network caused by attachment of denatured whey proteins to casein micelles. The denatured whey proteins may sterically hinder the close approach and contact between casein micelles, resulting in a weaker, looser network due to reduced cross linking (Vasbinder et al. 2003).

It is generally agreed that the secondary phase of rennet coagulation is more adversely affected by heating than the enzymatic phase. Denatured whey proteins on the surface of casein micelles sterically hinder the aggregation of rennet-altered micelles, resulting in prolonged rennet coagulation time (Singh and Kanawjia 1988). Other factors, such as the formation of heat-induced colloidal calcium phosphate (CCP), may also affect

the rate of aggregation of renneted micelles in heated milk (Darling and Dickson 1979; Schmidt and Poll 1986).

2.5.6.3 Proteolysis properties of heated cheese milks

Limited information is available on the effects of heat treatment of cheese milk on the proteolysis during cheese ripening. Benfeldt et al. (1997) reported that cheeses (Danbo) made from heated milks show decreased proteolysis of caseins, which resulted in slower formation of small peptides and free amino acids. Also, examination of various cheeses by Scanning Electron Microscopy (SEM) showed that as the intensity of heat treatment increases, the protein matrices of cheese become coarser and less homogeneous in appearance and contain numerous small holes or cracks. As a result, cheeses made from heated milk differ from raw milk cheese in body, texture and flavor profiles (Singh and Waungana 2001).

2.5.7 PEF effect on milk and cheese quality

In recent years, with the demand of high quality milk and milk products, more and more researchers have focused on studies of loss of organoleptic and physicochemical characteristics in milk and milk products following treatment with pulsed electric field (Dunn 1996; Qin et al. 1995a; Bendicho et al. 1999; Michalac et al. 1999; Yeom et al. 2001; Evrendilek et al. 2001; Sepulveda et al. 2005b; Li et al. 2003; Shin et al. 2007; Shamsi et al. 2008). As for the PEF effect on cheese process and quality, limited research work was found (Sepulveda-Ahumada et al. 2000).

Dunn (1996) reported that milk treated with PEF (E = 20-80 kV/cm) suffered less flavor degradation when compared to raw milk. The author proposed the possibility of manufacturing dairy products such as cheese, butter and ice cream using PEF treated milk although limited information was given in his report.

Qin et al. (1995a) carried out a study on the shelf-life, physicochemical properties, and sensory attributes of milk with 2% milk fat, treated with 40 kV/cm electric field and 6-7 pulses. No physicochemical or sensory changes were observed after treatment, in comparison with a sample treated with thermal pasteurization.

Bendicho et al. (1999) studied the destruction of riboflavin, thiamine (water-soluble) and tocopherol (liposoluble) in milk by treatment with PEF (E = 16-33 kV/cm; N = 100 pulses). The vitamin concentrations before and after treatment were determined by HPLC. The authors observed no destruction of vitamins by treatment with pulses.

Michalac et al. (1999) studied the variation in color, pH, proteins, moisture, and particle size of UHT skim milk subjected to treatment with PEF (E = 35 kV/cm; W = 3 μs and Time = 90 μs). The authors saw no differences in the parameters studied (color, pH, proteins, moisture, and particle size) before and after treatment.

Sepulveda-Ahumada et al. (2000) compared the textural properties and sensory attributes of Cheddar cheese made with heat-treated milk, PEF-treated milk (E = 35 kV /cm, N = 30 pulses), and untreated milk. In the hardness and springiness study, the cheeses made from milk pasteurized by any method were harder than those made from untreated milk. In the sensory evaluation, the panelists also found differences between the cheeses made from untreated milk and milk treated by PEF or heat. Regardless of the differences, the authors still considered using PEF treated milk to obtain cheese as a feasible option in order to improve product quality.

Yeom et al. (2001) studied a commercial, plain, low-fat yogurt mixed with strawberry jelly and strawberry syrup. They observed the changes in physical attributes (pH, color, and °Brix) and sensory attributes during storage at 4°C after treatment with both PEF (E = 30 kV/cm, t = 32 μs) and HEAT (T = 65°C, 30 s). The sensory evaluation

indicated that there were no changes between the control samples and the treated samples. There was also no variation in the color, pH, and °Brix.

Evrendilek et al. (2001) studied color, pH, °Brix, and conductivity at 4, 22, and 37°C in chocolate milk using treatment with PEF (E = 35 kV/cm; W = 1.4 μs; Time = 45 μs), and PEF + HEAT (112 and 105°C, 33 s). They compared the results with a control sample not treated by PEF or heat. Measurement of the a, b, and L parameters at 4°C revealed that the treatments of PEF at 105°C and PEF at 112°C did not cause changes in color.

Sepulveda et al. (2005b) treated HTST pasteurized milk with electric field of 35 kV/cm and 2.3 μs of pulse width, at a temperature of 65°C for less than 10 s. PEF treatments were applied either immediately after thermal pasteurization to produce an extended-shelf life product, or eight days after thermal pasteurization to simulate processing after bulk-shipping. Application of PEF immediately after HTST pasteurization extended the shelf life of milk from 45 d to 60 d, while PEF-processing after eight days caused a shelf life extension of 78 d, both was proving to be successful strategies to extend the shelf life of milk.

Li et al. (2003) investigated the effects of pulsed electric fields and thermal processing on the stability of bovine Immunoglobulin G (IgG) in enriched soymilk. PEF at 41 kV/cm for 54 μs caused a 5.3 log reduction of natural microbial flora, with no significant change in bovine IgG activity. Analysis using circular dichroism spectrometry revealed no detectable changes in the secondary structure or the thermal stability of secondary structure of IgG after the PEF treatment (Li et al. 2005). However, in an experiment investigating the effect of temperature on the stability of IgG during PEF

treatment (30 kV/cm, 54 μs), up to 20% of IgG was inactivated when the temperature was increased to 41°C (Li et al. 2003).

Shin et al. (2007) applied pulsed electric fields with square wave pulse to whole milk inoculated with *Escherichia coli*, *Pseudomonas fluorescens*, and *Bacillus stearothermophilus*. The samples were exposed to 30-60 kV/cm electric field intensity with 1μs pulse width, and 26-210 μs treatment time in a continuous PEF treatment system. Eight log reductions were obtained for *E. coli* and *P. fluorescens* and 3 logs reduction for *B. stearothermophilus* under 210 μs treatment time, 60 kV/cm pulse intensity at 50°C. There was no significant change in pH and titration acidity of milk samples after PEF treatment.

Shamsi et al. (2008) determined the effects of PEF treatments on the inactivation of Alkaline Phosphatase (ALP), Total Plate Count (TPC), *Pseudomonas* and *Enterobacteriaceae* counts in raw skim milk at field intensities of 25 - 37 kV/cm and final PEF treatment temperatures of 15°C and 60°C. At 15°C, PEF treatments of 28 to 37 kV/cm resulted in 24 - 42% inactivation in ALP activity and < 1 log reduction in TPC and *Pseudomonas* count, whereas the *Enterobacteriaceae* count was reduced by at least 2.1 log units to below the detection limit of 1 CFU/mL. PEF treatments of 25 to 35 kV/cm at 60°C resulted in 29 - 67% inactivation in ALP activity and up to 2.4 log 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. Kinetic studies suggested that the effect of field intensity on ALP inactivation at the final PEF treatment temperature of 60°C was more than twice that at 15°C. A combined effect was observed between the field intensity and temperature in the inactivation of both ALP enzyme and the natural microbial flora in raw skim milk. The results of this study suggest that PEF as

a non-thermal process can be employed for the treatment of raw milk in mild temperature to achieve adequate safety and shelf life while preserving the heat-sensitive enzymes, nutrients and bioactive compounds.

CONNECTING TEXT

A comprehensive review of literature demonstrated the need for further studies regarding the effect of fat content in milk on the microbial inactivation by PEF treatment. Also, in order to match the resistance of the food with the impedance of the pulse forming network, the electrical conductivities of milk with different fat content at different temperatures need to be measured.

Part of this research was presented at the 2007 NABEC Annual Conference, July 31 to August 2, Wooster, OH, USA.

CHAPTER III: INACTIVATION OF *SALMONELLA ENTERITIDIS* IN MILK BY PULSED ELECTRIC FIELD (PEF) TREATMENT: EFFECTS OF MILK FAT AND TEMPERATURE

3.1 ABSTRACT

The effects of milk fat and temperature on microbial inactivation in milk by pulsed electric field (PEF) treatment were examined. The electrical conductivity of milk was measured by on-line PEF treatment (15kV/cm, pulse width of 2 μs, 2 Hz) with temperatures varied from 5 to 55°C and milk fat content from 0 to 3.25%. Fat content had no significant effect on the electrical conductivity of milk, whereas temperature had significant effect on the electrical conductivity. The measured electrical conductivities were used to determine the size of the PEF treatment chamber. The inactivation of *Salmonella enteritidis* by a continuous PEF process (30kV/cm, treatment time of 128 μs) was studied as a function of milk fat content (0 to 3.25%) and temperature (5 to 55°C). Fat content showed different behaviours on microbial inactivation at different temperature levels. At 5 to 35°C, fat content did not have significant effect on microbial reduction by PEF treatment. However, when temperature was over 45°C, fat content seemed to hinder the PEF effect on microbial inactivation. The maximum bacterial reduction (5.25 logs) occurred in skim milk processed at 55°C, and the minimum reduction (0.89 log) was in milk treated at 5°C regardless of the milk fat content.

Keywords: Pulsed electric field, Fat content, Electrical conductivity, Milk, *Salmonella enteritidis*

3.2 INTRODUCTION

Milk is one of the most popular and commercialized liquid food products. Canadian milk production reached 7.6 million tons in 2008 (Statistics Canada 2009), indicating the enormous size of the market.

Raw milk must be pasteurized as required by regulations in Canada and in most other countries. Conventional heat pasteurization could be accomplished by two schemes: low temperature long time (LTLT 63 – 65°C, 30 min) and high temperature short time pasteurization (HTST 72°C, 15 s) (Fox et al. 2000). However, apart from bacterial inactivation, heat treatment can adversely affect flavor, taste and nutritional quality of milk. Therefore, there is a need to develop alternative processing technologies based on non-thermal techniques.

Pulsed electric field (PEF) is a non-thermal technology with potential to replace or supplement thermal treatment. It involves the application of high voltage pulses to a food placed between two electrodes for very short duration (normally less than 1 s). The pulsed energy destroys the bacterial cell membrane by mechanical effects with minimal heating of the food (Barbosa-Cánovas et al. 1999). The most widely accepted theory involves accumulation of free charges at membrane surfaces when a cell is exposed to an electric field and the resulting formation of a transmembrane potential. Membrane disruption and/or membrane breakdown occurs when the induced transmembrane potential exceeds a critical value (Barbosa-Cánovas et al. 1999). When the size and number of pores become large in relation to the membrane surface, there will be an irreversible breakdown associated with mechanical destruction of the cells (Zimmermann 1986).

The inactivation effect of PEF has been demonstrated with several microorganisms (Hülsheger et al. 1983; Pothakamury et al. 1996; Sale and Hamilton 1967; Zhang et al. 1994a, b) on a range of food products (Dunn and Pearlman 1987; Qin et al. 1995; Martin-Belloso et al. 1997; Bazhal et al. 2006). A number of researches on PEF have focused on inactivation of microorganisms in milk due to the importance of the dairy industry (Dunn and Pearlman 1987; Grahl and Markl 1996; Pothakamury et al. 1996; Sensoy et al. 1997; Dutreux et al. 2000; Floury et al. 2006).

Mild temperature effect on milk inactivation by PEF has been investigated by Evrendilek et al. 2001, Floury et al. 2006; Shin et al. 2007 and Shamsi et al. 2008. The results of these studies suggested that PEF combined with mild temperature could be used to achieve adequate safety and shelf life of milk while preserving the heat-sensitive nutrients and flavors.

The influence of milk ingredients, such as fats on microbial inactivation by PEF has also been studied, although the results are conflicting. Some investigators observed a protective effect of fats (Grahl and Markl 1996; Picart et al. 2002) or proteins (Martin et al. 1997); others did not report differences (Reina et al. 1998; Dutreux et al. 2000; Manas et al. 2001; Sobrino-Lopez et al. 2006) between microbial inactivation in buffers or milk with different fat contents.

So far, the information of milk fat effect on microbial inactivation by PEF combined with mild temperature is still lacking.

The electrical conductivity of a food medium, defined as the ability to conduct electric current, is an important variable affecting the PEF treatment (Barbosa-Cánovas et al. 1999). Normally, foods with high electrical conductivities are difficult to work with since they generate low peak electric fields across the treatment chamber as a result of the

high current they typically generate during PEF treatment (Barbosa-Cánovas et al. 1999). For a square wave PEF unit, the size of PEF treatment chamber needs to be carefully designed by matching the food medium resistance with the characteristic impedance of the PEF unit to allow higher energy transfer and proper treatment of the food (Barbosa-Cánovas et al. 1999). Therefore, the measurement of electrical conductivity is the prerequisite to select proper PEF treatment chamber size.

Electrical conductivity increases with an increase in temperature (Ruhlman et al. 2001). Thus determination of electrical conductivity of liquid foods over a wide range of temperature is critical for the design and optimization of PEF processes (Amiali et al. 2006a). Electrical conductivity can be measured using conductivity meter, LCR meter or calculated based on parameters tested on-line during PEF treatment.

$$R_l = \frac{V_l}{I} \tag{3.1}$$

$$\sigma = \frac{d}{AR_l} \tag{3.2}$$

where R_l (Ω), V_l (V), I (A), σ (S/m), A (m^2), and d (m) are the load resistance, voltage, current across the food, electrical conductivity of the food, electrode surface area and gap between the electrodes, respectively.

Based on the introduction above, the objectives of this study were to (1) measure the electrical conductivity of milk with different fat contents under different temperatures and select suitable PEF treatment chamber size; (2) investigate the effects of temperature and fat content in milk on microbial inactivation by PEF treatment.

3.3 MATERIALS AND METHODS

3.3.1 Milk product

Three pasteurized milk products, namely skim milk, 2% fat content milk and 3.25% fat content milk (whole milk) were used for electrical conductivity test, as well as microbial inactivation test. The pasteurized milk products were obtained from a local supermarket. The products were stored at 4^{o}C prior to PEF treatment.

3.3.2 Electrical conductivity measurement

Electrical conductivities of the milk were measured on-line with voltage and current across a continuous PEF treatment chamber developed for egg products (Amiali et al. 2006b). The 1.45 mL chamber consisted of two parallel stainless steel electrodes with 0.5 cm gap surrounded with a Derlin® polyoxymethylene insulator. The pulsed electric field system used was a 30 kV generator with the output impedance of 100 Ω. The output voltage had a bi-phasic instant reversal square waveform with pulse duration of 2 μs.

The product temperature was maintained at different set values between 5 and 55°C by passing the milk through a heat exchanger before it entered the treatment chamber. During the experiments, the high voltage leads from the generator were connected to the treatment chamber and the milk product was circulated through the treatment chamber. The flow rate was set at 6 mL/s using a peristaltic pump. More detailed information can be found in Amiali et al. (2006a). Electrical resistance and conductivity were calculated according to Equations 3.1 and 3.2, respectively. Three replicate measurements were conducted at each temperature.

3.3.3 Microbial inactivation test

3.3.3.1 Cultivation of microorganisms

S. enteritidis (ATCC 13076) was selected as the targeted pathogenic microorganisms in this study. Cultures were grown at 37°C with gentle agitation to the early stationary phase (about 15 h) in 50 mL of Brain Heart Infusion Broth (BHIB, DIFCO, 0037-17-8). Cells were harvested by centrifugation at 10,000 x *g* for 10 min (4°C) and the cell pellets were washed three times by re-suspension in 10 mL of sterile distilled water. The washed pellets were finally re-suspended in milk samples to obtain an initial cell concentration of ~10^7 CFU/mL.

3.3.3.2 Bacterial enumeration

Viable cells were counted before and after PEF treatment by plating on *Salmonella-Shigella* Agar (SSA, BD, 274500). The PEF treated milk samples were maintained at 4°C for about 6 h before plating on the selective media, in order to repair any injured cells. This method has been shown to be an effective technique for revival of injured cells compared to plating on a non-selective medium and followed by overlaying with the appropriate selective media (Mussa et al. 1999 and Amiali et al. 2006b). PEF-treated samples were diluted serially in 0.1% peptone water (DIFCO 1807-17-4), plated (0.1 mL) in triplicate on Petri dishes and incubated at 37°C for 18 to 24 h. Dilutions giving 30 to 300 colonies per plate were counted using a Darkfield Quebec colony counter (Model 3327, AO Scientific Instruments, Keene, NH). The counted results were expressed as Log (N/N_o), where N_o is the concentration of initial cells, and N is the cell concentration after PEF treatment.

3.3.3.3 PEF system used for microbial inactivation test

The continuous-flow PEF treatment chamber used in the milk microbial inactivation test consists of two parallel stainless steel electrodes enclosed in Derlin® polyoxymethylene. The size of the chamber was calculated based on Equation 3.1 and 3.2 using the measured electrical conductivity of milk.

For PEF treatments, a square wave pulse generator was used as described earlier. Milk samples were exposed to 30 kV/cm pulses at a frequency of 2 Hz and pulse width of 2 μs for a total of 64 pulses at 5, 15，25，35, 45 and 55°C. A cooling system was used to maintain constant temperature. Liquid flow rate through the chamber was set at 6 mL/min using a peristaltic pump (Masterflex 77521-40, Cole-Parmer Instruments Co., Vernon Hills, IL). Milk in the treatment chamber was circulated for every 8 pulses. After exposure to 80 pulses, proximately 2 mL of milk was aseptically transfer from the circuit to a sterile test tube and kept at 4°C for 6h before analysis. Treatment temperature was monitored at the inlet and outlet of the treatment chamber using K type thermocouples (OMEGA^{TM}, Stamford, CT). Less than 1°C difference was maintained between inlet and outlet temperature for each treatment cycle. The apparatus was thoroughly cleaned with alcohol (70%) and rinsed with sterile distilled water after each treatment.

3.3.4 Statistical analysis

Analysis of variance (ANOVA) was performed using the General Linear Model procedures (GLM) of the Statistical Analysis System (SAS, Version 8.02, 2001, Cary, NC, USA). Experiments were done in triplicates and the means of the three data were presented.

3.4 RESULTS AND DISCUSSION

3.4.1 Fat effect on electrical conductivity

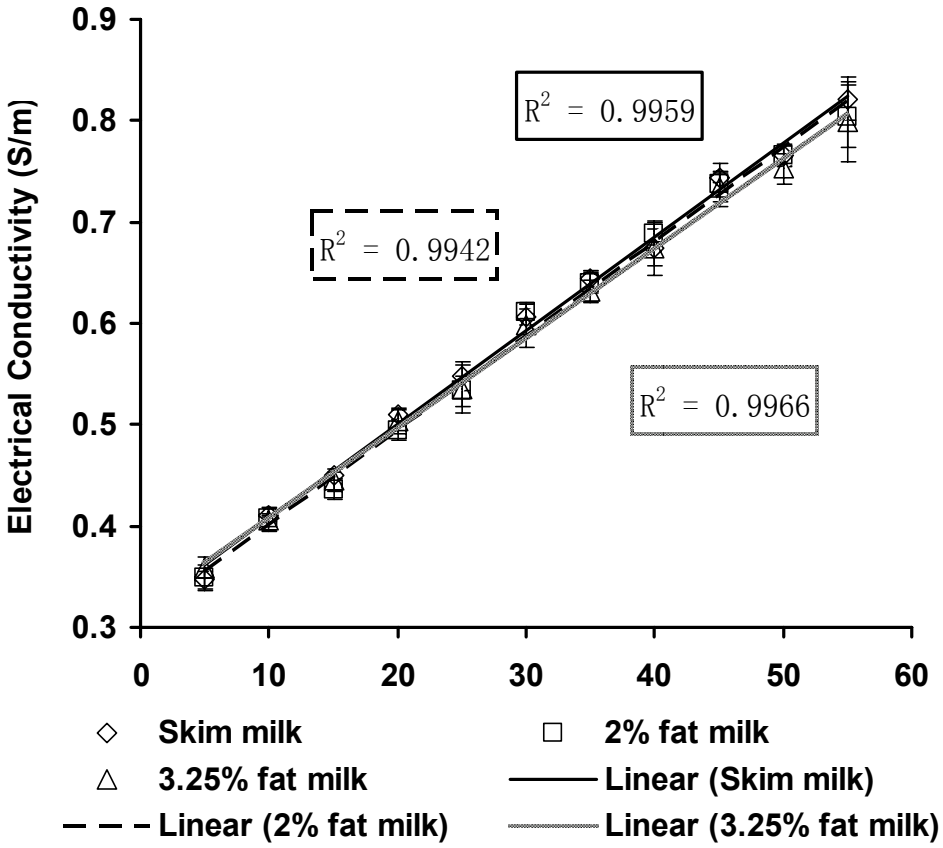

Figure 3.1 Comparison of electrical conductivity of milk with three fat contents at different temperatures (5 to 55°C). The applied electric intensity is 15kV/cm, pulse width is 2 μs, and frequency is 2Hz. The error bar was based on three replicates

Electrical conductivities of milk with three fat contents at different temperatures are presented in Figure 3.1. The analysis of variance of the data showed significant effects ($p<0.05$) of temperature on electrical conductivity (Appendix I). Electrical conductivity increased linearly with an increase in temperature (Figure 3.1). This conclusion is consistent with previous reports (Dunn and Pearlman 1987; Kent 1987; Ruhlman et al.

2001; Picart et al. 2002). Conductivity data from Dunn and Pearlman (1987) for milk was in the range of 0.32 to 0.46 (S/m) for temperatures from 17 to 43°C. Our results were in the range of 0.44 to 0.67 (S/m) for whole milk at temperatures from 15 to 40°C, a little higher than the results of Dunn and Pearlman (1987) but similar to the results of Ruhlman et al. (2001) measured by an electrical conductivity meter.

Fat content had no significant effects ($p > 0.05$) on electrical conductivity (Appendix I). Picart et al. (2002) and Ruhlman et al. (2001) also confirmed that the conductivity of milk products (whole milk and skim milk) is within a small range regardless of the fat content.

Amiali et al. (2006a) tested electrical conductivity of liquid egg and found that egg white had the highest electrical conductivity followed by whole egg and egg yolk. The authors attributed the results to the greater mineral concentration in egg white and dielectric non-conductive compounds such as fats in egg yolk, which act as insulators, thereby preventing ion migration. However, in this study, because of the much lower difference in milk fat content (0 to 3.25%) compared to egg, the fat effect on the electrical conductivity value in milk was not as significant as in egg products.

Considering the temperature effect on the electrical conductivity, the electrical conductivity at the intermediate temperature (30°C) was used to calculate the size of the treatment chamber used in milk microbial inactivation study. The distance between the electrodes was 5 mm, the total exposed electrode surface area was 102 mm^2 and the total chamber volume was 0.51 mL.

3.4.2 Fat effect on microbial inactivation

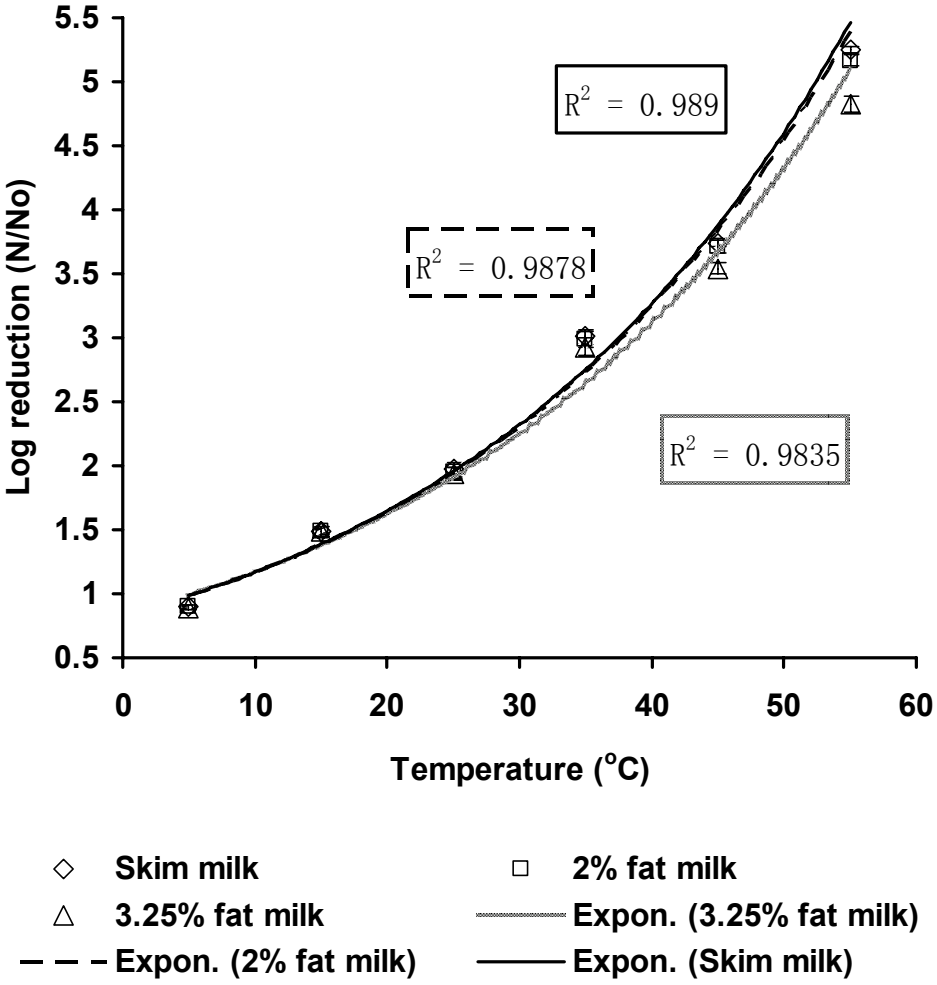

Figure 3.2 Comparison of *S. enteritidis* reduction in skim, 2% fat and whole milk exposed to square wave continuous PEF treatment at different processing temperatures (5 to 55°C). The applied electric intensity is 30kV/cm, pulse width is 2 μs, pulse number is 80, and frequency is 2Hz.

Figure 3.2 presents the microbial reduction data of *Salmonella enteritidis* in skim, 2% fat and whole milk exposed to PEF treatment at different processing temperatures (5 to 55°C). The analyses of variance are presented in two temperature ranges (5 to 35°C in

Appendix IIa and 45 to 55°C in Appendix IIb) considering the different behavior of fat content on milk microbial inactivation at different temperature levels.

Temperature had significant effect on microbial inactivation in milk (Appendix II). An increase in treatment temperature led to an exponential raise in microbial log reduction (Figure 3.2). Fat content showed different behaviors on microbial inactivation in different temperature ranges.

From 5 to 35°C, fat content did not have a significant effect on microbial log reduction ($p > 0.05$). The average log reductions of *Salmonella enteritidis* for all types of milk at 5, 15, 25 and 35°C were 0.89, 1.49, 1.94 and 2.93, respectively (Figure 3.2). These results were consistent with the findings by Reina et al. (1998) and Manas et al. (2001). Reina et al. (1998) inoculated *Listeria monocytogenes* in whole and skim milk samples, which were treated under 25°C at 30 kV/cm and frequency of 1700Hz, and no significant difference in microbial reduction was found between these two milk samples. Manas et al. (2001) used 33% emulsified fat cream to test fat effect on the inactivation of *E. coli* NCTC 9001 by PEF treatment. The treatment was conducted under 34kV/cm with a pulse frequency of 1.1Hz and temperatures less than 30°C. The result showed that the emulsified lipids do not appear to protect against microbial inactivation by electric pulses. Sobrino-Lopez et al. (2006) also presented that fat content of the milk did not modify the resistance of *Staph. aureus* to a PEF treatment, where three types of milk (whole, 1.5% and skim) were treated under 25°C, 30-35kV/cm and frequency of 100Hz.

From 45 to 55°C, fat content showed significant effect ($p < 0.05$) on microbial log reduction (Appendix IIb). At 45°C, the average reduction of *Salmonella enteritidis* in whole milk was 3.54 logs, while in 2% fat milk was 3.74 logs and in skim milk was 3.71 logs (Figure 3.2). At 55°C, the reduction of *Salmonella enteritidis* in whole milk, 2% fat

milk and skim milk were 4.82, 5.16 and 5.25 logs. The significant effects of fat content on microbial inactivation were also found by other researchers. Picart et al. (2002) claimed that whole milk with a higher fat content (3.6%) appeared to reduce *Listeria innocua* inactivation compared to skim milk at temperatures between 25 and 45°C, a pulse frequency of 1.1Hz and electric intensity of 29kV/cm. Grahl and Markl (1996) also indicated in their work that the fat particles of milk seemed to protect bacteria against electric pulses when the authors compared PEF inactivation effect of *E. coli* in 1.5% fat milk with that in 3.5% fat milk. The treatment conditions were 5-15kV/cm, 1-22Hz, and the temperatures did not exceed 45 -50°C.

The contradicting results of fat effect on milk microbial inactivation depends on many individual factors or their combined effects, such as treatment temperature, pulse frequency, the physical state of fat content and the concentration of the fat content. Picart et al. (2002) found that a higher fat content appeared to enhance microbial inactivation at 100Hz and to reduce it at 1.1Hz. The results of our study showed that the combined temperature and fat effects could lead to different behaviour of fat content on microbial inactivation in milk by PEF at different temperature levels.

3.5 CONCLUSIONS

The electrical conductivity of milk measured by PEF treatment was significantly affected by treatment temperature. However, fat content demonstrated no significant effect on the electrical conductivity of milk. The microbial inactivation in milk by PEF was significantly affected by treatment temperature. Fat content did not have significant effect on milk microbial reduction by PEF at 5 to 35°C. However, when temperature was

up to 45°C, fat content seemed to hinder the PEF effect on microbial inactivation. Further studies are needed to investigate the mechanism behind this.

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CONNECTING TEXT

Results in chapter 3 indicated that milk fat hindered microbial inactivation in combination with mild temperature between 45 and 55°C. Thus the microbial inactivation in whole milk was more difficult by pulsed electric field treatment at mild temperature. In chapter IV, an extensive study was carried out to investigate the effects of electric field intensity, treatment time as well as mild temperature on the inactivation of two pathogenic microorganisms, *Salmonella enteritidis* and *E. coli* O157:H7, in whole milk using a kinetic approach.

Part of this research was presented at the 4th NIZO Dairy Conference, June 15-17, 2005, Arnhem, Netherlands.

CHAPTER IV: INACTIVATION OF *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA ENTERITIDIS* IN WHOLE MILK BY PULSED ELECTRIC FIELD TREATMENT AND MODERATE TEMPERATURE

4.1 ABSTRACT

The effects of electric field intensity, treatment time and temperature on the inactivation of *Escherichia coli* O157:H7 and *Salmonella enteritidis* in whole milk (3.25%) by pulsed electric field (PEF) treatment were examined using a kinetic approach. Whole milk, inoculated with ~10^7 CFU / mL of either *E. coli* O157:H7 or *S. enteritidis* was treated in a continuous flow process at 20, 35, 45 and 50°C in combination with pulsed electric field intensities of 20 and 30 kV/cm. A biphasic instant reversal PEF waveform with a 2 μs pulse width was used and a maximum of 120 pulses were applied. Increasing the applied electric field intensity, treatment time and process temperature enhanced the bacterial inactivation. The maximum reduction of *E. coli* O157:H7 and *S. enteritidis* was 4.1 and 5.2 logs at 30 kV/cm and 50°C, respectively. The inactivation kinetics for both bacteria was primarily exponential, although tailing occurred in some cases. The inactivation rate constant increased from 0.016 to 0.104 μs^{-1} for *S. enteritidis* and 0.035 to 0.075 μs^{-1} for *E. coli* O157:H7 as processing temperature increased from 20 to 50°C. *E. coli* O157:H7 seemed more PEF-heat resistant than *S. enteritidis* under the treatment conditions.

Keywords: Pulsed electric field, Microbial inactivation kinetics, Whole milk, *Escherichia coli* O157:H7, *Salmonella enteritidis*

4.2 INTRODUCTION

Milk is a complex mixture of many nutrients (Martin et al. 1997). Whole milk, which contains 3.25% fat or more, has been used to produce cheeses for many decades. Milk is mostly produced on farms and easily contaminated by spoilage and pathogenic microorganisms. *Escherichia coli* O157:H7 is one of the most important pathogens found in unpasteurized milk products. It has been implicated in sporadic outbreaks of hemorrhagic colitis and hemolytic-uremic syndrome in humans. *Salmonella enteritidis* is also a dangerous food-borne pathogenic bacterium. It may contaminate milk and cheese products and cause salmonellosis in humans (Fox et al. 2000).

The control of these microorganisms is the most important issue in the handling and manufacturing of milk and dairy products. In most countries, raw milk must be pasteurized as required by strict regulations. Conventional heat pasteurization could be accomplished by two schemes: low temperature long time (LTLT 63 - 65°C, 30 min) and high temperature short time pasteurization (HTST 72°C, 15 s) (Fox et al. 2000). However, apart from bacterial inactivation, heat treatment can adversely affect flavor, taste and nutritional quality of milk. Therefore, there is a need to develop alternative processing technologies based on non-thermal techniques.

Pulsed electric field (PEF) is a non-thermal technology with potential to replace or supplement thermal treatment. It involves the application of high voltage pulses to a food placed between two electrodes for very short duration (in the order of 1 μs) using electric field strength in the range from 20 to 80 kV/cm (Barbosa-Cánovas and Rodríguez 2002).The pulsed energy destroys the bacterial cell membrane by mechanical effects with minimal heating of the food (Barbosa-Cánovas et al. 1999).

A number of studies have demonstrated the effectiveness of microbial inactivation by PEF for various milk-microorganism combinations (Dunn and Pearlman 1987; Pothakamury et al. 1996; Sensoy et al. 1997; Martin et al. 1997; Qin et al. 1998; Dutreux et al. 2000; Floury et al. 2006). However, the inactivation kinetics of whole milk inoculated with food-borne pathogenic microorganisms such as *E. coli* O157:H7 and *Salmonella enteritidis* requires further study.

Application of mild sub-lethal thermal conditions in combination with PEF has the potential to enhance microbial inactivation (Dunn and Pearlman 1987; Reina et al. 1998; Bazhal et al. 2006, Sepulveda et al. 2005). To balance the safety and quality of foods, accurate mathematical models are needed to ensure effective microbial inactivation of foods without over-processing. Different kinetic models have been used to describe microbial inactivation kinetics during PEF treatment (Hülsheger et al. 1981; Martin-Belloso et al. 1997; Alvarez et al. 2003). Several authors have reported rapid inactivation of microorganisms within early pulses and subsequent tailing phenomena (Smelt et al. 2002; Amiali et al. 2004). A two phase kinetic model has been proposed by Amiali et al. (2004) which sufficiently fitted their experimental data for whole egg and egg yolk.

The objectives of this study were to (1) investigate the effects of temperature, electric field intensity and treatment time on *E. coli* O157:H7 and *Salmonella enteritidis* in whole milk; (2) use a two phase kinetic model to relate the survival fraction of microorganisms to treatment time and temperature.

4.3 MATERIALS AND METHODS

4.3.1 Milk product

Pasteurized whole milk (3.25% fat content) was obtained from a local supermarket. The milk was stored at 4°C prior to PEF treatment.

4.3.2 Bacterial cultures

E. coli O157:H7 (ATCC 43894) and *S. enteritidis* (ATCC 13076) were grown at 37°C with gentle agitation to the early stationary phase in Brain Heart Infusion Broth (BHIB, DIFCO, 0037-17-8) as described by Amiali et al. (2006). Cells were harvested by centrifugation at 10,000 x *g* for 10 min (4°C). Cell pellets were washed three times by re-suspension in 10 mL of sterile distilled water. The washed pellets were then re-suspended in milk samples to obtain an initial cell concentration of ~10^7 CFU /mL.

4.3.3 Microbial enumeration

Viable cells were counted before and after PEF treatment by plating on Violet Red Bile Agar (VRBA, DIFCO, 0012-17) for *E. coli* and *Salmonella-Shigella* Agar (SSA, BD, 274500) for *S. enteritidis,* respectively. The PEF treated milk samples were maintained at 4°C for about 6 h before plating on the selective media, in order to repair any injured cells. This method has been shown to be an effective technique for revival of injured cells compared to plating on a non-selective medium and followed by overlaying with the appropriate selective media (Mussa et al. 1999 and Amiali et al. 2006). PEF-treated samples were diluted serially in 0.1% peptone water (DIFCO 1807-17-4), plated in triplicate on Petri dishes and incubated at 37°C for 18 to 24 h. Dilutions giving 30 to 300 colonies per plate were counted using a Darkfield Quebec colony counter (Model 3327, AO Scientific Instruments, Keene, NH). The counted results were expressed as Log

(N/N_o), where N_o is the concentration of initial cells, and N is the cell concentration after PEF treatment.

4.3.4 PEF apparatus

The continuous-flow PEF treatment chamber consisted of two parallel stainless steel electrodes enclosed in Derlin® polyoxymethylene. The distance between the electrodes was 5 mm and the total exposed electrode surface area was 102 mm^2. A 30 kV pulse generator with a matched output impedance of 100 Ω was used as described by Amiali et al. (2006). The output voltage had a bi-phasic instant reversal square waveform with pulse duration of 2 μs. Both voltage and current across the treatment chamber were monitored simultaneously using a 2-channel digital oscilloscope (TDS3000, Tektronix, Wilsonville, OR).

For PEF treatments, milk samples were exposed to 20 or 30 kV/cm pulses at a frequency of 2 Hz for a total treatment time of 0 to 240 μs at 20, 35, 45 or 50°C. A cooling system was used to maintain constant temperature. The circulation of samples during the treatment was described by Amiali et al. (2006). Liquid flow rate through the chamber was set at 6 mL/min using a peristaltic pump (Masterflex 77521-40, Cole-Parmer Instruments Co., Vernon Hills, IL). Milk in the treatment chamber was circulated for every 8 pulses. After exposure to the desired number of 40, 80 and 120 pulses, approximately 2 mL of milk were aseptically transferred from the circuit to a sterile test tube and kept at 4°C for about 6 h before plating on the selective media. Treatment temperature was monitored at the inlet and outlet of the treatment chamber using K type thermocouples (OMEGA™, Stamford, CT). Less than 1°C difference was maintained between inlet and outlet temperature for each treatment cycle. The apparatus was

thoroughly cleaned with alcohol (70%) and rinsed with sterile distilled water after each treatment.

4.3.5 Kinetic modeling

Survival fractions as a function of treatment time at each electric field treatment may be expressed by a two phase kinetic model (Equation 4.1 (Amiali et al. 2004)). The rate constants are dependent on electric field strength and temperature. Effect of temperature was described using the Arrhenius model (Equation 4.2).

$$s = s_e + (1 - s_e)e^{-kt} \tag{4.1}$$

$$k = k_0 \cdot e^{-\frac{E_a}{RT}} \tag{4.2}$$

where s is survival fraction, s_e is the tailing survival fraction, k is the kinetic rate constant and t is the treatment time (μs), k_0 is a constant (1/μs), E_a is activation energy (J / mol), R is gas constant (8.31 J / °K mol), T is the treatment temperature (°K).

4.3.6 Statistical analysis

Regression analyses were performed by Sigmaplot software (Sigmaplot, version 6.00, 2000, SPSS Inc, Chicago, Illinois). Experiments were conducted in triplicates and the means of the three data were presented.

4.4 RESULTS AND DISCUSSION

4.4.1 Microbial inactivation

The inactivation of *E. coli* O157:H7 and *S. enteritidis* at different process temperatures, treatment times and applied electric field intensities are shown in Figure 4.1 and 4.2. The maximum reduction of *E. coli* O157:H7 and *S. enteritidis* was 4.1 and 5.2 logs, respectively, at 30 kV/cm and 50°C with 240μs treatment time.

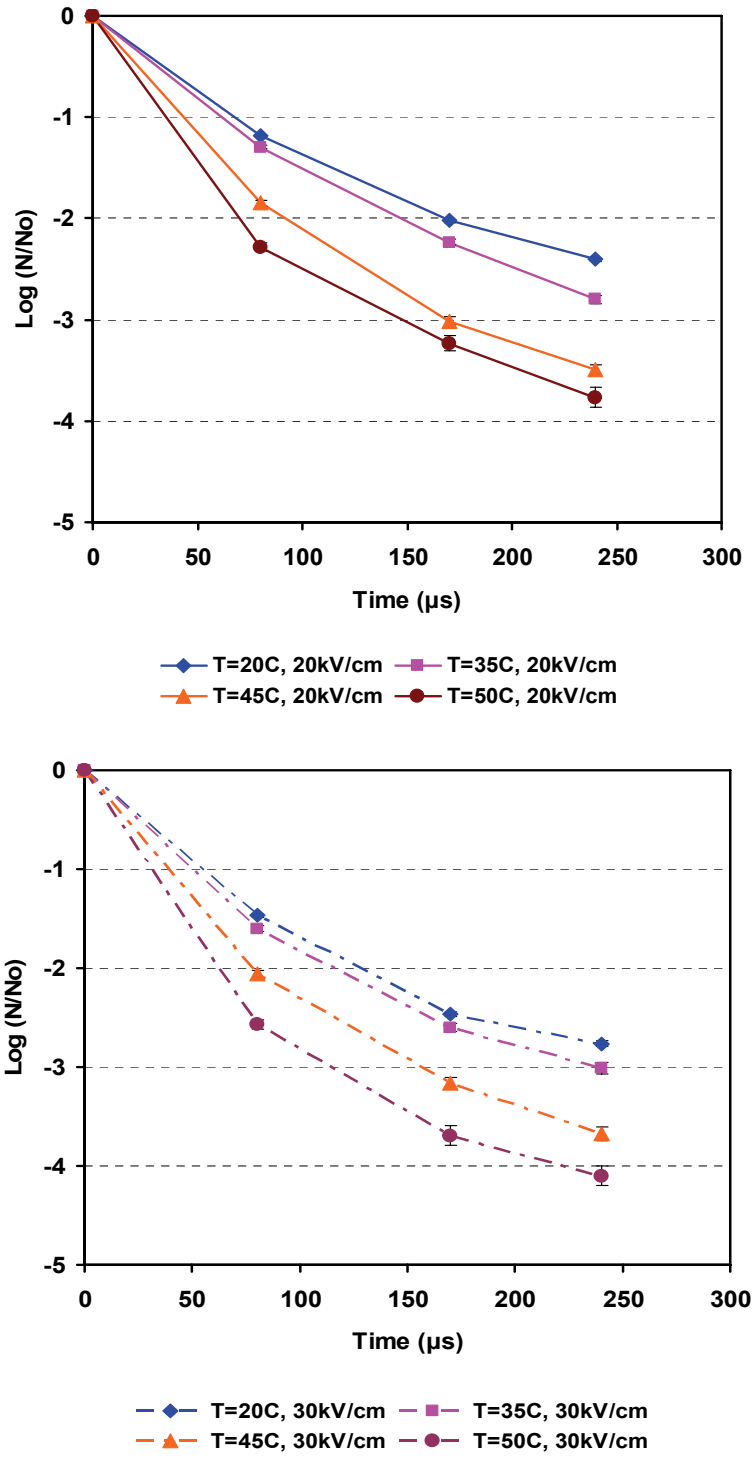

Figure 4.1 Survival fractions of *E. coli* O157:H7 in whole milk as a function of PEF treatment time and temperature at (a) 20 and (b) 30 kV/cm of electric field intensity

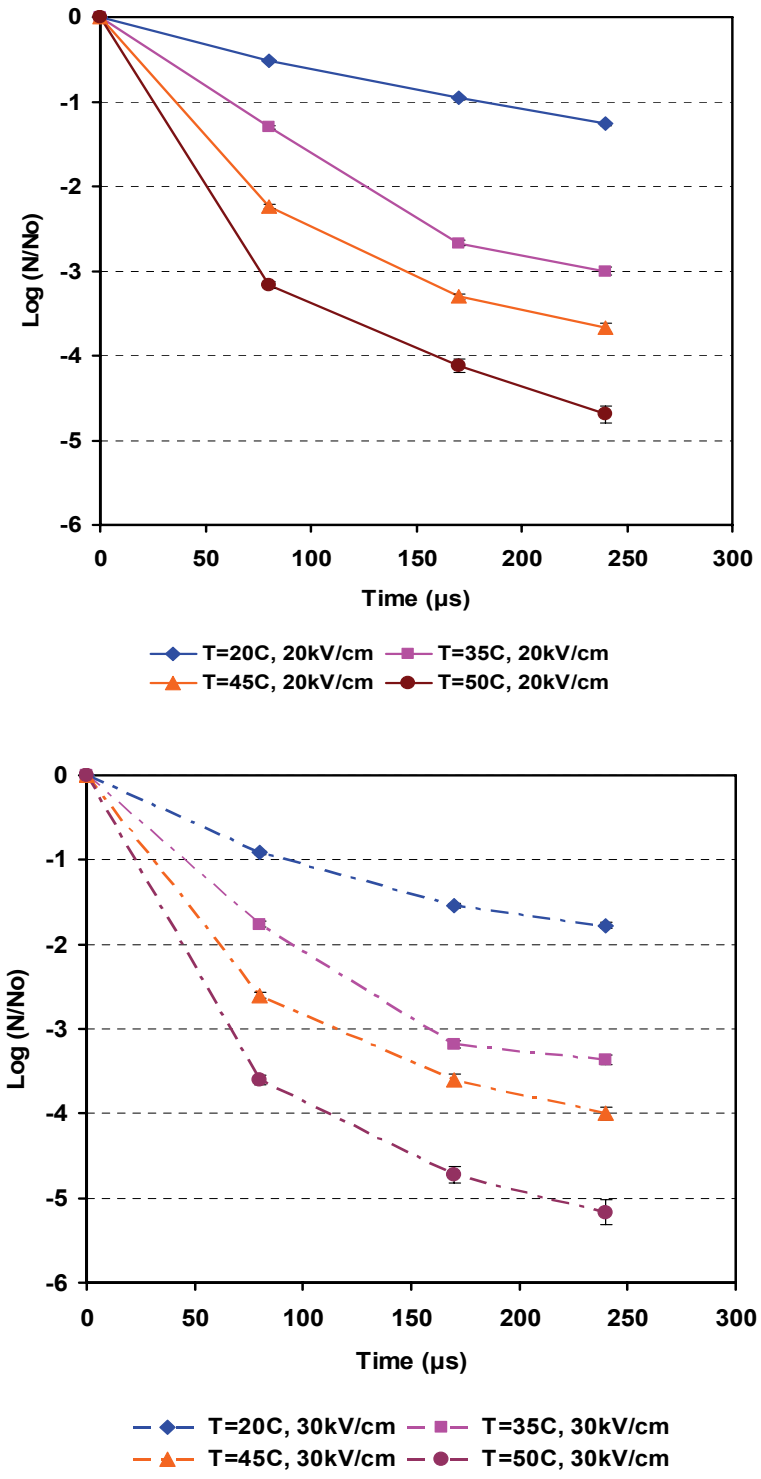

Figure 4.2 Survival fractions of *S. enteritidis* in whole milk as a function of PEF treatment time and temperature at (a) 20 and (b) 30 kV/cm of electric field intensity

As expected, the survival fraction of *E. coli* or *Salmonella* (initially 10^7 CFU/mL in whole milk) decreased with increasing temperature (Figure 4.1 and 4.2), which is consistent with the findings of previous studies (Pothakamury et al. 1996; Sensoy et al. 1997; Reina et al. 1998). Pothakamury et al. (1996) studied Simulated Milk Ultrafiltrate (SMUF) inoculated with *E. coli* and treated with PEF (36kV/cm, 40 pulses). The authors found that microbial reduction increased from 4 to 5 logs when temperature increased from 7 to 20°C. Sensoy et al. (1997) treated *Salmonella dublin* inoculated in skim milk by PEF (25kV/cm, 100 pulses) and observed that when temperature increased from 25 to 50°C, the microbial reduction increased by 1 log. Reina et al. (1998) also proposed that the increase in treatment temperature (from 25 to 50°C) contributed to higher effectiveness in the inactivation of *Listeria monocytogenes* in whole milk. At high temperatures, the increased inactivation of microorganisms could be attributed to the less ordered liquid crystalline structure of the microbial cell membrane, which makes the bacterial cells more susceptible to breakdown (Stanley 1991; Russell 2002).

For *E. coli* O157:H7 exposed to 240μs of 30kV/cm, increasing the temperature from 20 to 35°C, increased the microbial reduction by 0.24 log cycle, whereas for *S. enteritidis* the corresponding increase was 1.6 logs cycle. Similar trends were obtained as the temperature increased from 45 to 50°C. However, as the temperature increased from 35 to 45°C, the increase in survival fraction did not follow the same trend. So at most *S. enteritidis* seemed to be more sensitive to temperature change compared to *E. coli* O157:H7. Similar trends were obtained by Amiali et al. (2006) in liquid egg treated at 10, 20 and 30°C using electric field intensity of either 20 or 30kV/cm.

In Figure 4.1 and 4.2, it was shown that 30 kV/cm of electric field intensity resulted in greater bacterial inactivation than 20 kV/cm. This result was also

demonstrated by other studies although the treatment conditions may vary (Hülsheger et al. 1983; Zhang et al. 1994; Martin et al. 1997; Barbosa-Cánovas et al. 1999; Amiali et al. 2006). For *E. coli* O157:H7 at 20°C, increasing the field intensity decreased the survival fraction by 0.3 log cycle whereas for *S. enteritidis* the corresponding decrease was 0.5 log cycle. Similar trends were obtained at all other temperatures of 35, 45 and 50°C. Therefore, *S. enteritidis* was more sensitive to changes in electric field at the same temperature conditions. Amiali et al. (2006) obtained similar results for liquid egg treated at 10, 20 and 30°C using electric field intensity of either 20 or 30kV/cm.

4.4.2 Inactivation kinetics

The two phase kinetic model (Equation 4.1) adequately described the survival fraction of *E. coli* O157:H7 and *S. enteritidis* in whole milk within the experimental range of the present study (R^2 = 0.999 for all treatment levels). The kinetic rate constants (k) and tailing survival fraction (S_e) determined from Equation 4.1 were used to compare the behavior of *E. coli* O157:H7 to *S. enteritidis* (Table 4.1 and 4.2). The value of the kinetic rate constant (k) varied from 0.035 to 0.075 μs^{-1} and 0.016 to 0.104 μs^{-1} for *E. coli* O157:H7 and *S. enteritidis*, respectively. An increase in treatment temperature led to an exponential raise in k values. Sensoy et al. (1997) inactivated *S. dublin* in skim milk using 25kV/cm field intensity at temperatures varying from 10 to 50°C and obtained k values in the range from 0.044 to 0.083 μs^{-1}. At electric field of 26 kV/cm and temperature of 37°C, using batch treatments at different pulse rate and pulse duration, Martín-Belloso et al. (1997) obtained kinetic constant values in the range of 0.029 to 0.088 μs^{-1} for *E. coli* (ATCC 11229) in liquid egg. Amiali et al. (2006) inactivated *E. coli* O157:H7 and *S. enteritidis* in egg yolk using 20 to 30kV/cm field intensity at temperatures varying from 20 to 40°C and obtained k values in the range from 0.009 to 0.039 μs^{-1} for *E. coli*

O157:H7 and 0.004 to 0.098 for *S. enteritidis*. The values of kinetic constants obtained in this study were within the range as those reported in literature by various authors although variations existed due to the differences in media, temperature range, treatment chamber, microbial variation, type of flow, experimental setup, and so on.

Table 4.1 Parameters of two phase kinetic model* for inactivation of *E. coli* O157:H7 in whole milk at different electric field intensity and temperatures

Electric field (kV/cm)	Temperature (^oC)	Parameter k	Parameter S_e	R^2
20	20	0.035	0.01	0.999
	35	0.038	0.01	0.999
	45	0.054	0.00	0.999
	50	0.067	0.00	0.999
30	20	0.043	0.00	0.999
	30	0.047	0.00	0.999
	45	0.060	0.00	0.999
	50	0.075	0.00	0.999

*: $S = s_e + (1 - s_e)e^{-kt}$

The Arrehnius equation was used to describe the changes in kinetic rate constants with respect to temperature. The data for *E. coli* O157:H7 yielded Equation 4.3 and 4.4 for electric field intensity of 20 and 30kV/cm, respectively.

$$k = 25.87 \cdot e^{\left[\frac{-16.28 \cdot 10^3}{8.31 \cdot T}\right]} \tag{4.3}$$

$$k = 10.37 \cdot e^{\left[\frac{-13.51 \cdot 10^3}{8.31 \cdot T}\right]} \quad (4.4)$$

The data for *S. enteritidis* fit Equation 4.5 and 4.6 at the electric field strengths of 20 and 30kV/cm, respectively.

$$k = 1.66 \cdot 10^6 \cdot e^{\left[\frac{-44.97 \cdot 10^3}{8.31 \cdot T}\right]} \quad (4.5)$$

$$k = 2.49 \cdot 10^4 \cdot e^{\left[\frac{-33.42 \cdot 10^3}{8.31 \cdot T}\right]} \quad (4.6)$$

Table 4.2 Parameters of two phase kinetic model* for inactivation of *S. enteritidis* in whole milk at different electric field intensity and temperatures

Electric field (kV/cm)	Temperature (^oC)	Parameter K	Parameter S_e	R^2
20	20	0.016	0.04	0.999
	35	0.038	0.00	0.999
	45	0.065	0.00	0.999
	50	0.092	0.00	0.999
30	20	0.028	0.02	0.999
	30	0.051	0.00	0.999
	45	0.076	0.00	0.999
	50	0.104	0.00	0.999

*: $s = s_e + (1 - s_e)e^{-kt}$

The results (Table 4.1 and 4.2) showed that the kinetic rate constants increased with increasing temperature, as expected. Higher values of inactivation rate constants were obtained at lower temperature of 20°C for *E. coli* O157:H7 compared to *S. enteritidis*. However, at the higher temperature of 45 or 50°C, the rate constant for *E. coli* O157:H7 was considerably lower than for *S. enteritidis*. The rate of change of kinetic rate constant with respect to temperature was lower for *E. coli* O157:H7 than for *S. enteritidis*. Apparently, *S. enteritidis* was more resistant to PEF inactivation at lower temperature but more susceptible to PEF inactivation at higher temperatures. The conclusion was similar to that of Amiali et al. (2006), who found that *S. enteritidis* inoculated in egg yolk was more resistant at lower temperature (20°C) but more susceptible to PEF inactivation at higher temperatures (40°C) compared to *E. coli* O157:H7.

The tailing survival fraction (s_e) only showed up at lower temperature and electric intensity combinations (Table 4.1 and 4.2). For *E. coli* O157:H7, the s_e at 20kV/cm and 20 and 35°C were 0.01, while at all other treatment conditions was zero. For *S. enteritidis,* the s_e at 20kV/cm and 20°C was 0.04, and at 30kV/cm and 20°C was 0.02, while at all other treatment conditions were zero. It seemed that the inactivation kinetics for both bacteria was primarily exponential (a special case of the two phase model with $s_e = 0$), except in some cases with some tailing.

4.5 CONCLUSIONS

PEF treatment inactivated *E. coli* O157:H7 and *S. enteritidis* in whole milk. The extent of bacterial inactivation is a function of electric field intensity, treatment time and treatment temperature. The maximum reduction of *E. coli* O157:H7 and *S. enteritidis* was 4.1 and 5.2 logs, respectively, at 30 kV/cm and 50°C. The inactivation kinetics for both

bacteria was primarily exponential, except in some cases with some tailing. The temperature effect on the microbial inactivation by PEF was expressed by Arrhenius equation. Lower kinetic rate constant (*k*) was obtained for *E. coli* O157:H7 in whole milk, indicating greater resistance to heat-PEF treatment compared to *S. enteritidis*.

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CONNECTING TEXT

Chapter IV demonstrated the effectiveness of pulsed electric field and mild temperature on the inactivation of pathogenic microorganisms (*Salmonella enteritidis* and *E. coli* O157:H7) inoculated in whole milk. In Chapter V, the coagulation properties of milk treated by PEF and mild temperature were investigated, and compared with those from raw and thermally pasteurized milk.

Part of this research was presented in 2005 IFT annual meeting (New Orleans, USA) and was among the five finalists of the George Stewart International Research Paper Competition.

CHAPTER V: EFFECTS OF TEMPERATURE AND PULSED ELECTRIC FIELD TREATMENT ON RENNET COAGULATION PROPERTIES OF MILK

5.1 ABSTRACT

The objective of this study was to determine and compare the coagulation properties of pulsed electric field (PEF) treated milk with raw and thermally pasteurized milk. PEF treatment was achieved in a continuous chamber using a bipolar square waveform. Electric field intensity (E) of 20 to 30 kV/cm, pulse number (N) from 40 to 120, and treatment temperature of 20 to 50°C were applied. Dynamic rheological test was conducted to analyze milk coagulation properties. Results indicated that E, temperature, and N significantly impaired milk coagulation properties in terms of rennet coagulation time and curd firmness, and pasteurized milk from PEF combined with mild heat showed better rennetability compared to thermally pasteurized milk.

Keywords: Pulsed electric field, Rennet coagulation, Milk

5.2 INTRODUCTION

Raw milk cheeses possess unique flavor and texture not typically obtainable in cheeses made from pasteurized milk. However, raw milk cheeses have been involved in the majority of cheese related foodborne illness outbreaks (IFST 1998). Pasteurization of milk can be effectively achieved by heating using appropriate temperature-time schemes (Fox et al. 2000). However, apart from bacterial inactivation, heat treatment can adversely affect the flavor, taste and nutrients of the product. Therefore, applying non-thermal pasteurization, such as pulsed electric field (PEF) in cheese making is attracting attention.

PEF processing uses short bursts of electricity for microbial inactivation at relatively low temperature and causes minimal effect on food quality. A great number of studies on PEF have targeted the inactivation of microorganisms in milk due to the importance of the dairy industry (Zhang et al. 1995; Grahl and Märkl 1996; Martin et al. 1997; Calderón-Miranda et al. 1999; Dutreux et al. 2000; Fernández-Molina 2001; Evrendilek and Zhang 2005). However, limited research has focused on studies on PEF effects related to cheese making. Dunn (1996) reported that milk treated with PEF (E = 20-80 kV/cm) suffered less flavor degradation when compared to raw milk. The author proposed the possibility of manufacturing dairy products such as cheese, butter and ice cream using PEF treated milk although no detailed information was given in the report. Qin et al. (1995) carried out a study of shelf-life, physicochemical properties, and sensory attributes of milk with 2% milk fat, treated with 40 kV/cm of electric field and 6-7 pulses. No physicochemical or sensory changes were observed after treatment, in comparison with a sample treated with thermal pasteurization. However, Sepulveda-Ahumada et al. (2000) found some differences between the textural properties and sensory attributes of

Cheddar cheeses made with heat-treated milk, PEF-treated milk (E = 35 kV /cm, N = 30 pulses), and untreated milk. In terms of the hardness and springiness, the cheeses made from milk pasteurized by any method were harder than those made from untreated milk. As for the sensory, differences between the cheeses made from untreated milk and milk treated by PEF or heat were also found.

Rennet coagulated cheese represents about 75% of total cheese production and includes most major cheese varieties (Fox et al. 2000). Rennet induced milk coagulation is one of the important steps in rennet coagulated cheese making. Understanding the effect of PEF treatment on the rennet coagulation properties of milk is necessary for applying PEF for milk pasteurization prior to cheese making. However, the information on this issue is still lacking.

The rennet coagulation of milk under still conditions involves the conversion of milk from a colloidal dispersion of stable micelles to a gel network of aggregated micelles, which forms a continuous phase, entrapping moisture and fat globules in its pores. The gel formation is accompanied by a number of physicochemical changes, including the hydrolysis of κ-casein, the increase of the glycomacropeptide concentration; the aggregation of sensitized para-casein micelles and the increases of the viscosity and elasticity of the milk (Fox et al. 2000). Numerous methods, based on the detection of the above changes, have been developed for monitoring cheese formation. Major types of measuring instrument include (1) formagraph: measures the viscous drag of gelling milk by suspending a pendulum in the milk and it determines the tilt of the pendulum over time; (2) rotational viscometers: measure the apparent viscosity of gelled milk after a given time at a fixed shear rate; (3) hot wire probe: measures thermal conductivity of the gelling milk sample; (4) near infrared diffuse reflectance probe:

measures reflectance of near infrared light of the gelling milk sample and (5) controlled strain or controlled stress rheometers: measure dynamic parameters such as viscosity, elastic modulus (G'), viscous modulus (G"), and phase angle (δ) by applying a low-amplitude oscillating strain or stress to the milk sample (Fox et al. 2000).

Dynamic rheometer has been used increasingly as a research tool for the continuous measurement of the viscoelastic properties of renneted milks as a function of time following rennet addition (Samuelsson et al. 1997; Curcio et al. 2001; Lucey et al. 2001; Srinivasan and Lucey 2002). This approach gives parameters that are rheologically precise and accurately quantify the dynamic rheological changes that occur during renneting without altering the process of gel formation (Bohlin et al. 1984). Hence, it accurately reflects the changes in curd firmness that occur upon renneting milk in the cheese vat under quiescent conditions. G' and δ are major rheological parameters. Curd Firmness (CF) is derived from the G'-time and the Rennet Coagulation Time (RCT) is derived from δ-time curves and they are the two mostly used dynamic rheological parameters representing rennet coagulation properties (Fox et al. 2000; O'Callaghan et al. 1999).

The objective of this study was to determine the rennet coagulation properties of PEF treated milk using the dynamic rheological measurement approach.

5.3 MATERIALS AND METHODS

5.3.1 Milk product

Raw milk with 3.14 ± 0.02 % (w/w) protein and 3.81 ± 0.06% (w/w) fat was provided by the dairy farm of Macdonald campus, McGill University (Ste Anne de Bellevue, QC). It was filled in sterile plastic bottles and stored at 4°C for less than 4 h

prior to PEF or heat pasteurization. Thermally pasteurized milk (i.e. heat treated milk) was prepared by batch heating raw milk at 63°C for 30 minutes in a water bath.

5.3.2 PEF equipment and treatment conditions

A 30 kV pulse generator with a matched output impedance of 100 Ω and a continuous treatment chamber system were used in the experiment. The output voltage profile was bi-polar instant reversal square waveform. The treatment chamber consists of two parallel stainless steel electrodes, separated by a 50 mm thick polyoxymethylene Derlin® insulator, with 102 mm^2 of surface area. The voltage and current across the treatment chamber were captured simultaneously using a 2 channel digital oscilloscope (TDS3000, Tektronix, Wilsonville, OR).

The samples were treated following the procedure described by Amiali et al. (2006). A cooling system was used to maintain minimum temperature change during the treatment (less than 1°C difference between inlet and outlet temperature for each treatment cycle).

5.3.3 Experimental parameters

A full factorial design was used in this study. The parameters and selected levels were: electric field intensity (E) of 20 and 30 kV/cm; outlet temperature of 20, 35, 45 and 50 ± 1°C; pulse number of 40, 80 and 120 pulses. The pulse width was 2 μs and the frequency of pulses was set at 2 Hz. Treatment time was calculated as the product of pulse number and pulse width. Each treatment condition was conducted in triplicate.

5.3.4 Coagulation test

To follow the coagulation of PEF treated milk, dynamic rheological measurements were performed with a controlled stress rheometer (AR2000, TA

Instruments, New Castle, DE) in an oscillatory mode using a concentric cylindrical geometry. Milk sample was initially adjusted to pH 6.4 by adding 2% (v/v) liquid starter culture (Institute Rossel Inc., St-Laurent, Quebec, Canada). Then 0.02% (w/v) liquid rennet (Institute Rossel Inc., St-Laurent, Quebec, Canada) was added to the milk sample, the sample was transferred to the cylinder of the rheometer and the test began instantly. The amplitude of oscillation was kept at 0.01 of strain to ensure linear behavior; the frequency of oscillation was 1 Hz. Measurements were taken at 0.5 min interval for 1 h.

Parameters obtained included rennet coagulation time (RCT), which was the time at which phase angle (δ) fell to unity, and curd firmness (CF), which was the G' value after 1 hr of the test.

5.3.5 Statistical analysis

Analysis of variance (ANOVA) was performed using the General Linear Model procedures (GLM) of the Statistical Analysis System (SAS, Version 8.02, 2001, Cary, NC, USA). Experiments were conducted in triplicates and the means of the three data are presented.

5.4 RESULTS AND DISCUSSION

Figure 5.1 presents the typical CF profile derived from G'-time curve. CF was defined as the G' value after 1 h of the rheometry test. Higher CF normally means better rennetability of milk, Figure 5.2 presents the typical RCT profile derived from the δ-time curve. RCT was defined as the time at which phase angle (δ) fell to unity. Lower RCT is a sign of better gelation potential of milk.

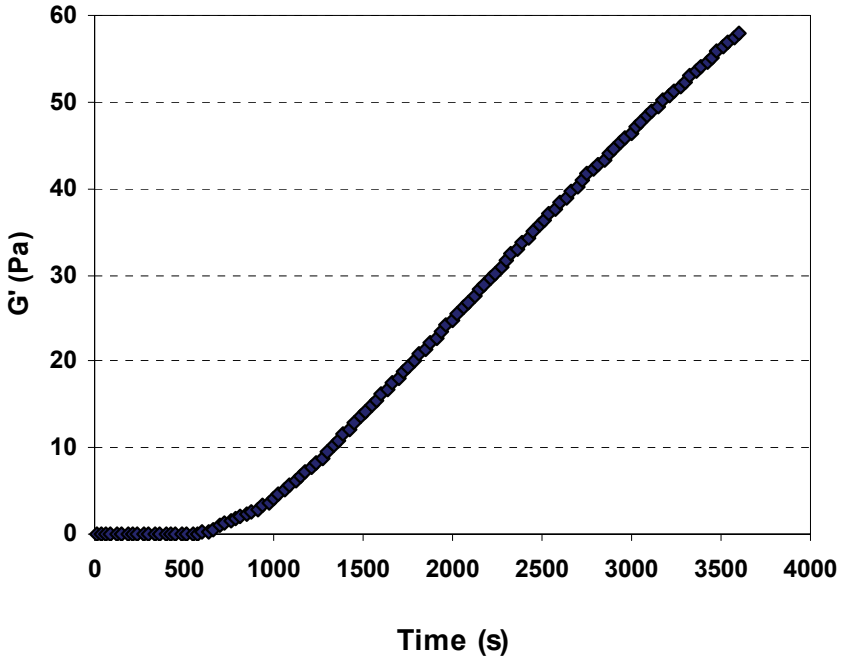

Figure 5.1 Typical Curd Firmness (CF) profile measured by rheological test (CF was the G' value after 1 hr of the test)

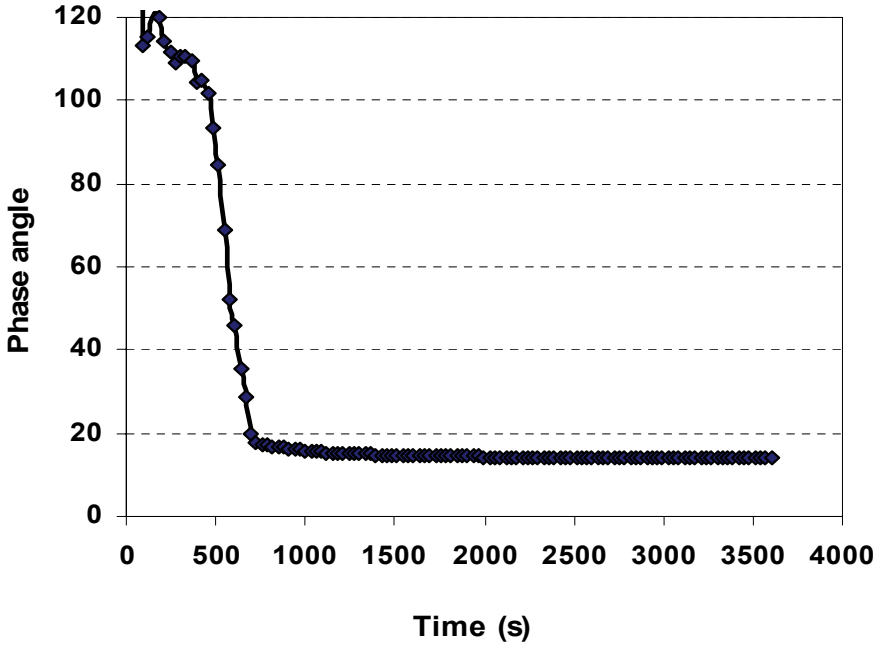

Figure 5.2 Typical Rennet Coagulation Time (RCT) profile measured by rheological test (RCT was the time at which phase angle (δ) fell to unity)

The results of changes in CF and RCT as a function of electric field intensity, pulse number and treatment temperature are presented in Figure 5.3 to Figure 5.6. Appendix III and IV are ANOVA results for CF and RCT, respectively.

5.4.1 Effects of PEF treatment and temperature on curd firmness

The analysis of variance (Appendix III) indicated that electric field intensity (E) was the most significant ($p<0.05$) factors for changes in CF, followed by treatment temperature. Increasing E and temperature decreased CF.

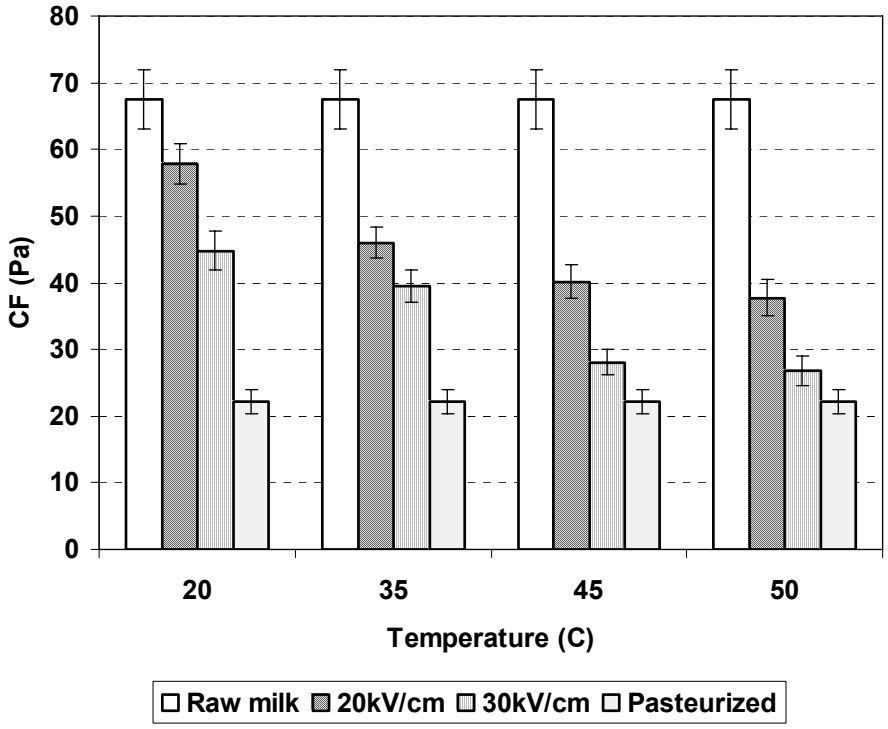

Figure 5.3 Effects of electric field intensity and temperature on curd firmness (CF). The applied pulse width is 2μs, pulse frequency is 2Hz, and pulse number is 120.

Raw milk coagulum showed the highest CF value, pasteurized milk gave the lowest CF value and PEF treated milk gave intermediate values (Figure 5.3). For PEF treated samples, all the CF values obtained at 20kV/cm were higher than the CF values

for pasteurized milk samples. All CF values obtained at 30kV/cm were also higher than those of pasteurized milk samples.

Dunn (1996) studied enzyme activity, fat integrity, starter growth, rennet clotting yield, cheese production, calcium distribution, casein structure, and protein integrity in PEF treated milk (E = 20-80 kV/cm; pulse width = 1-10 μs; T = 55°C). The author observed no significant differences between raw and PEF treated milk. Using 2% fat milk, Qin et al. (1995) compared physicochemical properties and sensory attributes of PEF treated milk (E = 40 kV/cm; pulse width = 2 μs; 6–7 pulses; T < 55°C) and thermal pasteurized milk. No significant differences between the two samples were observed.

Fox et al. (2000) showed that heat treatment of milk at temperatures above 65°C adversely affected its rennet coagulability. In our case, although the maximum temperature of 50°C was lower than 65°C, the combined effect of long pulse PEF and heat treatment may have led to a minor impairment of milk coagulation properties. This indicated that in the purpose of cheese production, the treatment temperature under higher electric field intensity (30 kV/cm) and longer pulses (120 pulse numbers) should not exceed 50°C in order to obtain curd formation better than thermally processed samples.

Pulse number (N) was the third significant factor ($p<0.05$) for CF (Appendix III). With the increase of pulse number, the CF decreased (Figure 5.4). The combination effect of N and temperature was also found significant (Appendix III), although it was not that much significant compared to E, temperature and N.

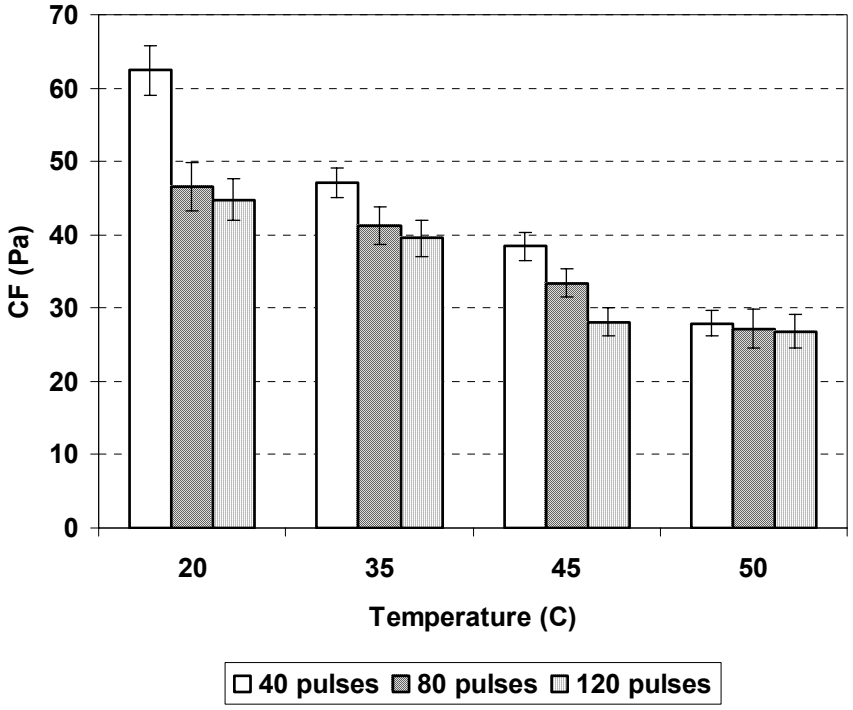

Figure 5.4 Effects of pulse number and temperature on curd firmness (CF). The applied pulse width is 2μs, pulse frequency is 2Hz, and electric intensity is 30kV/cm.

5.4.2 Effects of PEF treatment and temperature on rennet coagulation time

The analysis of variance results (Appendix IV) showed that treatment temperature, pulse number (N) and electric field intensity (E) significantly affected RCT ($p<0.05$). Higher E, N and temperature led to longer RCT (Figure 5.5, and 5.6). RCT is a good index of the gelation potential of milk. A low RCT usually shows potentially good gel formation and high gel strength. Raw milk obtained the lowest RCT. Pasteurized milk obtained highest RCT. While PEF treated samples obtained intermediate RCT values.

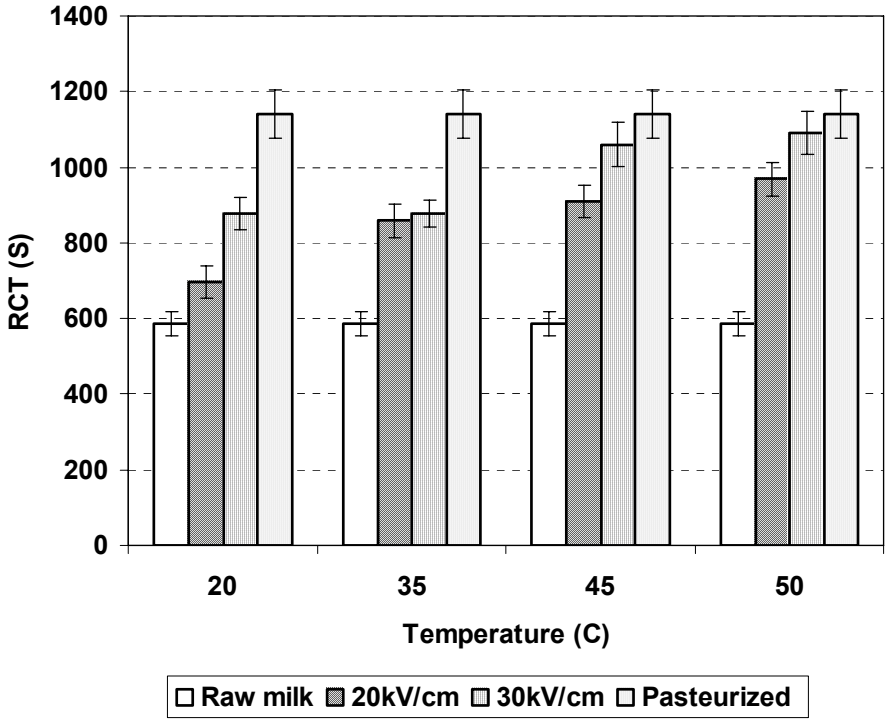

Figure 5.5 Effects of electric field intensity and temperature on rennet coagulation time (RCT). The applied pulse width is 2μs, pulse frequency is 2Hz, and pulse number is 120.

The values of RCT obtained using an electric field intensity of 20 kV/cm and temperatures from 20 to 50°C were significantly lower than those of heat pasteurized samples (Figure 5.5). The RCT values obtained at 30kV/cm were also higher than those of heat pasteurized milk samples. The RCT results again confirmed that milk treated by PEF combined mild temperature gave better rennetability compared to thermally pasteurized milk.

Evrendilek et al. (2001) studied a yogurt drink prepared using combined PEF (E = 30kV/cm, treatment time = 32 μs) and heat (60°C, 32s). The authors reported no significant differences between the control sample and the treated samples in terms of

color, soluble solids and pH. However, when milk was subjected to long duration pulses (Perez and Pilosof 2004), or high intensity electric fields (45-55 kV/cm) as described by Floury et al. (2006), the structure of milk protein may have been modified.

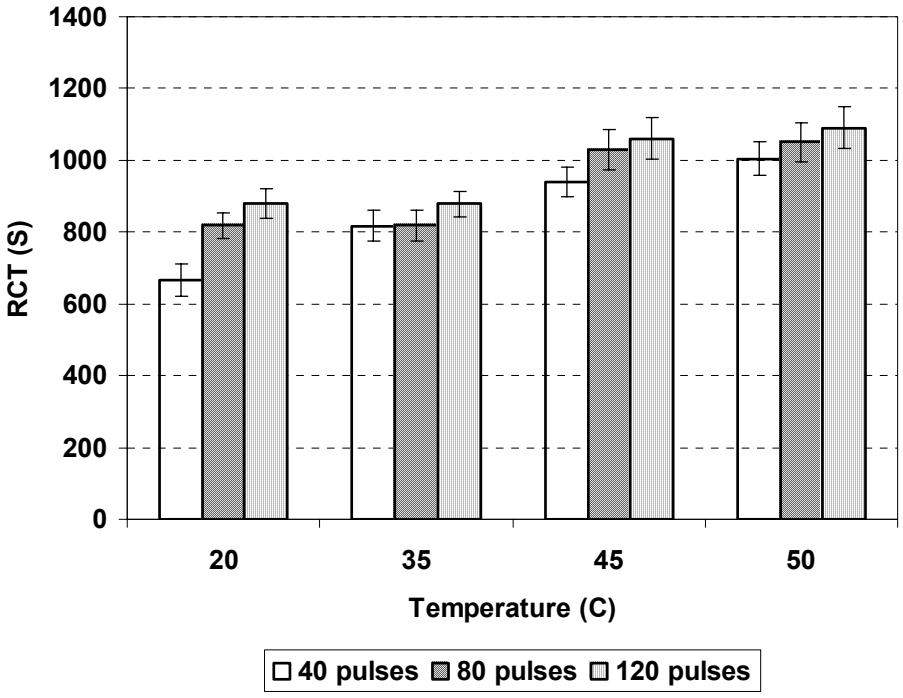

Figure 5.6 Effects of pulse number and temperature on rennet coagulation time (RCT). The applied pulse width is 2μs, pulse frequency is 2Hz, and electric intensity is 30kV/cm.

Xiang et al. (2007) treated whole milk at 20°C with the electric field intensity ranging from 18 to 22kV/cm and the number of pulses ranging from 0 to 80. By using differential scanning calorimetry (DSC) and fluorescence spectroscopy (FS), the authors found partial denaturation (25%) of milk protein with an electric field intensity of 22kV/cm and 80 pulses.

Perez and Pilosof (2004) attributed the effects of PEF on milk proteins as due to polarization of the protein molecule; dissociation of non-covalently linked protein subunits involved in quaternary structure; changes in the protein conformation so that buried hydrophobic amino acids or sulfydryl groups are exposed. And if the duration of the electric pulse is high enough, hydrophobic interactions or covalent bonds may occur, forming aggregates. Similarly, Floury et al. (2006) explained the effect of PEF on milk protein as due to the modification of the apparent charge after exposure to intense electric fields and then modification of ionic interactions between the proteins.

The modification of milk protein structure may lead to changes in milk functional properties such as coagulation, foaming and emulsifying. Different authors have reported varying levels of electric field strengths and temperatures beyond which changes in milk properties will occur. This study shows that coagulation properties of raw milk may be better preserved by using lower electric field strength (≤30 kV/cm) and temperature (≤50°C) combinations compared with thermally pasteurized milk.

5.5 CONCLUSIONS

Electric intensity, temperature and pulse number significantly affect milk coagulation properties such as Curd Firmness and Rennet Coagulation Time. Coagulation properties of raw milk may be better preserved by using electric field strength (≤30 kV/cm) and temperature (≤50°C) combinations compared with thermally pasteurized milk.

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CONNECTING TEXT

The results in chapter V indicated that the coagulation properties of milk treated by pulsed electric field and mild temperature were intermediate between that of raw milk and that of thermally pasteurized milk. Chapter VI investigated the ripening characteristics of cheese curd made from pulsed electric field treated milk and compared with those made from raw and thermally pasteurized milk.

Part of this research was published at the 2006 ASABE Technical Library, paper No. 06-140.

CHAPTER VI: PROTEOLYSIS IN CHEDDAR-TYPE CHEESE MADE FROM PULSED ELECTRIC FIELD TREATED MILK

6.1 ABSTRACT

Raw milk cheeses have unique flavor and texture characteristics not obtainable in cheeses made from pasteurized milk. However, cheeses made from pasteurized milk are widespread, primarily for public health reasons. Pulsed electric field (PEF) treatment as a non-thermal pasteurization method has shown its ability to keep the flavor and natural characteristics of food samples, thus providing advantage over conventional heat processing. In this study, PEF treatment was performed in a continuous treatment chamber, consisting of two parallel stainless steel electrodes separated by a 50 mm thick insulator. A 30 kV pulse generator was used to deliver bi-polar square waveform electric field to milk sample. Pulse width was 2 μs, pulse frequency was 2 Hz and up to 120 pulses were applied. Cheddar-type cheese curds were made from raw milk, pasteurized milk and PEF-treated milk and their proteolysis processes were compared using curd slurry incubated at 30°C for 5 days. The profiles of water-soluble peptides were measured using an RP-HPLC system. The concentration of free amino acids was measured by Cd-Ninhydrin method. Results indicated that PEF treated milk has similar proteolysis profiles as raw milk, and superior to pasteurized milk in terms of peptide composition and free amino acid concentration. The results showed the potential of making high quality cheeses by PEF treatment without sacrificing the natural characteristics of the cheeses.

Keywords: pulsed electric field, proteolysis, cheddar-type cheese

6.2 INTRODUCTION

Pasteurization of milk for cheese making became widespread after about 1940, primarily for public health reasons (Fox et al. 2000). However raw milk cheeses are known to possess unique flavor and texture characteristics not obtainable in cheeses made from pasteurized milk. This may be due to the denaturation of indigenous enzymes, partial denaturation of whey proteins and their interaction with casein, and the destruction of some desirable non-starter lactic acid bacteria present in raw milk (McSweeney et al. 1993).

PEF involves the application of high voltage pulses at relatively low temperature to a food placed between two electrodes for very short time (normally less than 1 second). A great number of researches (Dunn and Pearlman 1987; Qin et al. 1995 and Martin et al. 1997) have demonstrated the possibility of pasteurizing milk by PEF treatment without sacrificing its quality. Dunn (1996) reported that milk treated with PEF suffered less flavor degradation when compared to raw milk. The author proposed the possibility of manufacturing dairy products such as cheese, butter and ice cream using PEF treated milk. Sepulveda-Ahumada et al. (2000) evaluated the quality of cheese produced from PEF treated milk in terms of sensory and texture evaluation, and compared with cheese made from heat pasteurized milk. They claimed that using milk pasteurized by PEF, the obtained cheese appeared to have improved quality.

Knowledge of PEF effects on major cheese making steps, such as ripening, is crucial to develop higher quality cheeses made from pasteurized milk. However, little studies have been reported so far on this aspect. Cheese ripening involves a complex series of biochemical events, which lead to the characteristic taste, aroma and texture of

each cheese variety. Major biochemical changes occurring in cheese ripening include proteolysis, glycolysis and lipolysis. Proteolysis is considered the most important issue in terms of flavor and texture development. It contributes to textural changes of the cheese matrix due to breakdown of the protein network. It also contributes to flavor and bitterness of cheese mainly through the formation of peptides and free amino acids (Sousa et al. 2001). Many cheese ripening studies have focused on the proteolysis process (Farkye et al. 1995; O'Shea et al. 1996; Albenzio et al. 2001; Benfeldt and Sorensen 2001; Verdini et al. 2003).

Cheese making experiments, even on a pilot scale, are expensive and time-consuming. Kristoffersen et al. (1967) developed a rapid method for producing cheese flavor. In this method, cheese curd slurries (semisolid paste) were prepared and incubated at 30°C for five days, which could yield flavors similar to that of cheeses ripened for 3 months. Therefore, cheese curd slurries can be used as a quick tool to evaluate the contribution of different components into the cheese (Farkye et al. 1995, Muehlenkamp-Ulate and Warthesen 1999). Briggs et al. (2003) successfully used cheese slurries to study the cheese ripening process accelerated by PEF treated lactic acid bacteria.

The objectives of this study were (1) to evaluate the proteolysis process of cheddar-type cheese curd slurries made from PEF treated milk and (2) to compare results with those obtained from raw milk and heat pasteurized milk.

6.3 MATERIALS AND METHODS

6.3.1 Raw milk and heat pasteurized milk

Raw milk with 3.14 ± 0.02 % (w/w) protein and 3.81 ± 0.06% (w/w) fat was picked up at the dairy farm of Macdonald campus, McGill University (Ste Anne de

Bellevue, QC, Canada). The microbial load measured from standard plate counts in the raw milk was < 5000 CFU/mL.

Raw milk was filled in sterile plastic bottles and stored at 4°C for less than 4 h prior to PEF or heat pasteurization. Heat pasteurization was carried out by batch heating raw milk at 63°C for 30 minutes in a water bath.

6.3.2 PEF treated milk

A 30 kV pulse generator with a matched output impedance of 100 Ω and a continuous treatment chamber system were used in the experiment. The output voltage profile was bi-polar instant reversal square waveform. The treatment chamber consists of two parallel stainless steel electrodes, separated by a 50 mm thick Derlin® polyoxymethylene insulator, with 102 mm^2 of surface area. The voltage and current across the treatment chamber were captured simultaneously using a 2 channel digital oscilloscope (TDS3000, Tektronix, Wilsonville, OR).

The samples were treated following the procedure described by Amiali et al. (2006). A cooling system was used to maintain minimum temperature change during the treatment (less than 1°C difference between inlet and outlet temperature for each treatment cycle).

Results of Chapter IV showed that PEF treatment at electric field intensity of 30 KV/cm, outlet temperature of 50°C and pulse number of 80 and 120 could effectively inactivate pathogenic microorganisms in milk in terms of *E. coli* O157:H7 and *S. enteritidis*. Chapter V showed that milk treated with electric field intensity of 30KV/cm, outlet temperature of 50°C and pulse number of 80 and 120 gave better coagulation properties compared to pasteurized milk. Therefore, the parameters and selected levels for

this chapter were: electric field intensity of 30KV/cm; outlet temperature of 50 ± 1°C; pulse number of 80 and 120 pulses. The pulse width was 2 μs and the frequency of pulses was 2 Hz.

6.3.3 Preparation of cheese curd slurries

The Cheddar-type cheese curd was made following the revised procedure of Farkye et al. (1995). The 2% (v/v) liquid starter culture (Institute Rossel Inc., St-Laurent, Quebec, Canada) and 0.02% (w/v) liquid rennet (Institute Rossel Inc., St-Laurent, Quebec, Canada) were added in sequence to 500 mL warm milk samples (30°C). After brief mixing, the rennet-treated milk was then left to coagulate under quiescent conditions. The coagulum was cut and cooked to 39°C for over 30 min and held at this temperature for 15 min. The whey was drained at pH 5.3 and the curd was kept for slurry preparation.

The slurry was prepared following the revised procedure of Farkye et al. (1995) by blending 65 g cheese curd, 5 g NaCl and 30g sterile distilled water in a sterile blender jar (Hamilton, Beach, model C54252, Mexico). The diluted slurry (100 g) was then transferred aseptically into sterile tubes, capped loosely and incubated at 30°C for 5 days. Each slurry preparation was replicated two times using freshly made cheese curd.

6.3.4 Extraction of water soluble fraction (WSF)

Water-soluble fraction (WSF) of slurries was made based on the method of Kuchroo and Fox (1982). A sample (10 g) of cheese slurry was homogenized with 20 mL of distilled water at maximum speed on a vortex for 30s. Resulting mixtures were tempered in a 40°C water bath for 20 min. Next, the extracts were centrifuged at 10000 X g for 20 min at 4°C. The fat layer was removed. The aqueous layer was then filtered

through Whatman No. 1 filter paper. The permeate was lyophilized in a ULT freezer (Thermo Electron Corporation, Waltham, MA) and stored for further analysis.

6.3.5 Peptides analysis by RP-HPLC

Peptide profiles in the WSF extracts were analyzed by RP-HPLC using a Varian system (Varian Associates, CA, USA) equipped with a photodiode array detector (model 330), an auto-sampler (model 410) and a quaternary solvent delivery module (model 240). The system is connected to a personal computer and data was processed using Varian Star Workstation 5.5 software (Varian Associates, CA, USA). Separations were carried out using a 150 X 4.6 mm, C-18, 90^{o} pore size column (Varian Associates, CA, USA). A guard column (Supelguard LC-18-DB, 20 x 4.6 mm ID) was used in all cases.

Samples (10 mg) of each lyophilized WSF extract were dissolved in 2 mL 0.1% aqueous trifluroacetic acid (TFA) and filtered through a 0.45-μm filter. A 100 μL loop was used to introduce the sample into the HPLC. The composition of solvent A was 0.1% trifluoroacetic acid (TFA) in water and that of solvent B was 0.1% TFA in acetonitrile: water (75:25, v/v). A stepwise gradient elution in the order: 0% B (100% A) for 5 min; 30% B over 40 min; 65% B over 15 min; 80% B over 10 min at a flow rate of 0.75 mL/min was used. The column was then rinsed and allowed to equilibrate for 25 minutes between injections. The eluate was monitored at 220 nm.

HPLC test for each WSF extract was performed in triplicates. The total integration area of peptides detected at 220 nm during the HPLC run was determined. The UV absorption peaks observed for the HPLC runs were divided into two groups to allow a quantitative hydrophobic-hydrophilic index analysis (Lau et al. 1991). The first group consisted of the peaks with retention times from 5 to 50 minutes, which was considered as the hydrophilic peptide portion. The second group of peptides with retention time from 50

to 70 minutes was the more hydrophobic peptide portion (Guo et al. 1986). The ratio of hydrophobic to hydrophilic peptides was obtained by dividing the total peak area of the hydrophobic peptide portion by that of the hydrophilic peptide portion.

6.3.6 Free amino acids analysis by Cd-ninhydrin method

The presence of free amino acids in the WSF was determined in triplicates based on the Cd-ninhydrin method of Folkertsma and Fox (1992). Lyophilized WSF extracts were reconstituted in distilled water (1% w/v). A portion (200 μL) of the reconstituted extract was diluted to 1 mL with distilled water and then 2 mL Cd-ninhydrin reagent (1 g of $CdCl_2$ dissolved in 1 mL of water plus 0.8 g ninhydrin, 80 mL of 90% ethanol, and 10 mL of glacial acetic acid) was added. The solution was heated at 84°C for 5 min, cooled and the absorbance was read at 507 nm (A_{507}) on a UV-VIS spectrophotometer (ThermoSpectronic UV1, Cambridge, Great Britain). The A_{507} was converted to mM Leucine from a standard curve prepared with L-leucine (0.1 to 10 mM). The results were expressed as mg Leucine / g WSF extracts.

6.3.7 Statistical analysis

Analysis of variance (ANOVA) was performed using the General Linear Model procedures (GLM) of the Statistical Analysis System (SAS, Version 8.02, 2001, Cary, NC, USA). Experiments were conducted in triplicates and the means of the three data are presented.

6.4 RESULTS AND DISCUSSION

6.4.1 Concentration of peptides in WSF

McGugan et al. (1979) identified the nonvolatile WSF in mild and aged Cheddar cheese as the major contributor of cheese flavor intensity. Aston and Creamer (1986)

confirmed that the nonvolatile WSF of cheese is an essential fraction for cheese flavor intensity. Nonvolatile WSF of cheese could be successfully detected by HPLC analysis (Lau et al. 1991, McSweeney et al. 1993). The present study used HPLC analysis to compare the peptide profile in the WSF extracted from Cheddar type cheese curd slurries made from raw milk (RM), pasteurized milk (PM) and PEF treated milks at 120 pulses (PEF120) and 80 pulses (PEF80).

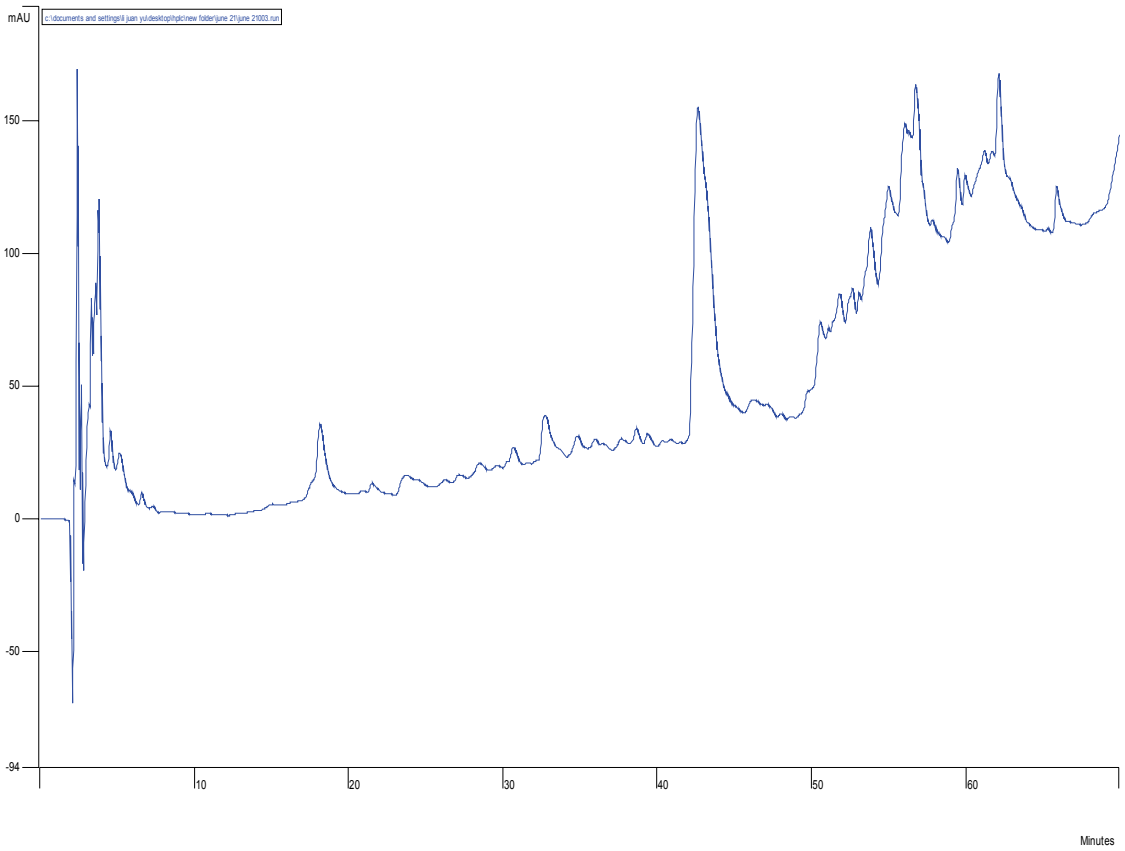

Figure 6.1 Typical HPLC profiles of Cheddar cheese curd slurry

Figure 6.1 shows the typical HPLC profiles of Cheddar cheese curd slurry. At 220 nm, the total area under the peaks on the HPLC profile represents the light absorbed by aromatic amino acids and peptide bonds present in the WSF of cheese (Lau et al. 1991). As cheese ages, more caseins and high molecular weight peptides are being broken down

into smaller peptides that may be water soluble. Therefore, it was expected that as the cheese aged, the total water-soluble peptide content increased.

The results of HPLC profiles for Cheddar type cheese curd slurries made from raw milk, PEF treated milk and heat pasteurized milk at the incubation time of 0, 3 and 5 days are presented in Figure 6.2 to Figure 6.5. ANOVA results for HPLC profiles are listed in Appendix V and VI.

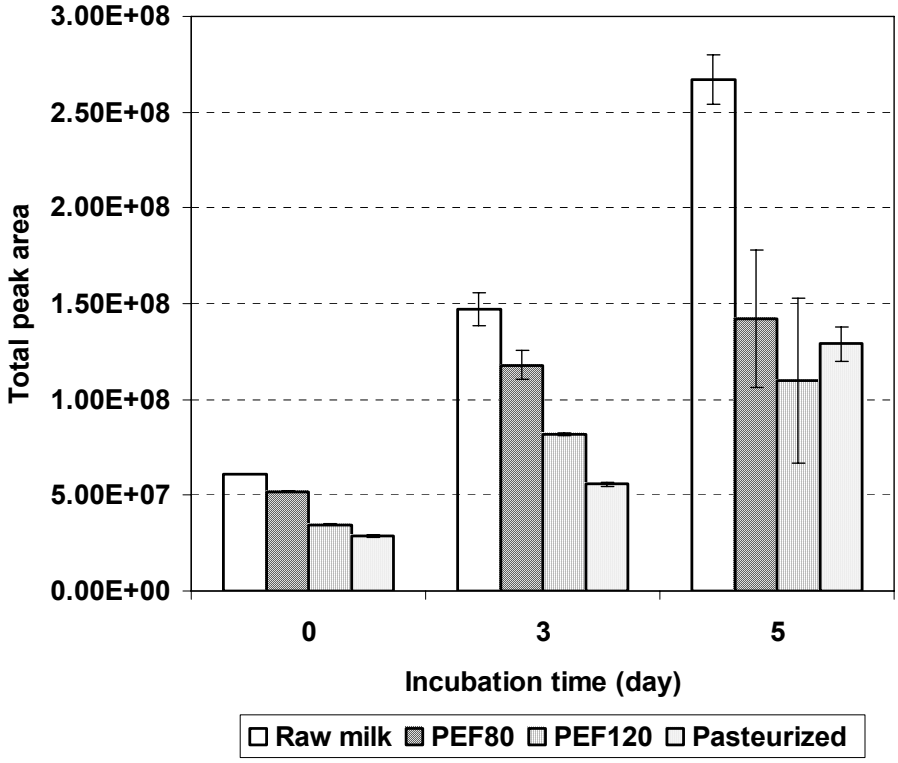

Figure 6.2 Total peak areas of HPLC profiles for Cheddar type cheese curd slurries made from (1) Raw milk (RM); (2) PEF treated milk at 80 pulses (PEF80); (3) PEF treated milk at 120 pulses (PEF120); (4) Pasteurized milk (PM) at the incubation time of 0, 3 and 5 days.

Figure 6.2 shows the total peak areas of HPLC profiles of Cheddar type cheese curd slurries made from RM, PM, PEF120 and PEF80. As expected, with increased

incubation time, the total peak areas increased. This implies that the total water-soluble peptide content increased. When comparing the results on day 0 and day 3, it can be found that RM samples had the largest peak areas, followed by the PEF80, PEF120 and PM samples. McSweeney et al. (1993) compared HPLC profiles of peptides in WSF of Cheddar cheeses made from raw and pasteurized milk and reported the differences between the two profiles. They deduced that the difference could be due to the non-starter microflora present in the raw milk. Although PEF treatment could inactivate the non-starter microflora, the indigenous enzymes could survive since they require more severe PEF treatment to yield a significant activity reduction (Ho et al. 1997). These enzymes could function in cheese aging and may have resulted in production of more water-soluble peptide. Peak areas of PEF80 were found more than that of PEF120, which was probably due to its lower severity in PEF treatment.

Lau et al. (1991) used the same HPLC approach as McSweeney et al. (1993), and reported that the amounts of water-soluble peptides were the same for RM and PM Cheddar cheeses.

On day 5, the trend was slightly different. RM still gave the largest peak area, while the peak areas of PM, PEF80 and PEF120 were not much different. It was interesting that for RM and PM, the peak area increased rapidly with the increase of the incubation time, while the peak area for PEF treated milk increased relatively slow. This needs more investigation probably in terms of protein structure changes during PEF treatment.

6.4.2 Analysis of hydrophilic and hydrophobic peptides

Figures 6.3 and 6.4 show the amounts of hydrophilic and hydrophobic peptides in WSF of Cheddar cheese curd slurries made from RM, PM, PEF80 and PEF120.

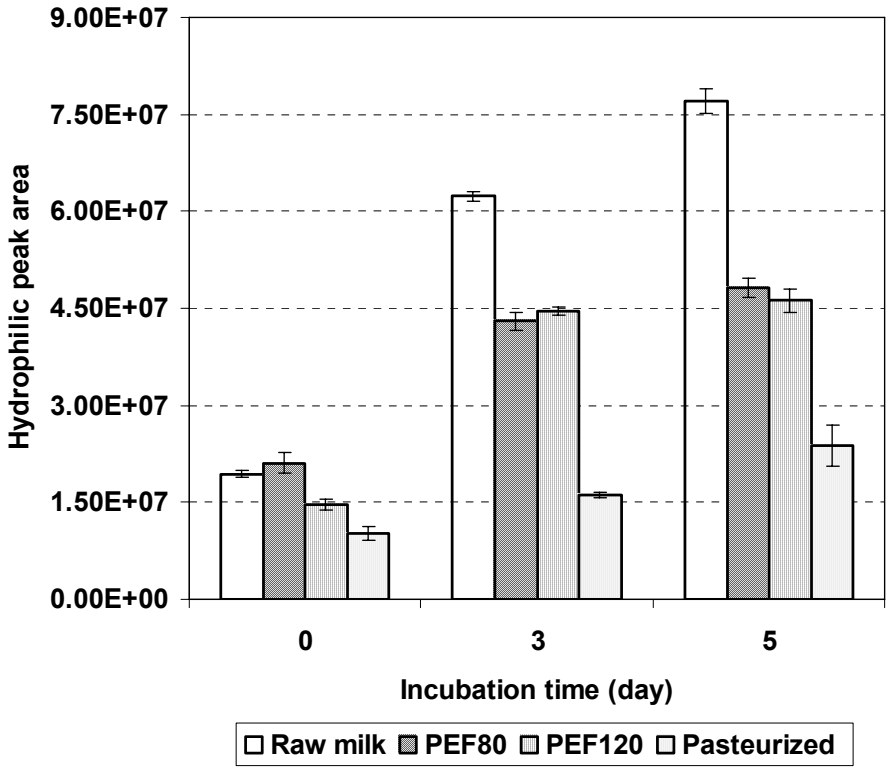

Figure 6.3 The amounts of hydrophilic peptides in WSF of Cheddar cheese curd slurries made from (1) Raw milk (RM); (2) PEF treated milk at 80 pulses (PEF80); (3) PEF treated milk at 120 pulses (PEF120); (4) Pasteurized milk (PM) at the incubation time of 0, 3 and 5 days.

It was found that both hydrophilic and hydrophobic peptides increased with the increase of the incubation time in all the test samples. This was consistent with the findings by Lau et al. (1991), who reported that hydrophilic and hydrophobic peptides increased during the Cheddar cheese aging for both raw and pasteurized milk cheeses.

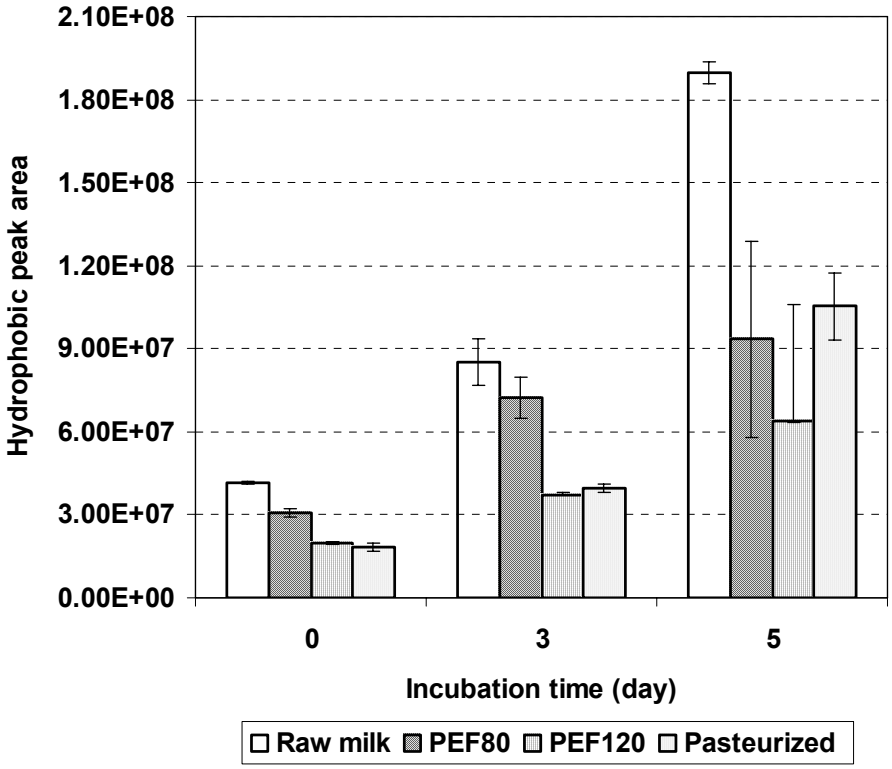

Figure 6.4 The amounts of hydrophobic peptides in WSF of Cheddar cheese curd slurries made from (1) Raw milk (RM); (2) PEF treated milk at 80 pulses (PEF80); (3) PEF treated milk at 120 pulses (PEF120); (4) Pasteurized milk (PM) at the incubation time of 0, 3 and 5 days.

Champion and Stanley (1982) stated that extracts of bitter Cheddar cheese contained a high proportion of hydrophobic peptides. Lau et al. (1991) found a high proportion of hydrophobic peptides present in the WSF of Cheddar cheese made from pasteurized milk than in cheese made from raw milk. The authors believed that a proper balance among the various water-soluble components is important for the development of a typical Cheddar cheese flavor. He proposed that the difference in the ratio of hydrophobic to hydrophilic peptides present in the WSF in Cheddar cheeses made from pasteurized and raw milk may cause flavor differences.

Figure 6.5 shows the ratio of hydrophobic to hydrophilic peptides in RM, PM, PEF80 and PEF120 Cheddar cheese curd slurries.

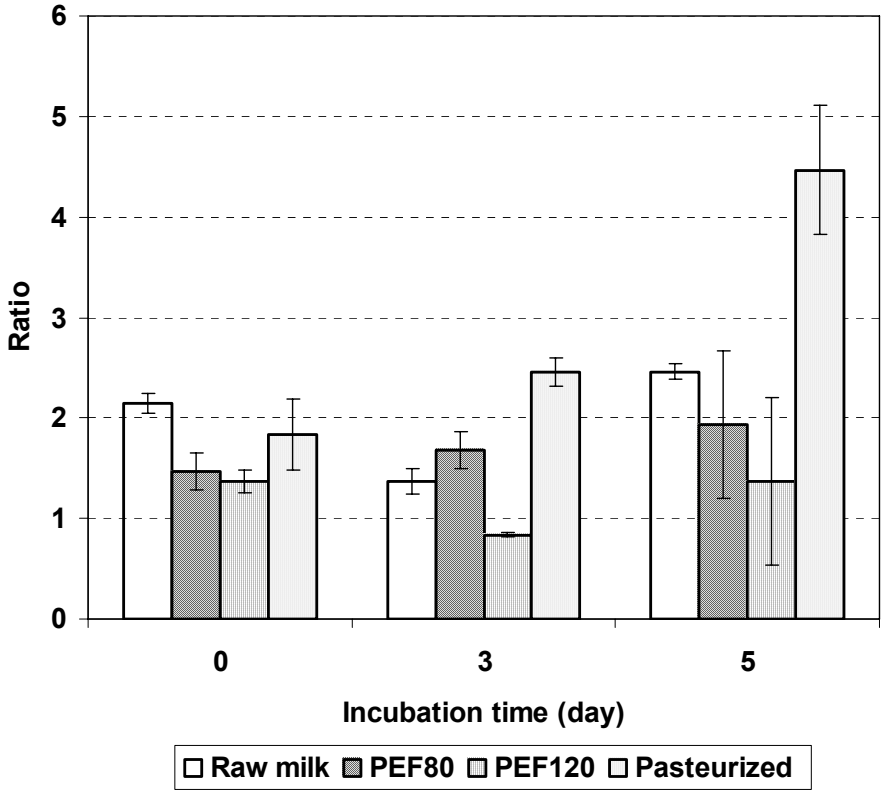

Figure 6.5 The ratio of hydrophobic to hydrophilic peptides in WSF of Cheddar cheese curd slurries made from (1) Raw milk (RM); (2) PEF treated milk at 80 pulses (PEF80); (3) PEF treated milk at 120 pulses (PEF120); (4) Pasteurized milk (PM) at the incubation time of 0, 3 and 5 days.

It was found that PM gave the highest ratio of hydrophobic to hydrophilic peptides during incubation (day 3 and day 5). Compared with PM, PEF treated milk gave similar ratios as RM, especially PEF80, which could imply that PEF treated milk could give similar flavor development in Cheddar cheese as for RM.

6.4.3 Analysis of Free amino acids

Figure 6.6 presents the concentration of free amino acids (FAA) in the WSF extracted from Cheddar type cheese curd slurries made from raw milk (RM), pasteurized milk (PM) and PEF treated milks at 120 pulses (PEF120) and 80 pulses (PEF80). ANOVA results for FAA are listed in Appendix VII.

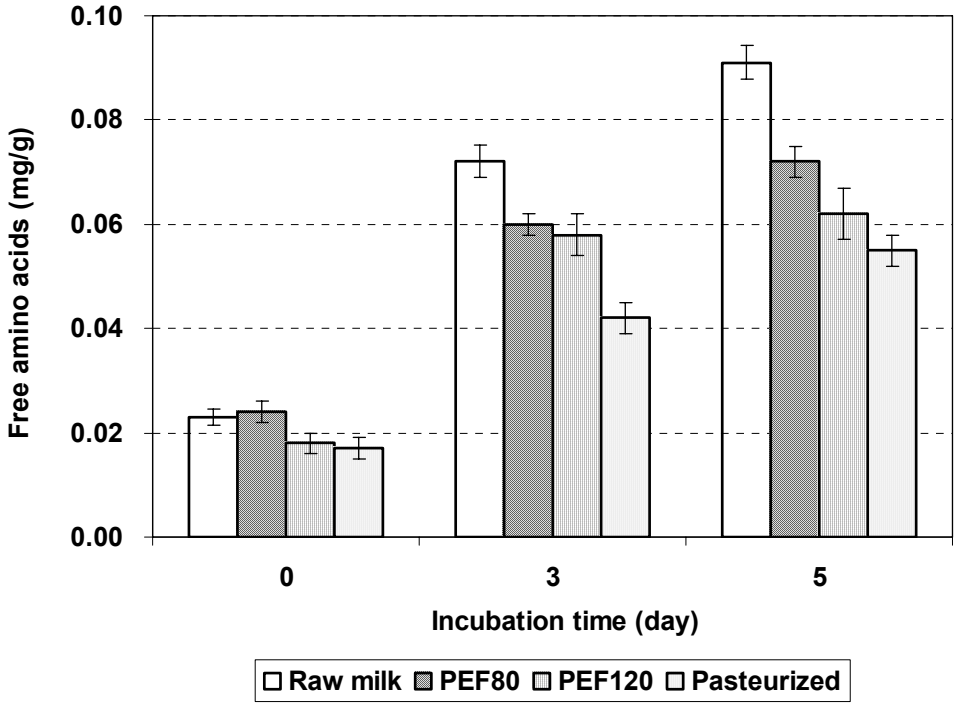

Figure 6.6 The concentration of free amino acids in WSF of Cheddar cheese curd slurries made from (1) Raw milk (RM); (2) PEF treated milk at 80 pulses (PEF80); (3) PEF treated milk at 120 pulses (PEF120); (4) Pasteurized milk (PM) at the incubation time of 0, 3 and 5 days.

As expected, with increased incubation time, the concentration of FAA increased. On day 0, RM and PEF80 samples had the same level of FAA, which was a little higher than that of PEF120 and PM samples. On day 3, the concentration of FAA in all four

samples increased dramatically compared to day 0. RM samples still had a higher level of FAA; followed by the PEF80 and PEF120 samples; PM samples lagged behind. The result that RM had higher FAA than PM is not surprising. McSweeney et al. (1993) and Shakeel-Ur-Rehman et al. (2000) found a higher concentration of amino acids in Cheddar cheese made from raw milk which had a higher and more heterogeneous NSLAB population than pasteurized milk cheeses. Albenzio et al. (2001) studied biochemical characteristics of Canestrato Pugliese cheese made from raw milk and pasteurized milk and also noticed consistent higher concentration of FAA in raw milk compared to pasteurized milk cheeses.

On day 5, the increase of FAA in all the samples was a little slower compared to the beginning of the ripening period. This was consistent with other similar work done by Muehlenkamp-Ulate and Warthesen (1999), who also presented a faster increase in Total FAA in cheese slurries ripened from 0-12 days. In our work, on day 5, RM still had the highest level of FAA; PEF80 samples ranked second, followed by PEF120 and PM samples which did not show much difference in FAA level. The FAA in PEF120 was consistently lower than that of PEF80, which was due to the greater severity of that PEF treatment.

It seems that PEF treated samples (especially PEF80) gave more similar FAA profile as RM samples in most cases and consistently gave higher FAA content than PM samples. This indicated that PEF treatment could possibly compete with traditional pasteurization process to provide safe cheeses while providing the cheese ripening quality of raw milk cheeses.

The content of FAA for all samples in our results varied from 0.018 to 0.091 mg Leucine g^{-1} WSF extracts during the five incubation days, which was within the

reasonable range of other researches (Farkye et al. 1995; Muehlenkamp-Ulate and Warthesen 1999; Briggs et al. 2003). Farkye et al. (1995) used starter-free Cheddar type cheese slurries to investigate proteolytic abilities of some lactic acid bacteria in a model cheese system. Their samples were ripened anaerobically at 32°C for five days and the concentration of FAA varied from approximately 0.034 to 0.101 mg Leucine g^{-1} WSF extracts. Muehlenkamp-Ulate and Warthesen (1999) evaluated ten strains of nonstarter lactobacilli for their proteolytic abilities in Cheddar cheese slurries by ripening anaerobically at 30°C for 12 days. The concentration of FAA in their studies varied from 0.1 to 0.5 mg Leucine g^{-1} WSF extracts from day 0 to day 6. The higher concentration of FAA in their work was due to an additional procedure for enhanced WSF fractionation: the WSF was further filtered through a 1000 molecular weight membrane before FAA analysis. Briggs et al. (2003) used cheese slurries to monitor the cheese ripening process accelerated by PEF treated lactic acid bacteria. The concentration of FAA in their studies was approximately 10 times lower than the values obtained in the present study and by Farkye et al. (1995). The lower concentration of FAA reported by Briggs et al. (2003) may be attributed to their direct use of liquid WSF instead of lyophilized WSF.

6.5 CONCLUSIONS

During the incubation of Cheddar cheese curd slurry, PEF treated milk gave greater total peak areas in HPLC profiles of the water-soluble fraction than pasteurized milk. Raw milk had even higher total peak areas. The analysis of hydrophobic and hydrophilic portions in HPLC profiles showed that PEF treated milk had similar hydrophobic and hydrophilic ratios as raw milk, which was significantly different from that of pasteurized milk. Results implied that PEF treated milk could develop similar

flavor to Cheddar cheese produced with raw milk. More studies are needed to investigate the mechanism behind this.

PEF treated samples (especially PEF80) gave similar FAA profile to RM samples in most cases and consistently gave higher FAA content than PM samples. This indicated that PEF treatment could have a chance to supplement or replace traditional pasteurization process with minimum impact on cheese proteolysis and the development of cheese flavors.

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CHAPTER VII: GENERAL SUMMARY AND CONCLUSIONS

7.1 GENERAL SUMMARY AND CONCLUSIONS

Researches conducted in the past decade have demonstrated the feasibility of using pulsed electric field (PEF) processing in the inactivation of microorganisms in milk and the minimization of loss of organoleptic and physicochemical characteristics in milk and milk products. However, as for the PEF effect on cheese process and quality, limited research work was found. This study demonstrated the potential of producing consumer-safe cheddar cheese with raw milk like flavor characteristics through PEF processing.

Milk fat content and treatment temperature showed combination effect on microbial inactivation in milk by PEF treatment. From 5 to 35°C, milk fat content did not have significant effect on microbial reduction by PEF treatment. However, when temperature was over 45°C, milk fat content seemed to hinder the PEF effect on microbial inactivation.

PEF treatment could inactivate *E. coli* O157:H7 and *S. enteritidis* in whole milk. The extent of bacterial inactivation is a function of electric field intensity, treatment time and treatment temperature. The maximum reduction of *E. coli* O157:H7 and *S. enteritidis* was 4.1 and 5.2 logs, respectively at 30 kV/cm and 50°C. The inactivation kinetics for both bacteria was primarily exponential, except in some cases with some tailing. Lower kinetic rate constant (k) for *E. coli* O157:H7 in whole milk indicated greater resistance to heat-PEF treatment compared to *S. enteritidis*.

The coagulation of milk is the first and most important step in cheese making. A dynamic rheological test was conducted to analyze cheese coagulation properties as affected by PEF and mild heat treatment. Results indicated that electric field intensity,

treatment time and treatment temperature significantly affected milk coagulation properties in terms of Curd Firmness and Rennet Coagulation Time. PEF treated milk showed better rennetability compared to thermally pasteurized milk.

Proteolysis is considered the most important issue in terms of cheese flavor development during ripening process. It contributes to flavor changes through the formation of peptides and free amino acids. The water soluble peptides and free amino acids in cheese curd were determined by RP-HPLC and Cd-ninhydrin methods, respectively. The analysis of hydrophobic and hydrophilic portions in HPLC profiles showed that PEF treated milk had the similar hydrophobic and hydrophilic proportion as raw milk, which was superior to that of pasteurized milk. Results implied that PEF treated milk could give similar flavor to Cheddar cheese as raw milk. The free amino acid (FAA) analysis showed that PEF treated samples gave more similar FAA profile to RM samples in most cases and consistently gave higher FAA content than PM samples. Results indicated that PEF treatment could have a chance to supplement or replace traditional pasteurization process with minimum impact on cheese flavor development.

7.2 CONTRIBUTIONS TO KNOWLEDGE

This is the first time that a comprehensive study has been carried out to investigate the effect of pulsed electric field treatment on cheese processing. PEF can be used as a single technology or as a complementary step with mild thermal processes to produce high quality cheese products. The results provide useful information for the food scientists and cheese processing industries to design and develop fresh-like cheese products without a sacrifice on the safety. The contributions to scientific knowledge are summarized as follows:

1. The inactivation kinetics of *E. coli* O157:H7 and *S. enteritidis* inoculated in whole milk by PEF treatment over a wide range of electric field intensities, number of pulses and treatment temperature was modeled;
2. The effect of milk fat content on the microbial inactivation by PEF treatment was determined;
3. For the first time, the rennet coagulation properties of PEF treated milk was evaluated and compared with those from raw milk and thermally pasteurized milk;
4. For the first time, the proteolysis process of cheddar-type cheese curd made from PEF treated milk was determined and compared with those from raw milk and thermally pasteurized milk.

7.3 RECOMMENDATIONS FOR FUTURE RESEARCH

Further investigation is required to study the effect of PEF on the micro structure of casein and the effect of PEF on the interaction of casein and β-lactoglobulin as well as with other ingredients to explain the effect of coagulation and gel characteristics. The design of a system of PEF process to avoid dielectric breakdown of the liquid food and provide more uniform distribution of the electric field is recommended. Scale up of cheese production using PEF treated milk is needed.

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APPENDICES

Appendix I: Analysis of variance (ANOVA) for effects of treatment temperature and milk fat content on the electrical conductivity of milk

Source	Type III Sum of Squares	Mean Square	F	Sig.
Temperature	0.000203	0.000101	0.06311	0.000
Fat content	2.070965	0.207096	128.8253	0.939
Temperature * Fat content	0.003457	0.000173	0.107525	0.999
Error	0.1061	0.001608		

Appendix IIa: Analysis of variance (ANOVA) for effects of treatment temperature (5 to 35°C) and milk fat content on the microbial log reduction by PEF

Source	Type III Sum of Squares	Mean Square	F	Sig.
Temperature	20.749	6.916	3766.868	0.000
Fat content	0.006	0.003	1.572	0.228
Temperature * Fat content	0.006	0.001	0.573	0.748
Error	0.044	0.002		

Appendix IIb Analysis of variance (ANOVA) for effects of treatment temperature (45 to 55°C) and milk fat content on the microbial log reduction by PEF

Source	Type III Sum of Squares	Mean Square	F	Sig.
Temperature	9.031	9.0313	4233.398	0.000
Fat content	0.334	0.167	78.378	0.000
Temperature * Fat content	0.0439	0.022	10.289	0.003
Error	0.0256	0.0021		

Appendix III Analysis of variance (ANOVA) for effects of electric field intensity (E), pulse number (N) and treatment temperature (T) on Curd Firmness

Source	Type III Sum of Squares	Mean Square	F	Sig.
E	2106.005	2106.005	267.161	0.000
N	1063.75	531.875	67.472	0.000
T	5391.886	1797.295	227.998	0.000
E * N	1.505833	0.753	0.0955	0.909
E * T	33.77833	11.259	1.428	0.245
N * T	229.0486	38.175	4.843	0.001
Error	425.6775	7.883		

Appendix IV Analysis of variance (ANOVA) for effects of electric field intensity (E), pulse number (N) and treatment temperature (T) on Rennet Coagulation Time

Source	Type III Sum of Squares	Mean Square	F	Sig.
E	258516.5	258516.5	108.472	0.000
N	167320.2	83660.12	35.103	0.000
T	845173	281724.3	118.209	0.000
E * N	448.006	224.003	0.0939	0.91
E * T	2909.465	969.822	0.407	0.749
N * T	27318.1	4553.016	1.911	0.0958
Error	128695.8	2383.256		

Appendix V Analysis of variance (ANOVA) results for total peak areas of HPLC profiles as a function of incubation time and method of milk treatment*

Source	Type III Sum of Squares	Mean Square	F	Sig.
Time	8.363E+16	4.181E+16	141.409	0.000
Treatment	4.348E+16	1.449E+16	49.012	0.000
Time * Treatment	1.840E+16	3.066E+15	10.371	0.000
Error	7.097E+15	2.957E+14		

*: Method of treatment: raw milk, PEF treated milk at 80 pulses, PEF treated milk at 120 pulses and heat pasteurized milk; Incubation time: 0, 3 and 5 days

Appendix VI Analysis of variance (ANOVA) results for the ratio of hydrophobic to hydrophilic peptides of HPLC profiles as a function of incubation time and method of milk treatment*

Source	Type III Sum of Squares	Mean Square	F	Sig.
Time	6.715	3.357	21.202	0.000
Treatment	14.204	4.735	29.899	0.000
Time * Treatment	7.394	1.232	7.782	0.000
Error	3.801	0.158		

*: Method of milk treatment: raw milk, PEF treated milk at 80 pulses, PEF treated milk at 120 pulses and heat pasteurized milk; Incubation time: 0, 3 and 5 days

Appendix VII Analysis of variance (ANOVA) results for free amino acids as a function of incubation time and method of milk treatment

Source	Type III Sum of Squares	Mean Square	F	Sig.
Time	0.0161	0.00806	898.839	0.000
Treatment	0.00277	0.000923	102.839	0.000
Time * Treatment	0.00095	0.000158	17.65	0.000
Error	0.00022	8.972E-06		

*: Method of treatment: raw milk, PEF treated milk at 80 pulses, PEF treated milk at 120 pulses and heat pasteurized milk; Incubation time: 0, 3 and 5 days

McGill University

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

Projects involving potentially biohazardous materials should not be commenced without approval from the Environmental Safety Office. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: James P. Smith PHONE: 398-7923

DEPARTMENT: Food Science & Ag. Chemistry FAX: 398-7977

ADDRESS: Macdoanls Campus of McGill E-MAIL: James. P. Smith @McGill.ca

PROJECT TITLE: SAFETY STUDIES ON NEW FOOD PROCESSING TECHNOLOGIES

2. EMERGENCY: Person(s) designated to handle emergencies

Name: James P. Smith Phone No: work: 398-7923 home: 457-2262

Name: Bernard Cayouette Phone No: work: 398-8623 home: 254-2434

3. FUNDING SOURCE OR AGENCY (specify): NSERC

Grant No.: 24871 (223093) Beginning date: 15/04/04 End date: 15/04/09

4. Indicate if this is

☐ Renewal: procedures previously approved without alterations.

Approval End Date: _____

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency: _____ Approval End Date: _____

☑ New project: project not previously reviewed.

☐ Approved project: change in biohazardous materials or procedures.

☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: The Environmental Safety Office approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in Health Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

Containment Level (select one): ☐ 1 ☐ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: _____ SIGNATURE date: 20 (day) 04 (month) 04 (year)

Approved by: Environmental Safety Office: _____ SIGNATURE date: 22 (day) 04 (month) 04 (year)

Expiry: 15 (day) 04 (month) 09 (year)

*as defined in the "McGill Laboratory Biosafety Manual"