IMMOBILIZED ENZYMES: TIME TEMPERATURE INDICATORS FOR DIELECTRIC PASTEURIZATION PROCESSES

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IMMOBILIZED ENZYMES: TIME TEMPERATURE INDICATORS FOR

DIELECTRIC PASTEURIZATION PROCESSES

Abstract

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Alpha-amylase from *Aspergillus oryzae* and phytase from *Aspergillus ficuum* entrapped in 20% polyacrylamide gel were developed as time-temperature indicators (TTI) for dielectric pasteurization processes. The dielectric properties of the TTI can be altered to match the food by adding salt. The recovered activity of both enzymes following immobilization exceeded 85% after storage for 2 months at 8 ºC. After 6 hours of incubation at 25 ºC in 200 ml of 0.05 M phosphate buffer (pH 7.1) for immobilized α-amylase and 200 mM glycine buffer (pH 2.8) for immobilized phytase, the enzymatic activity decreased to around 40%.

D-values (min) (55-70 ºC) ranged between 66.22 and 0.43 in ground meat (beef, 30% fat), 66.60 and 0.57 in mashed potatoes and 33.89 and 0.55 in ground shrimp were obtained for the immobilized α-amylase. D-values ranged between 6.42 and 0.08 in ground meat (beef, 30% fat), 5.13 and 0.06 in mashed potatoes and 4.60 and 0.05 in ground shrimp for *Listeria monocytogenes.*

D-values (53-63 ºC) ranged between 555.00 and 10.40 in ground meat (beef, 30% fat), 312.00 and 6.63 in mashed potatoes and 222.00 and 5.41 in ground shrimp for immobilized phytase, and 33.00 to 0.54 for *Salmonella typhimirium* and *Escherichia coli* O157:*H*7 in ground meat, mashed potatoes and 20.40 in ground shrimp. Thermal inactivation

kinetics for both, the α-amylase and phytase TTI followed first order kinetics with z-values falling within the recommended range for pathogen inactivation. The thermal inactivation of the TTI and reduction of pathogens in the tests foods could be correlated and predictive equations established, providing a simple and fast method for validating dielectric pasteurization processes.

Aspergillus oryzae α-amylase and *Aspergillus ficuum* phytase immobilized in polyacrylamide gel can be effective and applicable time temperature indicators for mapping heat distribution during a 915 MHz Microwave Water Combination (MCWC) dielectric processing. TTIs can be used to determine heat distribution during microwave heating when the direct measurement is impractical or costly. The assays for these particular TTIs provides a fast, inexpensive and simple approach which could be implemented in industrial settings.

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DEDICATION

First and foremost I would like to dedicated this dissertation to my GOD

Your Holy Spirit gave me comfort and strength in all moments

All things are possible with you

My faith in your power and guidance brought wonderful light during difficult times

MY HUSBAND

You are a wonderful person

The best that could happen in my life

MY FAMILY

Your prayers, phone calls and emotional support kept

me going throughout this journey

Thank for all your love and care during these years

You could never imagine how much I missed you all

CHAPTER 1

INTRODUCTION

Food pasteurization processes

Food preservation is design to enhance or protect food safety while maintaining the sensory attributes of food. Inactivating or inhibiting the growth of undesirable microorganisms is very important for the successful and acceptable preservation of food. While a large number of preservation processes are available to food processors, the use of adequate heat treatment to destroy pathogenic and spoilage microorganisms is one of the most effective food-preservation processes in use today and has been used for centuries. Pasteurized foods are an important food market segment because they meet consumer demand for convenient food and fill important niches in the home-meal replacement market, and as heat-and-serve products for food service. The increase in consumption of these convenient products is motivating food processors to develop products that serve this market segment. However, several food-borne disease outbreaks have been traced to retail chilled foods. The risk is widespread since the largest food-borne disease outbreaks are with low-level contamination of widely distributed foods (Tauxe 1997).

Pasteurized food can cause illness if not properly processed. Failing to accurately verify a process increases legal liability (Rasco 1999; Buzby and others 2000). Heat treatment design to achieve a specific lethality for food-borne pathogens is a critical control point in food processing and is fundamentally important for assuring that foods are shelf life and microbiologically safe. A key to optimization of the heating step is defining the target pathogen heat resistance. Recently, the importance of understanding the thermal cooking systems for elimination of food-borne pathogens has been studied (Chantarapanont and

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others 2000, D'Sa and others 2000 and Hyes and others 1999). Depending upon the specific chemical and physical characteristic of the food, microbial inactivation characterization can differ (Veeramuthu and others 1988). For example, in meat systems, factors such as fat content can affect the inactivation of food-borne pathogens (Juneja and others 1998; Heddleson and others 1996). Unfortunately, over-estimating the heat resistance and then overtreating the food negatively impacts product quality by altering the sensory attributes and nutritional qualities of a food. Under-estimating heat resistance increases the likelihood that a contaminating pathogen will persist after heat treatment or cooking. Inadequate heat treatment or undercooking is an important contributing factor in food-poisoning outbreaks (Roberts 1991).

Thermal inactivation of microorganisms

The higher the initial microbial population in a food, the longer the processing/heating time at a given temperature required to achieve a specific lethality of microorganisms is. Accordingly, the thermal process is designed based on the expected microbial load in the raw product. As such, the heat resistance of bacteria is described by two parameters: D and z value. The Decimal Reduction Time (D-value) is the time of heating, in minutes, at a particular temperature necessary to destroy 90% of the viable cells or spores of a specific organism (the time for the curve to span on log cycle). It is a measure of the death rate or the heat sensitivity of the organism. If bacterial spores or organisms are exposed to heat at a constant temperature and the surviving fraction plotted against time the resulting curve is generally considered to follow a logarithmic course with equal percentages of surviving cells dying in each successive unit of time. The graph obtained by plotting the logarithmic of the number of viable cells against the time of heating is known as a survivor

curve or thermal death-rate curve. The slope of the survivor curve determines the D value (Hersom and Hulland 1981).

If the D values equivalent to a number of temperatures are plotted on a logarithmic scale against their corresponding temperature (thermal-death-time curve; TDT curve) a straight line is normally obtained, the slope is designated z. The z-value is the change in heating temperature needed to change the D-value by 90% or is the number of degrees for the line to traverse one log cycle of thermal death-time. The z-value provides information on the relative resistance of an organism at different destructive temperatures. The value of z varies with the organism and the medium in which the heating and recovery is carried out. D and z values are used for designing heat-processing requirements for desirable destruction of microorganisms in a particular food.

Generally, the rate of destruction of bacteria follows first-order kinetics. When a microbial population is heated at a specific temperature, the cells die at a constant rate with the log number of survivors declining in a linear manner with time (Stumbo 1973; Tomlins and Ordal 1976). This traditional first-order kinetics model of thermal inactivation forms the basis for the calculations used in thermal processing and this model has served the food industry and regulatory agencies for decades.

An appropriate heat treatment design to achieve a specific lethality of microorganisms is influenced by many factors, some of which can be attributed to the inherent resistance of microorganisms, while others are due to environmental influences. Examples of inherent resistance include the differences among species and between different strains or isolates of bacteria (assessed individually or as a mixture) and the differences between spores and vegetative cells. Environmental factors include those affecting the

microorganisms during growth and formation of cell or spores (e.g., stage of growth, growth temperature, growth medium, previously exposure to stress) and those active during the heating of bacterial suspension, such as the composition of the heating media (e.g., amount of carbohydrate, proteins, lipids, solutes), water activity (a_w), pH, added preservatives, method of heating, and methodology used for recovery of survivors.

The heat resistance of food-borne pathogens has been studied in different substrates. Comparing the heat resistance of some pathogens, such as *Listeria monocytogenes*, *Salmonella* spp., and *Escherichia coli* O157:H7, it appears that *L. monocytogenes* is relatively more heat resistant (Table 1)(Juneja and others 1997; Juneja and Marmer 1999; Juneja and Eblen 2000; Gaze and others 1989; Goodfellow and Brown 1978; Doyle and Schoeni 1984; Line and others 1991; Fain and others 1991). The pH of the heating menstruum is recognized as one of the most important factors influencing the heat resistance of bacteria. Microorganisms usually exhibit their maximum heat resistance at pH close to neutrality. A decrease in the pH of the heating medium usually results in a decreased D-value. Reichart (1994) provided a theoretical interpretation of the effect of pH on microbial heat destruction and described a linear relationship between pH and the logarithm of the D-values for *Escherichia coli*. The logarithm of the heat destruction rate increases linearly in the acid and alkaline range and has a minimum at the optimum pH for growth. High pH interacts synergistically with high temperatures to destroy Gram-negative food-borne pathogens (Teo and others 1996).

Media/Temperature	Escherichia coli O157:H7	Salmonella spp.	Listeria monocytogenes
Beef/60ºC	3.17[a]	5.48[c]	8.32[d]
Beef/57.2ºC	5.3[g]; 4.5[f]	5.4[e]	5.8[h]
Beef/62.8ºC	0.5[g]; 0.4[f]	0.7[c]	1.2[h]
Chicken/60ºC	1.63[a]	5.2[c]	5.29[d]
Turkey/60ºC	1.89[b]	4.82[c]	-
Pork/60º	2.01[b]	6.65[c]	-

[a] Juneja and others 1997; [b] Juneja and Marmer 1999; [c] Juneja and Eblen 2000; [d] Gaze and others 1989; [e] Goodfellow and Brown 1978; [f] Doyle and Schoeni 1984; [g] Line and others 1991; [h] Fain and others 1991.

Table 1. Heat resistance of three food borne pathogens in meat expressed as D-values in minutes.

The protective effect of fatty materials in the heating medium on the heat resistance of microorganisms is well documented (Ahmed and Conner 1995). Theories behind increased heat resistance in foods with higher fat contents relate to reduce water activity and poorer heat penetration (lower thermal conductivity) in the fat portion (Juneja and Eblen 2000). Doyle and Schoeni (1984) reported a D- value at 60 ºC of 0.75 minute for E. *coli* O157:H7 strain 932 in ground beef containing 17-20% fat. Ahmed and others (1995) reported D- values for E. *coli* O157:H7 in ground beef heated at 60 ºC ranged from 0.45 (beef, 7% fat) to 0.47 (beef, 20% fat) minutes. Ground beef contaminated with *S. typhimurium* DT 104 heated to an internal temperature of 58 ºC for 53.5 (7% fat) or 208.1 minutes (24% fat) resulted in a 7-D process for the pathogen; a heating time at 65 ºC achieved the same level of reduction in 7.1 and 20.1 minutes, respectively (Juneja and Eblen 2000). The authors reported that *S. typhimurium* DT 104 does not possess unique characteristics that would predispose it to survival during thermal processing. In another study, vacuum-packaged pasteurized salmon fillets (10.56-17.2%, w/w fat) had one to four

times higher D-values for *L. monocytogenes* than the lower fat (0.6-0.8%, w/w fat) cod fillets (Emarek Ben and Huss 1993).

Various solutes in the heating medium exert different effects on the heat resistance of microorganisms, depending upon the nature of the solutes and their concentration. The effects of solutes on thermal resistance have mainly been examined by determining the relationships between thermal resistance and either solute concentration or water activity of the heating media. In a study by Reichart and Mohacsi-Farkas (1994), heat destruction of seven food-borne microorganisms was studied as a function of temperature and water activity was assessed in synthetic heating media; the results showed that the heat destruction increased with increasing water activity.

Recovery of cells after heat treatment can vary. Both an increase in the number of viable cells capable of producing colonies and an increase in the estimated D-value are observed under optimum recovery conditions. Temperatures below the optimum for growth may enhance repair of heat damage (Katsui and others 1982). Bacterial cells/population in stationary phase or those that have experienced some sub-lethal stress undergo physiological changes that make them more resistant to subsequent heat treatment or any other potentially stressful condition (Smith 1995). For example, sub-lethal heat stress renders an organism more resistant to subsequent heat treatment that would otherwise be lethal.

Several methods are commonly used to measure heat stress and thermal inactivation. Existing methods for thermal inactivation determination of microorganisms include TDT (thermal death time) tubes, TDT pouch (nylon), TDT can, flask, thermoresistometer and capillary tube methods (Farkas 1997). All these containers, and some modifications, are being used to obtain data for thermal process calculations. Each has advantages and

disadvantages. In the TDT tube method, inoculated sample (water, buffer solution, culture medium, or food material) is distributed in small diameter (7 to 10 mm) test tubes, which are subsequently sealed. The volume of product per tube usually is from 1 to 4 ml. The sealed tubes of sample are heated in a thermostatically controlled bath. At predetermined intervals, replicate tubes are removed and plunged into ice water. After cooling, the tubes are aseptically opened and their contents transferred to sterile culture medium favorable for growth of the organism being studied. The chief advantages of the TDT tube method, relative to some other methods, are: 1) it employs simple, inexpensive equipment available to most laboratories, 2) bacterial growth in clear media and spoilage changes in some foods products may be observed visually without opening the tubes, 3) tubes may be easily opened for subculture with little danger of contamination and 4) space required for incubation of unopened TDT tubes is small. Chief disadvantages of the TDT method are: 1) filling, sealing, heating and sub-culturing of samples within the tubes are very time-consuming operations, thereby making labor costs high, 2) in transferring contents for subculture there is always the possibility of leaving some survivors in the TDT tubes, 3) generally only liquid products or homogenates can be used as the sample media, 4) and heating and cooling periods in the tube content are appreciable and difficult to evaluate with respect to lethal value (Al-Holy 2003; Stumbo 1973), however procedures for evaluating these heating and cooling periods have been proposed (Sognefest and Benjamin 1944).

The effectiveness of the individual effects of heat treatment, pH, salt etc., with regard to pathogen inactivation is maximized by conducting multiple factorial experiments in which the effects and interactions of these parameters in foods are assessed. Subsequently, inactivation kinetics or thermal death models are developed which predict the target

pathogens survival within a specific range of food formulations variables. These models can help either to establish an appropriate heat treatment or to understand and determine the extent to which existing/traditional thermal processes could be modified for a variety of cooked foods. The models can contribute to more effective evaluation and assessment of the impact of changes in food formulations that could affect their microbiological safety or the lethality of pathogens. These predictive models enable food processors and regulatory agencies to ensure critical food safety margins by predicting the combined effects of multiple food formulation variables. Using these models, food processors are able to design appropriate processing times and temperatures for the production of safe food with extended shelf life without adversely affecting the sensory quality of the product. However, it is of critical importance that the D-values predicted by the model first be validated with the resistance data obtained by actual experiments in specific foods before the predictive values can be used to design thermal processes for the production of safe food (Juneja and Sofos 2002).

Development of time-temperature indicators (TTI)

Since both the heating system and food composition affects the ability of a thermal process to kill harmful bacteria in ways that are not easy to predict, development of a rapid and accurate method for determining pasteurization effectiveness is critical. A common method for evaluating effectiveness of the thermal process is microbial testing (Pflug and others 1980). However, this method is time consuming, uneconomical and labor intensive (Mulley and others 1975: Pflug and Odlaug 1986). Microbial testing involves recovering microorganisms from the treated food and enumerating them, which takes a minimum of two days. Development of an inexpensive, fast and easy to use process time-temperature indicator

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that mimics how a target pathogen behaves in a food, and also provides a practical and accurate analytical method for process validation in the food industry.

Several alternative techniques have been studied as indirect assessments for thermal process validation. These include measuring the loss of heat labile compounds in foods such as thiamine and formation of specific Maillard reaction products (Prakash and others 1997); however, none of these methods is useful for pasteurization regimes as these chemical changes occur slowly, or do not provide a sensitive assessment of heat treatment at temperatures less than 100 ºC. One alternative method for determining process effectiveness is by assessing the impact of time-temperature heat exposure on time temperature indicators and directly correlating these to thermal inactivation data for target microorganisms. Various types of time-temperature indicators (TTI) have been developed in an attempt to provide simple indirect assessments of the cumulative time-temperature effects in thermal processes.

A time-temperature indicator is a device that responds to the combined effect of time and temperature (Singh and Wells 1987). The TTI mimics the changes of a target attribute undergoing equivalent variable temperature exposure (Taoukis and Labuza 1989a,b; De Cordt and others 1992; Hendrickx and others 1995; Van Loey and others 1996). According to the response mechanism, TTI are classified as either full or partial temperature history monitors. Full history TTI responds to the complete range of exposure temperatures and provides a means for comparing temperature histories. Partial history TTI responds only to temperature fluctuations that exceed a predetermined threshold and are most effectively used to detect severe temperature abuse (Manske 1983).

TTI systems can include microbiological, chemical, physical, and biochemical or enzyme indicators. The TTI should be a simple, fast, inexpensive, and precise device. The

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thermal inactivation of a TTI must correlate with the thermal inactivation of a target microorganism or other appropriate parameter (Hayakawa 1978; Hendrickx and others 1992).

Correlating thermal inactivation of enzymes with inactivation of microorganisms is employed as a technique of process validation. Thermal inactivation of an enzyme is generally first order, reaction kinetics that are relatively simple to model (De Cordt and others 1992, 1994; Van Loey and others 1997; Violet and Meunier 1989). Therefore, is possible to predict inactivation of microorganisms in heated foods by monitoring enzyme inactivation.

Microbiological TTIs have been specifically developed for determining process lethality during thermal processing of foods (Pflug and Odlaug 1986). *Bacillus anthreacis* suspended in polymethylmethacrylate in spherical shapes was used to determine the heat transfer coefficient between water and particles in a scraped surface heat exchanger (Hunter 1972). *Bacillus stearothermophilus* immobilized in calcium alginate was studied for the convective heat transfer coefficients at the boundary between a heated liquid and spherical particles (Heppel 1985). Several studies have employed microbiological TTIs for survival curve applications where the numbers of surviving organisms from the TTI are counted to verify if sterilization of the product has been achieved (Yawger 1978). Survival of microorganisms in the product suggests under-processing has occurred. Since thermal properties of microbiological TTI are greatly affected by carrier materials, many materials such as alginate (Pflug and others 1980; Brown and others 1984: Sastry and others 1988), plastic rods (Pflug and others 1980), glass (Hersom and Shore 1981),

polymethylmethacrylate (Hunter 1972), and polyacrylamide gel (Ronner 1990) have been studied.

Microbiological TTIs serve as the reference method to which others are compared (Pflug and others 1980) because of the temperature ranges in which the microbiological TTI and target microorganism are equally sensitive providing multipoint measurement capability. However, this approach has not been widely adopted for routine experiments because the analysis take two to ten days, and the assay could require a large amount of resources and time (Mulley and others 1975; Pflug and Odlaug 1986; Brown and others 1984).

To overcome the inherent disadvantages associated with microbiological TTIs, chemical TTIs have been developed (Mulley and others 1975). Thiamine and dextran have been investigated as a chemical TTI, but z- values of these chemical TTIs do not coincide very well with target microorganisms. Destruction of thiamine was found to be slower than bacterial inactivation (David and Merson 1990).

Intrinsic chemical markers, where yield of thermally produced substances that are formed naturally in the food during processing, have been developed by the US Army Natick Soldier Center (Toribio and Lozano 1987; Kim and Taub 1993; Kim and others 1995; Ross 1993). Such markers are 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (M-1), which is formed at sterilization temperatures from D-glucose and amines through 2,3-enolization under weakly acid or neutral conditions. A 4-hydroxy-5-methyl-3(2H)-furanone (M-2) is formed similarly from D-ribose or D-ribose-5-phosphate. Another thermally produced compound is 5-hydroxymethylfurfural (M-3). Formation of the markers is reported to be directly proportional to the heating time at a given temperature. The M-1, M-2 and M-3 were suggested for evaluating time temperature indicators in microwave and ohmic

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sterilization of meats and vegetables, meats, and fruits products, respectively. However, this approach has some critical deficiencies because it cannot be used for pasteurization applications since the compounds form at sterilization temperatures (110-130 ºC) and form slowly under 100 ºC, if at all.

Another strategy for TTIs is to measure a physical change that can assess the impact of heat treatment. One TTI system employs water vapor production to control the melting point of a colored chemical, then monitoring the color change of the TTI and correlating this to thermal exposure. This system is easy to prepare and evaluate; however, is large for imbedding into foods and cannot be applied to solid food or other types of heating media besides water because the color change is tied to water vapor generation (Witonsky 1977).

Change of capacitance before and after thermal treatment is the basis for another type of TTI, the thermal memory cell. It is a simple system, but the effect of food components on the system has not been evaluated (Swartzel and others 1991).

Biochemical based TTI are another system evaluated as TTI. Enzyme systems have a significant advantage over microbiological TTI. Depending upon the system, recovery of enzymes for assays is easier, faster, and safer than recovering microorganisms as a way to evaluate the effectiveness of thermal processes. Thermal characteristics of enzymes can be modified by several techniques such as enzyme immobilization (Zaborsky 1973; Klibanov 1983; Khare and others 1994), isolation or treatment with organic solvents (Zaks and Klibanov 1984; Klibanov 1986; Laane and others 1987; Reslow and others 1987; Weng and others 1991a, 1991b), or modifying water activity by polyols addition (Chang and others 1988).

Some studies used the thermal inactivation of endogenous enzymes in muscle foods as a means of determining whether cooking is adequate. These endogenous enzymes include pyruvate kinase in canned cured pork (Davis and others 1987), cathepsins (Spanier and others 1990), lactate dehydrogenase (Collins and others 1991; Stander and others 1991; Searcy and others 1995), and glutamic-oxaloacetic transminase in cooked beef (Searcy and others 1995), acid phosphatase (Davis and Townsend 1994; Davis 1998; Veeramuthu and others 1988), aspartate-oxoglutarate aminotransferase (Klinger and others 1982), and creatine kinase (Townsend and others 1994) in poultry. Similarly, several enzymes and proteins have been suggested as end point temperature indicators in the cooking of turkey products, including glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase (Wang and others 1995, 1996), creatine kinase, malic dehydrogenase (Bogin and others 1992), lactate dehydrogenase (Bogin and others 1992; Standler and others 1991), serum albumin (Smith and others 1996) and immunoglobin G (Smith and others 1996; Veeramuthu and others 1998).

However, end-point assays of endogeneous enzyme activity are poor candidates for the quantitative monitoring of thermal inactivation (Raviyan and others 2003) because no residual activity remains after the thermal treatment. In addition, the assays for many endogenous enzymes are labor intensive (Smith and others 1996), requiring immunochemical techniques such as enzyme-linked immunosorbent assay (ELISA) or Western blotting, expensive reagents and instrumentation uncommon in food quality assurance laboratories.

Employing exogenous enzyme systems for biochemically based TTI propose the use of enzymes not naturally present in the food. Enzyme based TTIs require slightly higher heat tolerance than the target pathogen, but similar temperature sensitivity and inactivation

at pasteurization temperatures. The use and characterization of an enzyme with a somewhat higher thermal resistance than the target pathogen makes the development of TTI realistic because measurable residual enzyme activity will remain after processing, providing an accurate indication of the degree of microbial inactivation compared to a system in which the target enzyme is non-detectable.

One example of a possible TTI candidate is α-amylase, an enzyme with a wide range of uses in the brewing, baking and starch industries (Yamamoto 1988). α-Amylase is an endo-enzyme which catalyses the hydrolysis of α-(1,4)-glycosidic bonds of amylose and amylopectin to a range of malto-oligosaccharides (Adams 1991). α-Amylase also hydrolyzes α-(1,4)-glycosidic bonds in smaller polyglucans, such as maltodextrin. These enzymes are of particular interest in TTI applications for the following reasons: 1) they are inexpensive, 2) commercially available, 3) and the enzymatic assay is fast and simple. Heat labile α-amylases from *Aspergillus oryzae* (Kunda and Das 1970), *Bacillus subtilus* (Yamane and Maruo 1974) and *Bacillus amyloliquefaciens* (Borgia and Campbell 1978), with temperature optima and stability falling within the range of pasteurization temperatures are candidates as TTI. Ideally, according to Van Loey and others (1997) an enzyme based TTI should have a z-value between 5 and 12 ºC to target pathogens of interest during pasteurization processes.

Another possible enzyme for biochemical TTI is phytase. Phytases (EC 3.1.3.8) belong to the family of histidine acid phosphatases (Mitchell and others 1997; Piddington 1993) and are found primarily in microorganisms and plants. These enzymes catalyze the release of phosphate from phytic acid (*myo*-inositol hexaphosphate), the major phosphorus storage form in plants. At temperatures between 50 and 55 ºC, *Aspergillus niger* (pH 2.5) phytase undergoes an irreversible conformational rearrangement that is associated with

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losses in enzymatic activity of 70 to 80% (Orta-Ramirez and others 1997). The temperature dependence of phytase is similar to those of both *Escherichia coli* O157:H7 and *Salmonella typhimurium,* suggesting that this enzyme could be used as a time-temperature indicator in food products where inactivation of these microbes is of particular concern.

Enzyme immobilization is the most promising technique for a TTI that would be incorporated into a food system. An effective TTI based upon immobilized enzymes requires that the TTI possess the following physicochemical characteristics: 1) high recovery of enzyme activity following immobilization, 2) high retention of enzyme activity over time, 3) slow diffusion of enzyme from the immobilization matrix, 4) and physicochemical compatibility with the food.

Immobilized enzymes have been used for many years in food processing. There are three principal techniques for enzyme immobilization including: entrapment, adsorption to a solid, and covalent attachment to a solid (Swaisgood 1985; Zaborsky 1973). Entrapment is often the most desirable method because results in high recovery of enzyme activity after immobilization, long enzyme stability in the immobilization matrix, and protection from microbial degradation (Cabral and Kennedy 1993).

Acrylamide is one of the support matrices commonly used for the enzyme immobilization. Polyacrylamide gel is a high molecular weight cross-linked three-dimensional porous polymer network. Polyacrylamide gel is insoluble, but can absorb solvent molecules, particularly in aqueous solutions, and results in a porous structure in which enzymes can be entrapped (Nottelmann and Kulicke 1991; Baker and others 1992). The pores of network gel have to be large enough to permit free internal diffusion and substrate accessibility, while small enough to inhibit enzyme leakage (Pizarro and others

1997). The polyacrylamide gel porous structure and pore size are affected mainly by the monomer and the cross-linking agent proportions (Siegel and Firestone 1988; Firestone and Siegel 1991; Pizarro and others 1997). High quantities of the monomer produce a small network structure. In smaller network structures, enzyme activity is reduced as a result of enzyme-network interactions and limitations to substrate or product diffusion (Pizarro and others 1997). According to the path of steepest ascent design, which permits a description of gel structure over a range of the monomer and cross-linking agent proportions, the optimum gel structure can be chosen that offers the maximum enzyme activity (Pizarro and others 1997).

Compared with other methods, polyacrylamide gel entrapment often results in enhanced thermostability. The increased thermostability results from the increased quantity of linkages that may form when an enzyme is held by multiple hydrogen or electrostatic bonds within a porous network. The effect of entrapment on enzyme stability depends upon many factors. An important factor is changing the enzyme conformation, which can occur as the result of microenvironmental binding effects. Enzyme localization is another important factor. Enzymes that are located within the cell wall or bind directly to the gel matrix tend to exhibit greater thermostability than the intracellular soluble form of the same enzyme (Wasserman 1984).

Target microorganisms

During food production proper measures are taken to ensure the safety and stability of the product during its entire shelf life. In particular, modern consumer trends and food legislation have made the successful attainment of this objective much more of a challenge to the food industry. The use of heat for the inactivation of microorganisms is the most

common process used in food preservation today (Juneja and Sofos 2002). Heat treatment is one of the fundamentally important strategies used to assure the microbiological safety of thermally processed foods. A food-borne illness occurs by eating food that has been contaminated with an unwanted microorganism or toxin. This condition is often called "food poisoning". There are a number of factors which contribute to food being unsafe and causing food poisoning. The principal causes of food poisoning can be summarized as: 1) poor personnel hygiene, 2) cross-contamination between raw and processed products, 3) and inadequate monitoring of processing, handling or storage processes.

Many cases of food-borne illness go unreported because their symptoms resemble influenza. The most common symptoms of food-borne poisoning include stomach cramps, nausea, vomiting, and diarrhea (Forsythe 2000). Only a small proportion of cases of food-borne poisoning are brought to the attention of food inspection or health agencies. This is partially because many food-borne pathogens cause mild symptoms and the victims may not seek medical help. Hence the notified number of cases is just the "tip of the iceberg" with regard to true numbers of food poisoning cases. Recently in the United States of America (USA) and England there have been studies to estimate the proportion of cases which are not recorded in an attempt to obtain a more accurate figure of food poisoning numbers (Table 2; Mead and others 1999).

Organism	Under-reporting factor
Bacterial pathogens	
Bacillus cereus	38
Clostridium botulinum	2
Brucella spp.	14
Campylobacter spp.	38
Clostridium perfringens	38
Escherichia coli O157:H7	20
STEC (VTEC) other than O157	Half as common as *E. coli* O157:H7 cases
Escherichia coli enterotoxigenic (ETEC)	10
Escherichia coli, other diarrhoegenic	Assumed to be as common as ETEC
Listeria monocytogenes	2
Salmonella typhi	2
Salmonella, non-typhoid spp.	38
Shigella spp.	20
Staphylococcus aureus	38
Streptococcus Group A	38
Vibrio cholerae 01 or 0139	2
Vibrio vulnificus	2
Vibrio spp. other than those above	20
Yersinia enterocolitica	38

Note: Real cases = Reported cases X Under-reporting factor

Table 2. Under-reporting of food-borne illness in the USA.

Despite the progress in food science and the technology of food production, illness caused by food-borne pathogens has continued to present a major problem of both health and economical significance. In 1990, an average of 120 cases of food-borne illness per 100,000 population were reported from 11 European countries and more recent estimates indicates that in some European countries there at least 30,000 cases of acute gastroenteritis per 100,000 population yearly (Notermans and van der Giessen 1993), much of which is thought to be food-borne.

Mead and others (1999) reported that 76 million illness, 323,000 hospitalizations and 5,000 deaths occurs each year in the USA due to food-borne poisoning (Table 3). Three

pathogens (*Salmonella*, *Listeria* and *Toxoplasma*) were responsible for 1500 deaths per year, which is more than 75% of those caused identified food-borne pathogens.

Disease or agent	Illnesses			Hospitalizations	Deaths
	Total No. of cases	Foodborne	% Foodborne Transmission	Foodborne	Foodborne
Bacterial					
Bacillus cereus	27360	27360	100	8	0
Botulism, foodborne	58	58	100	46	4
Brucella spp.	1554	777	50	61	6
Escherichia coli O157:H7	73480	62458	85	1843	52
Listeria monocytogenes	2518	2493	99	2298	499
Salmonella typhi	824	659	80	494	3
Shigella spp.	448240	89648	20	1246	14
Vibrio cholerae, toxigenic	54	49	90	17	0
Campylobacter spp.	2453926	1953141	80	10539	99
Staphylococcus food poisoning	185060	185060	100	1753	2
Streptococcus, foodborne	50920	50920	100	358	0
Yersinia enterocolitica	96368	86731	90	1105	2

Table 3. Estimated illnesses, hospitalizations, and deaths caused by known food-borne pathogens in the USA (Adapted from Mead and others 1999).

In addition to human suffering, food-borne diseases can also be costly. Buzby and Roberts (1997a, 1997b) estimated that in the USA the medical costs and work productivity losses are in the range of USA $6.6-37.1 billion. Of these costs, USA $2.9-6.7 billion are attributable to: *Salmonella* serovars, *Campylobacter jejuni*, *Escherichia coli* 0157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium perfringens.*

Salmonella typhimurium

Salmonella is a genus of the Enterobacteriaceae family. They are small, Gram negative, facultative anaerobic, non-sporeforming rods, and motile with peritrichous flagella. *Salmonella* spp. are widely distributed in nature with humans and animals being their

primary reservoirs. *Salmonella* serovars are typical of other Gram negative bacteria in that they are able to grow on a large number of culture media and produce visible colonies within 24 hours at about 37 ºC. *Salmonella* serovars are generally unable to ferment lactose, sucrose, or salicin, although glucose and certain other monosaccharides are fermented, with the production of gas. *Salmonella* spp. normally utilize amino acids as N sources, but in the case of *S. typhimurium*, nitrate, nitrite, and NH_3 will serve as sole sources of nitrogen.

The pH for optimum growth is around neutrality, with values greater than pH 9.0 and less than pH 4.0 being bactericidal. A minimum growth pH of 4.5 is recorded for some, but depending on the acid used to lower the pH, the minimum may be as high as pH 5.5 (Chung and Goepfert 1970). Aeration favors growth at the lower pH values. The parameters of pH, water activity, nutrient content, and temperature are all interrelated for *Salmonella* spp., as they are for most other bacteria (Troller 1976). Their optimum growth temperature is about 38 ºC. The lowest temperature at which growth has been reported is 5.3 ºC for *S. heidelberg* and 6.2 ºC for *S. typhimurium* (Matches and Liston 1968). Temperatures of around 45 ºC have been reported by several investigators to be the upper limit for growth (Jay 2000). Growth inhibition has been reported for a_w values below 0.94 in media with neutral pH, with higher a_w values being required as the pH decreases. *Salmonella* spp. are unable to tolerate high salt concentrations. Brine above 9% is reported to be bacterostatic. Nitrite is effective for *Salmonella* spp. control in foods, with the effect being greatest at the low pH values (Jay 2000).

Salmonella spp. are found in many foods. Among the contaminated foods were cake mixes, cookie doughs, dinner rolls, coconut meal, salad dressings, mayonnaise, milk, and cornbread mixes (Adinarayanan and others 1965). Eggs, poultry, meat, and meat products are

the most common food vehicle of salmonellosis to humans (Carramiñana and others 1997; Hendberg and others 1996; Hennessy and others 1996; Lammerding and others1988; Hobbs 1961). In a study of 61 outbreaks of human salmonellosis for the period 1963 to 1965, eggs and egg products accounted for 23 of the outbreaks, chicken and turkey for 16, beef and pork for 8, ice cream for 3, potato salad for 2, and other miscellaneous food for 9 (Steele and Galton 1967; Letellier and others 1999; Mahon and others 1997; Vought and Tatini 1988). In 1967, the most common food vehicles involved in 12,836 cases of salmonellosis were beef, turkey, eggs and egg products, and milk (Korsak and others 1998; Ercolani 1976). More recently, of 7,907 salmonellae isolations made by the Center for Disease Control (CDC) in 1996, 70% were from raw and processed food sources with turkey and chicken sources accounting for 42%.

Poultry is probably the most common source of salmonella (Mead 1982), with up to 70% of broiler carcasses found contaminated with salmonella. The 11 most frequently isolated serovars from clinical specimens in the USA for 1996-1997 are presented in Table 4. For latter years, *S. typhimurium* accounted for 29% and *S. enteritidis* for 16% of all isolates (CDC 1998).

Serovars	Rank by Year	
	1996	1997
Typhimurium	1	1
Enteritidis	2	2
Heidelberg	3	3
Newport	4	4
Montevideo	5	5
Agona	6	6
Braenderup	10	7
Infantis	7	8
Thompson	11	9
Saint-Paul	8	10
Oranienberg	8	13

Table 4. The 11 top-ranked Salmonellae serovars in 1996-1997 by isolation from clinical specimens in the five U.S. sites of the foodnet surveillance program of the U.S. Center for Disease Control and Prevention (Jay 2000).

With respect to heat destruction, *Salmonella* spp. are readily destroyed at milk pasteurization temperatures ($D_{62.8}$=0.06 min) (Forsythe 2002). Shrimpton and others (1962) reported that *S. senftenberg* 775W required 2.5 minutes for a 10^4- 10^5 reduction in numbers at 54.4 ºC in liquid whole eggs. *S. senftenberg* is the most heat resistant of all *Salmonella* serovars. This treatment of liquid whole egg produces a *Salmonella*-free product and destroy egg α-amylase. It has been suggested that the α-amylase test may be used as a means of determining the adequacy of heat pasteurization of liquid egg (compared with the pasteurization of milk and the enzyme phosphatase) (Brooks 1962). In a study on the heat resistance of *S. senftenberg* 775W, Ng and others (1969) found this strain to be more heat sensitive in the log phase than in the stationary phase of growth. These investigators also found that cells grown at 44 ºC were most heat resistance than those grown at either 15 ºC or 35 ºC. Although *S. senftenberg* 775W is 30 times more heat resistance than *S. typhimurium*,

the latter organism is more resistance to dry heat than the former (Goepfert and Biggie 1968). *Salmonella* spp. are quite sensitive to ionizing radiation, with doses of 5-7.5 kGy being sufficient to eliminate it from most foods and feed. The decimal reduction dose has been reported to range from 0.4 to 0.7 kGy for *Salmonella* spp. in frozen eggs.

Salmonella is the most commonly reported cause of food borne outbreaks, accounting for 28% of such outbreaks of known etiology and 45% of outbreaks associated cases during 1973-1987 (Bean and Griffin 1990). The *Salmonella* food-poisoning syndrome (salmonellosis) is caused by the ingestion of food that contains significant numbers of non-host-specific species or serotypes of the genus *Salmonella.* From the time of ingestion of food, symptoms usually develop in 12-14 hours, although shorter or longer times have been reported. The symptoms consist of nausea, vomiting, abdominal pain (not as severe as with staphylococcal food poisoning), headache, chills and diarrhea. These symptoms are usually accompanied by prostration, muscular weakness, faintness, moderate fever, restlessness, and drowsiness. Symptoms usually persist for 2-3 days. The average mortality rate is 4.1%. Although these organisms generally disappear from the intestinal tract, up to 5% of patients may become carriers of the organisms upon recovery.

The infective dose varies according to the age and health of the victim, the food and also the *Salmonella* strain. The infectious dose varies from 20 cells to 10^9 per gram according to serotype, food and vulnerability of the host (Forsythe 2000). According to the National Institute of Health, 1.4 million Americans suffer from salmonellosis every year and about 1,000 are believed to die from the condition annually (Tauxe 1991). The largest outbreaks of salmonellosis typically occur in banquets or similar functions. The *Salmonella* outbreaks, cases, and deaths associated with foods in the USA between 1983-1990 are listed in Table 5.

Summaries of eight outbreaks are presented in Table 6. The 10 leading food sources of salmonellosis outbreaks in the USA, 1973-1987 are listed in Table 7. The leading food sources were beef, turkey, chicken, ice cream, and pork products.

Year	Outbreak	Cases	Deaths
1983	72	2,427	7
1984	78	4,479	3
1985	79	19,660	20
1986	61	12,833	7
1987	52	1,846	2
1988	40	1,010	8
1989	77	2,394	14
1990	49	1,646	2

Table 5. *Salmonella* outbreaks, cases, and deaths traced to foods in the United States, 1983-1987 (Bean and others 1990).

Year	Vehicle Food	Location	Serovar	No. of cases
1989	Mozzarella cheese	MN (USA)	*Javiana*	136[a]
1989	Mozzarella cheese	MN (USA)	*Oranienburg*	11[a]
1994	Ice cream	USA	*Enteritidis*	224,000[b,c]
1994	Hollandaise sauce	DC	*Enteritidis*	56[d]
1995	Baked eggs	IN (USA)	*Enteritidis*	70[d]
1995	Caesar salad dressing	NY (USA)	*Enteritidis*	76[d]
1995	Alfalfa sprouts	USA/Finland	*Stanley*	242[e]
1998	Toasted oats cereal	USA	*Agona*	209[f]

[a] Hedberg and others 1996; [b] Hennessy and others 1996; [c] Vought and Tatini 1998; [d] Center for Disease Control and Prevention 1996; [e] Mahon and others 1997; [f] Center for Disease Control and Prevention 1998.

Table 6. Synopsis of some *Salmonella* spp. food-borne outbreaks.

Rank	Vehicle Food	Outbreaks	Percentage
1	Beef	77	9.7
2	Turkey	36	4.5
3	Chicken	30	3.8
4	Ice cream	28	3.5
5	Pork	25	3.2
6	Dairy products	22	2.8
7	Eggs	16	2
8	Bakery products	12	1.5
9	Mexican food	10	1.3
10	Fruits and vegetables	9	1.1

Table 7. Leading vehicle foods known for salmonellosis outbreaks in the United States, 1973-1987 (Bean and Griffin 1990). Note: An outbreak is defined as two or more cases.

Since the early 1990s, the case rate of salmonellosis has been cut by half, according to a study published in the January issue of Emerging Infectious Disease (CDC, 2004). In 1995, infections caused by *Salmonella* spp. reached a high of 3.9 per 100,000 people but that dropped to 1.9 per 100,000 in 1999. Health officials credited the reduction to extensive control efforts (farm-to-table), encourage the use of pasteurized eggs and teach people to avoid eating raw or runny eggs (http://vm.cfsan.fda.gov/~mow/chap1.html).

Listeria monocytogenes

Listeria are Gram positive, facultative anaerobic, catalase positive, oxidase negative, non-sporeforming bacteria. They are motile by means of flagella and grow between 0 and 42 ºC. A summary of thermal D- and z- values for some *Listeria monocytogenes* strains is presented in Table 8 (Jay 2000). The genus is divided into eight species of which *Listeria monocytogenes* is the species of primary concern with regard to food poisoning (Novak and others 2003). The first complete description of this bacterium dates back more than 60 years, when Murray and others (1926) isolated a short, rod causing disease in rabbits and guinea pigs (ICMSF 1996).

Strains Tested/State	Number of Cells/ml	Heating menstruum	Heating Temp (ºC)	D value (sec)	z value (ºC)	Reference
Scott A, free suspension	~10^5	Sterile skim milk	71.7	1.7	6.5	a
	~10^5	Sterile skim milk	71.7	2	6.5	a
	~10^5	Sterile skim milk	71.7	0.9	6.3	b
Scott A, intracellular	~10^5	Whole raw milk	71.7	1.9	6	c
Scott A, free suspension	~10^5	Whole raw milk	71.7	1.6	6.1	c
F5069, intracellular	~10^6	Sterile whole milk	71.7	5	8	d
F5069, free suspension	~10^6	Sterile whole milk	71.7	3.1	7.3	d
Scott A, free suspension	~10^5	Ice cream mix	79.4	2.6	7	a
	~10^8	pH 7.2 phosphate buffer	70	9	-	e
	~10^8	pH 5.9 meat slurry	70	13.8	-	e
	~10^7	Liquid whole egg	72	36	7.1	f
	~10^7	Irradiated ground meats	62	61	4.92	g
Ten strains	~10^5	Beef	70	6.6	7.2	h
Chicken, meat isolate	~10^5	Minced chicken	70	6.4	6.7	h

[a] Bradshaw and others 1987; [b] Bradshaw and others 1985; [c] Bunning and others 1986; [d] Bunning and others 1988; [e] Boyle and others 1990; [f] Foegeding and Stanley 1990; [g] Farber 1989; [h] Machey and others 1990.

Table 8. Summary of some findings on the thermal destruction of *L. monocytogenes*.

Listeria monocytogenes is widely distributed in the environment and is frequently isolated from soil, and from raw or processed vegetables such as lettuce, asparagus, broccoli, cauliflower and endive (Lovett and Twetd 1988). This bacterium has been found in the digestive tract and fur of animals; in mollusks, crustaceans, refrigerated uncooked pork, beef, turkey, processed meat products, sausage, ice cream and frozen yogurt (Papageorgion and others 1997). *L. monocytogenes* can grow over wide pH, a_w and temperature ranges. *L. monocytogenes* has been associated with such foods as raw milk, supposedly pasteurized fluid milk, cheeses (particularly soft-ripened varieties), ice cream, raw vegetables, ready-to-eat (RTE) food, fermented raw-meat sausages, raw and cooked poultry, raw meats (all types), and raw and smoked fish. Its ability to grow at temperatures as low as 0 °C permits multiplication under refrigeration, therefore it is classified as a psychotropic pathogen. *L.*

monocytogenes not only multiplies at refrigeration temperatures but is also highly salt tolerant.

Some studies suggest that 1-10% of humans may be intestinal carriers of *L. monocytogenes*. *L. monocytogenes* has been found in at least 37 mammalian species, both domestic and feral, as well as at least 17 species of birds and possibly some species of fish and shellfish. This ubiquitous bacterium has been isolated from various environments including decaying vegetation, soil, animal feed, sewage, water, and other environmental sources. *L. monocytogenes* is quite hardy and resists the deleterious effects of freezing, drying, and heat remarkably well for a bacterium that does not form spores. Most *L. monocytogenes* are pathogenic to some degree and are responsible for opportunistic infections; preferentially affecting individuals whose immune system is immunocompromised including: pregnant women, newborns, HIV infected individuals and the elderly (Forsythe 2000).

L. monocytogenes was first recognized as an emerging food borne pathogen in the 1980s (Farber and Peterkin 1991). Listeriosis is the clinical name of the general group of disorders caused by *L. monocytogenes*. Listeriosis is clinically defined when the organism is isolated from blood, cerebrospinal fluid, or an otherwise normally sterile site (e.g. placenta, fetus). The manifestations of listeriosis include septicemia, meningitis (or meningoencephalitis), encephalitis, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion (2nd/3rd trimester) or stillbirth. The onset of the previously mentioned disorders is usually preceded by influenza-like symptoms including persistent fever. Gastrointestinal symptoms such as nausea, vomiting, and diarrhea may precede more serious forms of listeriosis or may be the only symptoms expressed.

The time of onset to serious forms of listeriosis is unknown but may range from a few days to three weeks. The onset time to gastrointestinal symptoms is unknown but is probably greater than 12 hours. The infective dose of L. *monocytogenes* is unknown but is believed to vary with the strain and susceptibility of the victim. From cases contracted through raw or supposedly pasteurized milk, it is safe to assume that in susceptible persons, fewer than 1,000 total organisms may cause disease. *L. monocytogenes* may invade the gastrointestinal epithelium. Once the bacterium enters the host monocytes, macrophages, or polymorphonuclear leukocytes, it is bloodborne (septicemic) and can grow. Its intracellular presence in phagocytic cells also permits access to the brain and probably transplacental migration to the fetus in pregnant women. The pathogenesis of *L. monocytogenes* centers on its ability to survive and multiply in phagocytic host cells. Listeriosis has a very high mortality rate. When listeric meningitis occurs, the overall mortality may be as high as 70%. Cases of septicemia have a 50% fatality rate whereas in perinatal-neonatal infections the rate is greater than 80%. In infections during pregnancy, the mother usually survives. Infection can be symptomless, resulting in fecal excretors of infectious listeria.

USDA data from 1998 indicates that 2.5% of 3547 RTE samples tested (including salads, jerky, sausage, cooked poultry, ham, and roast beef) were positive for *L. monocytogenes* (http://www.fsis.usda.gov/oa/topics/lm_action.htm; Farber and Peterkin 1991).

Because of its heat resistance, *Listeria* inactivation is commonly used for validating pasteurization process. Microorganisms with z-values ranging from 5 to 12 °C, such as *Listeria monocytogenes*, are often used as reference microorganisms for monitoring the efficacy of pasteurization processes (Van Loey and others 1997). Thermal death times for

Listeria spp. have been evaluated in many foods. However, the results vary due to the different methodologies used. Furthermore, *Listeria* spp. exhibit heat shock response when exposed to sub-lethal temperature, resulting in a significantly higher survival during subsequent heat exposure. The thermal death times can increase 2.3 times when compared to the non-heat shocked controls.

Contamination of foods with *L. monocytogenes* has had a significant impact on the safety of foods. In some countries, currently including the USA, food regulatory agencies require zero tolerance, which is interpreted as none detectable per 25 g in ready to eat (RTE) food. However, this policy is rare in the international community, with Italy being the only other country besides the USA with a zero tolerance for all RTE foods. The next most stringent nation is Australia, which has a zero tolerance policy for foods that support the growth of *L. monocytogenes*. Germany and France each have a tolerance limit of less than 100 CFU per gram of food at the point of consumption for RTE foods. (Hu and Shelef 1996).

The minimal infection dose has been reported as either unknown or more than 100 viable cells (ICMSF 1994). While it is likely that exposure to fairly high doses is required for infection and disease, strain differences in virulence and differences in host susceptibility contributed to the fact that exposure to *L. monocytogenes* from contaminated foods rarely appears to cause disease (Notermans and Hoornstra 2000). However, because of the occurrence of *L. monocytogenes* in foods, as well as its ability to survive under harsh conditions, the risk of these microorganisms cannot be under estimated.

Recent estimates suggest that a total of 2500 human listeriosis cases occur annually in the USA, resulting in a total of 500 deaths (Mead and others 1999). Human listeriosis can occur as sporadic cases and as epidemic outbreaks. Most epidemic outbreaks have been

linked to consumption of contaminated foods and 99% of sporadic cases are also though to be food borne (Farber and Peterkin 1991; Schuchat and others 1991; Mead and others 1999). Data from the Foodborne Diseases Active Surveillance Network (FoodNet) found that, although human listeriosis is less common than many other food-borne diseases (e.g., those caused by *Escherichia coli* O157:H7, *Campylobacter*, or *Salmonella)*, it is by far the most severe. *L. monocytogenes* has a 90% hospitalization rate as well as the highest case fatality rate, resulting in the death for 20% of those infected. As an increasing segment of the population falls into high-risk groups for *L. monocytogenes* infection (e.g. the elderly), those with pre-existing diseases, and the immunocompromised (including AIDS patients, transplant recipients, and cancer patients), listeriosis will continue to be a major public concern.

Escherichia coli

Escherichia coli was established as a food-borne pathogen in 1971 when imported cheeses contaminated with an enteroinvasive strain that caused illness in nearly 400 individuals. Prior to 1971, at least five food borne outbreaks were reported in other countries, with the earliest being from England in 1947. As a human pathogen, evidence suggested that *Escherichia coli* was recognized as a cause of infant diarrhea as early as the 1700s. The organism, originally called *Bacterium coli*, was isolated from the feces of infants by Escherich over a century ago (Wilson and others 1983). In 1920, the organism was renamed *Escherichia*, and there was increasing evidence that the bacterium could occasionally cause gastroenteritis with significant mortality in infants. By the mid-1940s, the role of *Escherichia coli* as an enteropathogen was firmly established and control measures to reduce the incidence of the illness were subsequently taken in developed countries.

Escherichia coli are members of the Enterobacteriaceae family. As such, the organisms are Gram negative, catalase-positive, oxidase-negative, facultative anaerobic short rods. Most stains ferment lactose, although some are slow lactose fermenters and a few are anaerogenic. Strains of *E. coli* may be differentiated from one another serologically on the basis of somatic (O), flagellar (H), and capsular (K) antigens. Pathogenic strains of *E. coli* are divided according to clinical symptoms and mechanisms of pathogenesis into the following groups (Forsythe 2000):

- Enterotoxigenic *E. coli* (ETEC) are commonly cause an ailment known as traveler's diarrhea. ETEC cause watery diarrhea, rice water-like, and a low-grade fever. The organism colonizes the proximal small intestine.

- Enteropathogenic *E. coli* (EPEC) cause watery diarrhea of infants. EPEC cause vomiting, fever, and diarrhea which is watery with mucus but no blood. The organism colonizes the microvilli over the entire intestine to produce a characteristic attaching and effacing injury in the brush border microvillis membrane.

- Enterohaemorrhagic *E. coli* (EHEC) cause bloody diarrhea, haemotthagic colitis, haemolytic uraemic syndrome and thrombic thrombocytopenci purpura. This group includes the verotoxigenic *E. coli* serotypes 0157, 026 and 0111.

- Enteroaggregative *E. coli* (EaggEC) cause persistent diarrhea, especially in children, lasting more than 14 days. The EaggEC align themselves in parallel rows on tissue cells. This aggregation has been described as "stacked brick-like". These strains produce a heat-labile toxin. EaggEC adheres to the intestinal mucosa and elaborate the enterotoxins and cytotoxins, which result in secretory diarrhea and mucosal

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damage. Recent studies support the association of EaggEC with malnutrition and growth retardation in the absence of diarrhea.

- Enteroinvasive *E. coli* (EIEC) cause a fever and profuse diarrhea containing mucus and streaks of blood. The organism colonizes the colon and carries a 120-140 mD plasmid known as the invasiveness plasmid which carries all the genes necessary for virulence.

- Diffusely adherent *E. coli* (DAEC) have been associated with diarrhea in some studies.

EHEC were first described in 1977 and recognized as a disease of animals and humans in 1982 during an outbreak of severe bloody diarrhea. The outbreak was traced to contaminated hamburgers. Since then, most infections have come from eating undercooked ground beef. EHEC harbor plasmids of various sizes, the most common being 75-100 kbp. The EHEC belong to many serogroups. The serotype O157:H7 is the most important in the UK and USA. The 'O157' and 'H7' refer to the serotyping of the strain's O and H antigens respectively. EHEC can cause severe forms of food poisoning resulting in death. It has been postulated that *E. coli* O157:H7 evolved from EPEC and acquired the toxin gene from *Shigella dysenteriae* via a bacteriophage, and that the newly emerging pathogen arrived in Europe from South America (Coghlan 1998). The reported incidence and serotype vary from country to country (Table 9; Adapted from Forsythe 2000).

Country	EHEC infections	Millions of inhabitants	Per million inhabitants
Spain	4	39.6	0.1
Italy	9	57.1	0.2
Netherlands	10	15.4	0.6
Finland	5	5.1	1
Denmark	6	5.2	1.2
Austria	11	8	1.4
Germany	314	81.5	3.9
Belgium	52	10	5.2
Sweden	118	8.7	13.6
United Kingdom	1180	58.1	20.3
Northern Ireland	14	1.6	8.8
Wales	36	2.9	9.2
England	624	48.5	12.4
Scotland	506	5.1	99.2

Table 9. Incidence of EHEC in Europe (1996).

Some strains of pathogenic *E. coli* can grow at temperatures as low as 7 ºC and as high as 46 ºC, the optimum range being 35-40 ºC. Pathogenic *E. coli* strains generally survive well in foods at refrigeration temperatures (3-7 ºC). Thermal inactivation studies have revealed that *E. coli* O157:H7 is more sensitive to heat than typical *Salmonella* spp. Hence, heat treatments that are sufficient to kill *Salmonella* should also kill *E. coli* O157:H7.

The *E. coli* O157:H7 strain differs from the majority of *E. coli* strains because it does not grow, or grows poorly, at 44 ºC. *E. coli* O157:H7 is slightly more limited in its growth range, with a minimum temperature of growth of 8 ºC, a maximum of about 44-45 ºC and a optimum of 37 ºC. Also this strain does not ferment sorbitol or produce β-glucuronidase. The growth of *E. coli* O157:H7 in the human intestine produces a large quantity of toxin(s) that cause severe damage to the lining of the intestine and other organs of the body. These toxins, referred to as verotoxin (VT) or Shiga-like toxin (SLT), are very similar, if not

identical, to the toxins produced by *Shigella dysenteriae*. They are four subgroups VT1 or SLT1, VT2 or SLT2, VT2c or SLT IIc and VT2e or SLT IIe. Strains producing shiga toxins were initially recognized by the cytotoxicity towards Vero cells (green monkey kidney cell line) and subsequently the term "verotoxigenic *E. coli*" or VTEC arose. However, since the purified and sequenced verotoxin is nearly identical to shiga toxin, the organisms are referred to as "shiga toxin-producing *E. coli*" or STEC. The toxins destroy the intestinal cells of the human colon and may cause additional damage to the kidneys, pancreas and brain (Forsythe 2002).

Children and the elderly are the most vulnerable members of the population to *E. coli* O157:H7 infection and develop haemorrhagic colitis (HC) which may lead to haemolytic uraemic syndrome (HUS). Healthy adults suffer from thrombic thrombocytopenic purpura (TTP) where blood platelets surround internal organs, leading to damage of the kidneys and central nervous system. HC is a less severe form of *E. coli* O157:H7 infection than HUS. The first symptom of HC is the sudden onset of severe crampy abdominal pains follow by watery diarrhea. Vomiting occurs in about half of the patients during the period of non-bloody diarrhea and/or other times in the illness. After 1 or 2 days, the diarrhea becomes bloody and the patient experiences increased abdominal pain. Symptoms usually lasts between 4 and 10 days. In severe cases, fecal specimens are described as "all blood and no stool". In most patients, the bloody diarrhea resolves with no long-term impairment. Unfortunately, 2-7% of the infected individulas (up to 30% in certain outbreaks) will progress to HUS and subsequent complications. Acute renal failure is the leading cause of death in children, whereas thrombocytopenic is the leading cause of death in adults. The "Shiga-like toxins" are specific for the glycosphingolipid globotriaosylceramide (Gb3) which is present in renal

endothelial cells. Because Gb3 is found in the glomeruli of infants under 2 years of age but not in the glomeruli of adults, the presence of Gb3 I in the pediatric renal glomerulus may be a risk factor for development of HUS (Forsythe 2002).

With HUS, the patient suffers from bloody diarrhea, haemolytic anemia, kidney disorder and renal failure, and requires dialysis and blood transfusions. Central nervous system disease may develop which can lead to seizures, coma and death. The mortality rate of *E. coli* O157:H7 infection is 3 – 17 %. HUS is a leading cause of kidney failure in children, which often requires dialysis and may ultimately be fatal. Other systemic manifestations of illness due to *E. coli* O157:H7 include a central nervous system involvement, hypertension, myocarditis and other cardiovascular complications that may result in death or severe disability. In some cases, the illness is indicative of heart disease and has been responsible for strokes in small children. These complications are attributed to direct or indirect actions of verotoxins absorbed from the intestinal tract.

Escherichia coli O157:H7 is an emerging cause of foodborne illness. An estimated 73,000 cases of infection and 61 deaths occur in the USA each year (Forsythe 2002). Cattle appear to be the main reservoir of *E. coli* O157:H7. Transmission to humans is principally through the consumption of contaminated foods, such as raw or undercooked meat products and raw milk. Freshly pressed apple juice or cider, yogurt, cheese, salad vegetables and cooked maize have also been implicated. Fecal contamination of water and other foods, as well as cross-contamination during food preparation, are also implicated as transmission routes. There is evidence of transmission of this pathogen through direct contact between people. *E. coli* O157:H7 can be shed in feces for a median period of 21 days with a range of 5-124 days.

According to the US Food and Drug Administration (FDA), the infectious dose for *E. coli* O157:H7 is unknown (FDA Bad Bug Book; http://vm.cfsan.fda.gov/~mow/intro.html); however, a compilation of outbreak data indicates that it may be as low as 10 organisms. The data show that it takes a very low number of microorganisms to cause illness in young children, the elderly and immunocompromised people. Most reported outbreaks of EHEC infection have been caused by O157:H7 strains. This suggests that this serotype is more virulent or more transmissible than the other serotypes.

Consumers can prevent *E. coli* O157:H7 infection by thoroughly cooking ground beef, avoiding unpasteurized milk, and washing hands carefully. Because the organism lives in the intestines of healthy cattle, preventive measures on cattle farms and during meat processing are being investigated.

Microwave heating

The application of microwave energy to heat foods was patented in 1945 by Percy Spencer of Raytheon Corporation as an offshoot of radar technology developed during World War II (Buffler 1993). The first Radarange™ became available for foodservice use in 1947 and commercially ovens were introduced in 1955. Approximately 93% of USA households own a microwave oven, primarily for use in rewarming previously cooked, chilled, or frozen foods (Fusaro 1994). Microwave energy is more efficient and rapid than conventional heating, allowing food to be heated in as little as one quarter of the normal time.

There are additional commercial applications of microwave heating other than cooking and reheating operations typically used by consumers. Microwaves are used to inactive enzymes (Copson 1954; Pour-El and others 1981), blanch foods (Goldblith 1966), defrost, temper, or thaw products (Decareau 1985), bake, pasteurize, or sterilize (Olsen 1965;

Ayoub and others 1974; Kenyon and others 1971; Sale 1976), and evaporate, dry and freeze-dry (Sunderland 1982).

Like ultraviolet, visible light, infrared, and radio waves, microwave energy is a form of nonionizing electromagnetic radiation in the frequency range from 300 MHz (0.3 GHz) to 300 GHz that generate heat in a dielectric material (Risman 1991). Microwaves differ from other energy forms, in addition to frequency of oscillation, in wavelength and wave velocity (Chipley 1980; Clearly 1970; Clearly 1977; Curnutte 1980). In the USA, frequencies used for microwave heating in an industrial, scientific, and medical context are controlled by the Federal Communications Commission (FCC). Of the commonly used frequencies (915, 2450, 5800 and 22125 MHz), the US FDA currently allows only 915 and 2450 MHz for commercial and home ovens, respectively (Chipley 1980).

The magnetron or generator produces microwaves through interaction of strong electric and magnetic fields (Figure 1, Figure 2). The magnetron is a vacuum tube that uses a magnetic field to affect the flow of electrons from the cathode to the anode. When power is supplied, an electron-emitting material at the cathode becomes excited and emits electrons into the vacuum space between the cathode and anode. The anode is composed of resonant cavities that act as oscillators and generates electric fields. Microwave energy from the magnetron flows down the waveguide, a hollow metallic tube, into the oven. As the waves enter the cavity, they are dispersed by a mode stirrer, causing multiple reflections of the energy to minimize "hot and cold spots" in the oven cavity (Knutson and others 1987).

Microwaves can also be transmitted through containers and lids made of ceramic, glass, paper, and plastic materials, and can be absorbed by a medium such as food (Knutson and others 1987). Cookware remains cool unless heat from the cooking product is absorbed.

In the case of plastic, sufficient heat can soften or melt the container; absorption of moisture by paperboard may result in sogginess. Metals such as aluminum foil and steel reflect microwaves and can be used to shield parts of foods, but arcing can occur if metal touches metal or the oven walls (Curnutte 1980). Excessive reflection can harm the magnetron and damage the oven.

The magnetron produces an electromagnetic field with positive and negative charges (Mudgett 1989). Production of heat occurs during microwave heating primarily through dipolar rotation and ionic polarization (Chipley 1980), that is caused by these field oscillations. When microwave energy is absorbed by the food that the energy is converted to heat. Because of the way in which microwaves heat, some foods require reformulation to accommodate these changes (Schiffmann 1990).

Polar molecules such as water have negatively and positive charged ends. In the presence of a microwave electric field, the water molecules attempt to line up with the field. Since the microwave field is reversing polarity millions of times per second, the water molecules moves first in one direction and then reverses to move in the opposite direction. The friction caused by constantly oscillating charged particles produce heat. Most foods are composed of 50-90% water (Ohlsson 1983), and it is the unbound water that contributes to the heating effect. In addition, ions, because of their electric charge, flow in one direction, then in the opposite direction in an electromagnetic field producing heat by ionic conductivity. This effect leads to a higher temperature at the surface of water or foods containing salt.

The ability of microwaves to heat depends upon the dielectric properties of the food as described by the dielectric constant and the dielectric loss factor (Schiffmann 1990). The

dielectric constant (ε') describes the ability of the material to store electrical energy and varies significantly with temperature and frequency. The dielectric loss factor (ε'') describes the ability of the material to dissipate electrical energy as heat.

The air inside the microwave oven cavity is cool, which causes surface cooling of the microwave-cooked foods. Consequently, the maximum temperature is reached somewhat below the surface of the cooked foods, leaving the surface cooler and moister than those foods cooked conventionally. The speed of microwave cooking results from the ability of microwaves to deposit more energy at greater depths in the food than can be achieved by conventional heating techniques (Bakanowski and Zoller 1984). Therefore, is often recommended that final cooking of microwave-heated foods should include a "standing time" during which foods continue to transfer heat from the interior portion to the surface.

In early studies comparing microwave heating with conventional heating, a tremendous effort was focused on obtaining an accurate temperature analysis of heated samples. Although the time of heating could be easily determined, temperature measurement was available only at the end of the heating cycle (Juneja and Sofos 2002). The most common procedure was to use a mercury thermometer to gauge the endpoint temperature after heating. The sample was removed from the oven, mixed or stirred, and temperature measured. This temperature comprised a representative reading of the entire product with no delineation of any variations in heat distribution throughout the product. Thermocouples, while commonplace in other temperature-measurement settings, where observed to arc. Even with proper shielding, thermocouples did not provide accurate temperature measurements and often altered the heating patterns during cooking. Infrared imaging or thermography

provides a detailed map of surface temperature distributions but could not be used within the oven to map heating patterns as they were developing or changing.

With the advent of fluorescent fiberoptic or "fluoroptic" thermometry foods can be measured for temperature during heating, and somewhat detailed temperature profile can be characterized (Berek and Wickersheim 1988; Wickersheim and Sun 1987). The temperature pattern in foods, however, is dependent upon the number of probes used and their position in the product, as these probes only provide information on discrete points within the sample. The probes are constructed of a phosphor sensor attached to the tip of an optical fiber, neither of which adversely affects temperature measurement. Probes are generally inserted into the microwave through a small (1/8 inch) hole drilled in the oven wall, which does not affect microwave heating (Juneja and Sofos 2002). Process validation of microwave using thermal imaging, fluoroptic probes and fiber optics sensors are not practical for commercial continuous-flow units. Determination of final product temperature after a heating process is less reliable than the in-time temperature measurement because do not provide a measure of the cumulative temperature and time exposure that indicates process lethality. Consequently, one alternative method for assessment of temperature distribution within the packaged foods during microwave sterilization is by assessing the impact of cumulative heating with time temperature indicators.

Overall objectives

The objectives of this research were to:

- Develop two enzyme-based time-temperature indicators (TTI) employing α-amylase from *Aspergillus oryzae* and phytase from *Aspergillus ficuum* entrapped in 20% polyacrylamide gel;

- Investigate the effect of salt content on retention of enzyme activity, dielectric properties, diffusion and storage stability of the TTI;

- Study the thermal inactivation of the TTI and the thermal inactivation of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella typhimurium* in ground shrimp, ground meat (beef, 30% fat) and mashed potatoes;

- Construct prediction equations that correlate thermal inactivation of the TTI with the thermal inactivation of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella typhimurium*;

- Evaluate the use of the TTI as a tool to map heat distribution within ground shrimp, ground meat (beef, 30% fat) and mashed potatoes exposed to 915 MHz in a Microwave Circulated Water Combination (MCWC) heating system.

Significance

- TTI will be effective tools for developing and evaluating pasteurization solving a pressing need for quick and easy validation method.

- TTI can be directly incorporated into foods for heat distribution studies in industrial settings.

- TTI can be incorporated as part of a firm's Hazard Analysis and Critical Control Point (HACCP) verification program.

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CHAPTER 2

PHYSICOCHEMICAL PROPERTIES OF ENZYME-BASED TIME TEMPERATURE INDICATORS BASED UPON IMMOBILIZATION OF α-AMYLASE FROM *ASPERGILLUS ORYZAE* AND PHYTASE FROM *ASPERGILLUS FICUUM* IN POLYACRYLAMIDE GEL

ABSTRACT

Two enzyme based time-temperature indicators (TTI), suitable for pasteurization processes, were developed with *Aspergillus oryzae* α-amylase and *Aspergillus ficuum* phytase physically immobilized in 20% polyacrylamide gel. The activity of immobilized α-amylase with 0.00, 1.00 and 2.00% NaCl exceeded 85% and for immobilized phytase with 0.00, 0.50 and 1.00% NaCl exceeded 85% after storage for 2 months at 8 ºC. After 6 hours of incubation at 25 ºC in 0.05 M phosphate buffer (pH 7.1) for immobilized α-amylase and 200 mM glycine buffer (pH 2.8) for immobilized phytase, the enzymatic activity decreased to around 40%. The dielectric properties of the TTI can be altered to match the experimental foods by adding salt.

Key words: immobilized enzymes, dielectric properties, microwave, pasteurization and radio-frequency.

INTRODUCTION

Food safety has been at the forefront of societal concerns in recent years, and the major emphasis it has received is likely to continue into the future. The complexity of food safety issues has increased, as has the number of emerging pathogenic organisms. Some of these food-borne pathogens are resistant to antibiotics or to traditional preservation methods and sometimes cause illness with low infectious doses (Lederberg 1997; Tauxe 1997). In addition to microorganisms associated concerns, societal changes including changing consumer food preferences, lack of adequate food handling education, increases in human populations at-risk for food-borne illness, complex food distribution patterns, increase international trade, and greater demand for microbial testing and detection also emphasize the complexity of the safety concerns and the need for development of strategies to address these challenges.

It is of key importance for food companies to accurately measure the impact of heat processes in terms of food safety and quality. Failing to accurately verify a process increases legal liability (Rasco 1997, 1999; Buzby and others 2000). Process verification and mapping heat distribution in foods are difficult. This is particularly problematic for dielectric heating processes. The *in situ* method and the mathematical modeling are commonly used for this purpose. In the *in situ* method, the quality/safety attribute is measured in the food before and after heat treatment (*e.g.* microbial load, flavor, nutrient content) (Van Loey and others 1995), while in the physical mathematical method, a model is developed based on the knowledge of the time-temperature history of the product, combined with the knowledge of heat resistance parameters of the attribute under study (Guiavarc'h and others 2002). These two methods, however, have serious limitations with

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regard to modern heating processes (Hendrickx and others 1995). Such limitations include the fact that direct registration of the products time-temperature data is not possible under some processing conditions (*e.g.* continuous processes). In the case of dielectric heating, electronic sensors with metallic components cannot be used with microwave or radio frequency (RF) heating systems because they interact with electromagnetic waves giving misleading data. To overcome these limitations, time-temperature-indicators have been and continue to be proposed as an alternative tool in process design to measure the impact of heat processes in terms of food safety and quality. (De Cordt and others 1992, 1993, 1994; Maesmans and others 1993, 1994, 1995; Hendrickx and others 1995; Van Loey and others 1995, 1996a, 1996b, 1997a, 1997b; Stoforos and Taoukis 1998; Haentjens and others 1998; Raviyan and others 2003; Kim and Taub 1993; Taoukis and Labuza 1989a, 1989b; Weng and others 1991a; 1991b).

Foods are non-ideal capacitors which are able to store and dissipate electrical energy from electromagnetic fields (Mudgett 1986). The dielectric properties of foods are the principal parameters that determine the coupling and distribution of electromagnetic energy during dielectric heating (Nelson 1998; Schiffmann 1990). Dielectric properties are normally described in terms of the dielectric constant (ε') and the dielectric loss factor (ε"). The dielectric constant describes the ability of a material to store energy in response to an applied electric field. The dielectric loss factor describes the ability of a material to dissipate energy in response to an applied electric field, which results in heat generation (Nelson 1994; Lorrain and others 1988; Mudgett 1986; Nyfors and Vainikainen 1989). A suitable time-temperature indicator (TTI) system for microwave heating systems would match the dielectric properties [dielectric constant (ε') and dielectric loss factor (ε")], to the

target food product to ensure that the heating rate of the food product and the TTI are similar.

The dielectric properties for foods are related to the water and ion content. The heating mechanism of water relies on the fact that the water molecule is a dipole. Water molecules align with the direction of the electromagnetic field, which requires some energy absorption from the field for overcoming intermolecular forces among the water dipoles (Ohlsson and others 1974a; Mudgett 1986; Tong and Lund 1993: Ryynanen 1995). Most foods also contain dissolved ions that participate during dielectric heating. However, to obtain a better understanding of how a food will behave during dielectric heating, knowledge of dielectric properties of the target food is important. Knowing these properties is also important for understanding how to modify dielectric properties when producing time temperature indicators (TTI). Several techniques have been used for dielectric property measurement including: cavity perturbation technique (Bengtsson and Risman 1971; Ohlsson and Bengtsson 1975), dynamic cavity perturbation (Jain and Voss 1987), precision slotted line technique (Mudgett and others 1977; To and others 1974), short-circuited coaxial line technique (Mudgett and others 1979; Nelson 1982), slotted coaxial line (Harper and others 1962) and test cell Boonton RX-meter (Bengtsson and others 1963).

The dielectric constant and dielectric loss factor vary with frequency, temperature, density, moisture content, homogeneity, particle size, distribution and state of foods (Ohlsson and others 1974a, 1974b; Mudgett 1986; Tong and Jund 1993; Ryynanen 1995). Therefore, it is beneficial to know the dielectric properties of foods that undergo a dielectric heating process.

The dielectric loss factor increases with water and ion concentration. The elevation of the dielectric loss factor results from increases in ionic conductivity as salt concentration increases. The temperature dependence of the dielectric loss at different salt concentrations is explained by the relative effects of dipolar and ionic losses (Bengtsson and Risman 1971). Salts dissolved in aqueous solutions act as energy conductors in the electromagnetic field, by simultaneously depressing the dielectric constant and elevating the dielectric loss factor. Depression of dielectric constant results from the binding of free water by counter-ions of dissolved salt. The major effect of undisolved organic solids is an exclusion of dielectrically active materials, mainly water, from the total volume, thus depressing the dielectric constant (Mudgett 1986).

The objectives of this study were to: 1) develop enzyme-based time temperature indicators (TTI) employing α-amylase from *Aspergillus oryzae* and phytase from *Aspergillus ficuum* entrapped in 20% polyacrylamide gel, 2) determine the effect of NaCl content on the retention of TTI enzyme activity during refrigerated storage, 3) determine external diffusion of the enzymes from the TTI, and 4) evaluate the possibility of modifying the dielectric properties of the TTI to match those of target foods for microwave or RF processing.

MATERIALS AND METHODS

Enzymes

Aspergillus oryzae α-amylase (EC 3.2.1.1) from Sigma Aldrich Co. (St Louis, MO) with a specific activity of 39 units/mg was used. One unit will liberate 1.0 mg of maltose from 5 DE maltodextrin per minute at pH 6.9 at 30 °C. *Aspergillus ficuum* phytase (EC 3.1.3.8) from Sigma Aldrich Co. with a specific activity of 3.5 units/mg solid was used.

One unit will liberate 1.0 μmole of inorganic P from 4.2 X 10^2 M Mg·K phytate per minute at pH 2.5 at 37 ℃. The polyacrylamide and all of the other chemicals used were at least analytical grade (Sigma Aldrich Co.).

Preparation of enzymes solutions

For soluble enzyme measurement, ~ 15 mg/ml of soluble α-amylase enzyme was prepared in 20 ml of cold 0.05 M phosphate buffer (pH 7.1), and ~ 0.572 mg/ml of soluble phytase enzyme was prepared in 45 ml of 200 mM glycine buffer (pH 2.8). The solution was kept cool in an ice bath and used within 4 h.

Immobilization procedure

The specific activity of α-amylase solution for immobilization was 170 Sigma units mg^{-1} of protein. The specific activity of phytase solution for immobilization was 700 Sigma units mg^{-1} of protein.

The system for immobilization contained: 66.6% (v/v) of solution 1 [20% (w/v) acrylamide gel with 5.3% (w/v) bisacrylamide], 31.3% (v/v) of enzyme solution, 2.1% (v/v) of 3% (w/v) ammonium persulphate, and 60 μl of N,N,N',N'-tetramethyethylenediamine (TEMED). The components in solution 1 were made up to 100 ml with milliQ water and stored at 4 ℃ until used.

The immobilized enzyme-containing gel was prepared by blending the enzyme solution into the solution 1 while stirring on a magnetic stir plate with a Teflon®-coated stirring bar for 2 min at low speed. Ammonium persulphate was added, stirred for 1 min, followed by TEMED, and stirred for 0.5 min. The mixture was allowed to polymerize between two-glass plates separated with flexible rubber tubing around three sides (0.5 cm

spacing) for 4 h at 4 ºC. Gels were stored at 4 ºC in Ziplock® plastic bags until used. For routine experiments, the gel was cut into (ca.) 0.5 x 0.5x 0.5 cm pieces with a razor blade.

The gels containing sodium chloride (NaCl) for the diffusion and shelf life study were prepared as described previously; but NaCl was added to make the phytase polyacrylamide gel solution 0.00% to 1.00% (v/v) NaCl, and 0.00% to 2.00% (v/v) NaCl for the α-amylase polyacrylamide gel solution. The solution was stirred for 1 min, after the enzyme solution had been added.

Maltodextrin substrate preparation for α-amylase activity measurement

The maltrodextrin used as a substrate was MALTIN® M040 (Grain Processing Corporation, Muscatine, IW, USA).   This is a 5DE, bland, white, cold water soluble powder. To block reducing ends, the maltodextrin was treated with sodium borohydride. Five g of MALTIN®M040 was dispersed into 20 ml distilled water and heated for 20-30 sec for mixing. This suspension was diluted to 90 ml, and cooled in an ice bath. Then 10 ml of cold sodium borohydride solution (0.15 g $NaBH_4$/10 ml distilled water) was added and stirred using a magnetic stirrer for 2 min. The solution was stored under refrigeration. This stock solution of maltodextrin substrate was stable for several days (Strumeyer 1967; Raviyan and others 2003).

To prepare the substrate solution for enzyme assay, 0.4 ml acetone was added drop by drop into 20 ml of the $NaBH_4$ -treated MALTIN® M040 while shaking. The solution was allowed to stand for 20 min at 22-25 °C. Then the solution was brought to pH 7.0 with 1 M acetic acid. The volume was adjusted to 100 ml with 0.05 M phosphate buffer pH 6.9 (Strumeyer 1967). This solution was prepared fresh daily.

Phytic acid solution preparation for phytase activity measurement

A 44.1 mM phytic acid solution (pH 2.5) was prepared using phytic acid magnesium-potassium salt from Sigma Aldrich Co. The powder was dissolved using 200 mM glycine buffer (pH 2.8).

Assay of soluble enzymes

The activity of α-amylase (mg maltose/min) was measured spectrophotometrically (Bernfeld 1955; Raviyan and others 2003). This procedure is based upon the progressive hydrolysis of the α-1,4 glucosidic bonds in MALTIN$^®$ M040 at 540 nm. The amount of maltose liberated in the test solution was calculated using reagent grade maltose as a standard.

For measurement of α-amylase activity, the enzyme solution was pre-equilibrated to 30 °C for 15 min later, 1.0 ml substrate was added to 1.0 ml pre-equilibrated enzyme solution and kept at 30 ºC in a water bath with agitation for 5 min. The reaction was terminated by adding 1.0 ml of 3,5-dinitrosalicylic acid solution. The color was developed by placing the reaction mixture in a boiling water bath for exactly 10 min, then cooling on ice to 22-25 °C. Nine mililiters of deionized water was added to each reaction tube before recording the A_{540nm} using Ultrospec 4000 UV/VIS Spectrophotometer (Pharmacia Biotech, Cambridge, England).

The activity of phytase (P-9792) was measure spectrophotometrically according to Heinonen and others (1981). This procedure is based on the progressive release of inorganic phosphate from phytic acid (*myo*-inositol hexakisphosphate) at 37 ºC, which absorbs maximally at 400 nm.

Assay of immobilized enzymes

Three pieces of α-amylase gel were weighed, added to 3.0 ml of cold extraction buffer (0.05 M phosphate buffer, pH 7.1 for gel with 0.00 and 1.00% NaCl, and 0.05 M phosphate buffer, pH 7.1 with 1.00% NaCl for gel with 2.00% NaCl), homogenized using a hand-held tissue grinder while on ice and incubated (4-6 °C) for 30 min. The solution was centrifuged in a Eppendorf Centrifuge 5415 at 10,000 rpm for 4 min at room temperature to remove the precipitated gel. One ml of the liquid fraction was assayed as described for soluble enzyme.

A similar procedure was used for phytase gels except that 200mM glycine buffer (pH 2.8) was used as the extraction buffer.

Enzymatic activity

Enzymatic activities are expressed in terms of the change of optical density and reaction time. The initial activity was determined approximately one hour after polymerization of the polyacrylamide gel was complete. Three experiments were conducted in duplicate.

Enzyme diffusion study

Twenty-four hours following immobilization, thirty-six pieces of gel (0.5 X 0.5x 0.5 cm; approx. weight 0.2 g/piece) were incubated in 250 ml beaker containing 200 ml of 0.05 M phosphate buffer (pH 7.1) for α-amylase and 200 ml of 200 mM glycine buffer (pH 2.8) for phytase. After the desired time of incubation (one to six hr) at 25 °C, three pieces of gels were collected and excess buffer solution was removed by blotting with Whatman #1 filter paper. The weight of the gel following incubation was measured. The enzyme activity following incubation was determined as described previously. Three experiments were conducted in duplicate.

Storage stability study

Three samples for each immobilized gel treatment were sealed in a Ziplock® plastic bags (total 3 bags/treatment). An open ended glass tube (diameter of 0.9 cm and 2.75 cm in length) containing a roll of sterile cotton soaked with buffer was placed inside of each bag to prevent gel desiccation. There was no direct contact between the gel and buffer. Samples were stored at 8 °C and enzyme activity determined in duplicate after 1, 3, 6, 10, 20, 40, 60 days of storage (N=3).

Dielectric properties measurement

Materials

Immobilized α-amylase in polyacrylamide gels (with 0.00, 0.50, 0.75, 1.00, 2.00, and 3.00% NaCl), and immobilized phytase in polyacrylamide gel (with 0.00, 0.50, 1.00 and 2.00% NaCl) were prepared 24 h before dielectric measurements. The gels were placed in Ziplock® plastic bags and maintained at 4 °C until tested. Phosphate buffer 0.05 M (pH 7.1) with 0.00, 0.50, 0.75, 1.00, and 2.00% NaCl, and glycine buffer 200 mM (pH 2.8) with 0.00, 0.50 and 1.00% NaCl were used as model foods along with shrimp (*Penaeus monodon)*, ground meat (beef, 30% fat) and mashed potatoes as target foods. The shrimp, mashed potatoes and ground meat (beef, 30% fat) were obtained from a local supermarket (Pullman, WA) and kept at –35 °C until used. Twenty hours before the experiment, shrimp and ground meat (beef, 30% fat) were thawed under refrigeration conditions. The frozen shrimp headless with intact exoskeleton were peeled, deveined, ground in a food processor, placed in Ziplock® plastic bag and kept in a refrigerator at 4 °C until used. Mashed potatoes were prepared by mixing ½ cup of instant potato flakes (100% Idaho Russet

mashed potatoes, Safeway Inc., Oakland CA) with ½ cup of boiling water and kept at 4 °C until used.

Methods for dielectric measurement

The dielectric properties measurement system used in this study consisted of an Agilent (formerly Hewlett Packard) 4291B impedance analyzer with a calibration kit (Agilent Technologies, Palo Alto, CA), a custom-build test cell, a VWR Model 1157 programmable circulator (VWR Science Products, West Chester, PA), a high temperature coaxial cable, and the dielectric probe included in the Hewlett Packard 85070B dielectric probe. A digital thermometer (model 600-1020, Barnet 115, Bernet Co., Barrington, IL) was used for temperature recording.

Before the measurements, the impedance analyzer was warmed up for at least 30 min, following the manufacturer's recommendations. All electrical connections were checked and cleaned with pure ethanol to remove any residue. The system was calibrated before each set of measurements using four calibrations standards: an open, a short, a 50 Ω load, and a low-loss capacitor. Following this, an 85070B dielectric probe was used to calibrate the testing probe, tests included a short circuit (a gold-plated precision shorting block), an open circuit (air) and a known load (pure water at 25 ºC) Once the system was calibrated, the sample was loaded into a custom build test cell. The probe was then sealed into the loaded test cell and kept in contact with the sample during the measurement.

The test cell was constructed of two coaxial sections of 1 in. and 1 and 1.5 in. OD 304 stainless steel sanitary tubing welded to a 1 in. sanitary ferrule at each end, to serve as sample holder and water jacket. The dielectric probe was installed through a solid sanitary

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end cap and sealed with an o-ring. The probe and end cap mated with the top end of the water-jacketed sample holder, sealed with a gasket and held in place using a sanitary clamp. A thermocouple port was mounted through the bottom sanitary end cap covering the other end of the sample holder. A stainless steel piston provided constant pressure on the sample, maintaining close contact between the sample and the probe tip throughout the whole measurement sequence. A thin 1.02 mm rigid stainless steel thermocouple probe passed through a pressure-tight gland in the thermocouple port, through the center of a spring and piston, and into the center sample to determine temperature of the sample. The programmable circulator was used to pump a temperature-controlled liquid (90% ethylene glycol and 10% water by volume) through the water jacket of the test cell, allowing the cylindrical sample inside to be heated or cooled (Wang and others 2003). Measurements of dielectric constant and dielectric loss factor were conducted on samples at 10 ºC increments from 20 to 70 ºC at 2 FCC-approved frequencies for food treatments (915 and 2450 MHz). Intervals of 10 min were allowed to achieve each 10 ºC increment in the samples before dielectric properties were measured. This period was adequate for the sample to reach a stable temperature. Three experiments were conducted in duplicate.

Statistical analysis

All experiments in this study were repeated at least three times and results are reported as means. General Linear Model procedures for analysis of variance and regression (Proc GLM) were determined using Statistical Analysis System version 8 (SAS Institute, Inc., Cary, N.C, 1999). Arithmetic means were compared by the Fisher LSD grouping test at the 95% confidence level ($p \leq 0.05$). Interaction effects were analyzed by the least square means model.

RESULTS AND DISCUSSION

Enzyme activity during storage at refrigerated conditions.

Figure 1 shows the changes in enzyme activity as a function of storage time for α-amylase TTI. There is a significant effect of the amount of NaCl on the storage stability. The residual activity of the immobilized enzyme with 0.00, 1.00 and 2.00% NaCl exceeded 85% after storage for 2 mo at 8 °C. The significant reduction in the activity of the enzyme immobilized in polyacrylamide with 2.00% NaCl indicates that enzyme stability is affected by ionic strength variations created in the microenvironment of gel matrix as the storage time increases.

The concentration of NaCl in the extracted buffer may also play a role in enzyme recovery. When NaCl (1%) was added to the extraction buffer, a loss of activity of about 20 and 10% was observed in gels immobilized with 0.00 and 1.00% NaCl, respectively. In contrast, the activity of gel immobilized with 2.00% NaCl remained unchanged under the same extraction conditions. Chloride ion may assist with enzyme substrate binding to α-amylase (Whitaker 1996) and help to optimize ionic strength of the extraction media for enzyme recovery.

Figure 2 shows the changes in enzyme activity as a function of storage time for phytase TTI. There was not a significant effect on the amount of NaCl in the gel matrix and the storage stability of the TTI. The residual activity of the immobilized enzyme with 0.00, 0.50 and 1.00% NaCl exceeded 85% after storage for 2 months at 8°C. No visible microorganism growth was observed on the gels during storage.
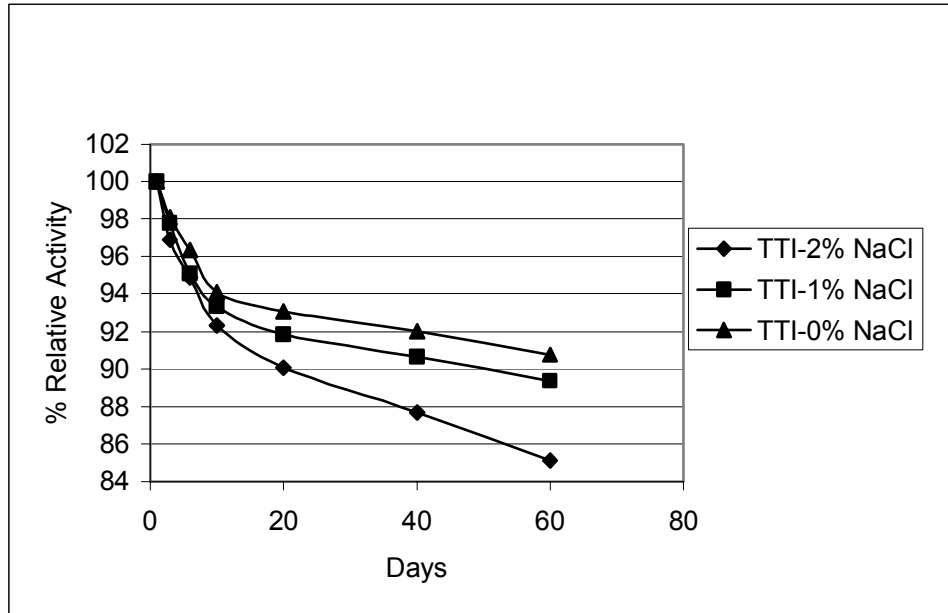
Fig. 1 – Residual activity of α-amylase immobilized in polyacrylamide gel, stored at 8 ^{o}C.
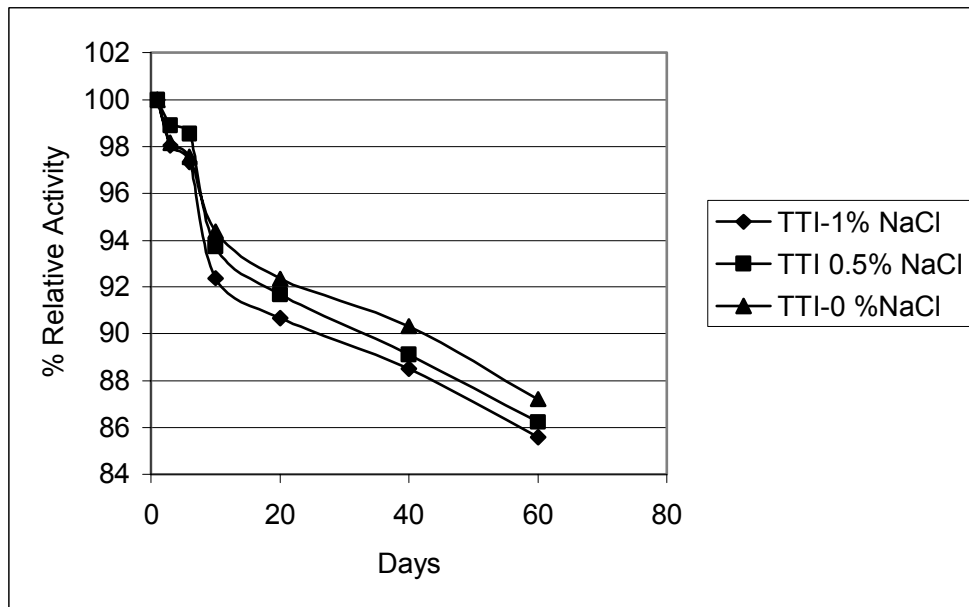
Fig. 2 – Residual activity of phytase immobilized in polyacrylamide gel, stored at 8 ^{o}C.

Acrylamide polymerization is an exothermic reaction, where the amount of heat generated depends significantly upon the level of initiator (ammonium persulphate and TEMED) (Yamamoto and Tatsumi 1986). However, because a constant quantity of initiator was used, and the temperature during polymerization did not increase significantly, this effect was predicted to have little effect on enzyme recovery. The difference in enzyme recovery was primarily due to inactivation through reaction with polymerization reagents, and changes to the gel structure (Pizarro and others 1997). Exposure to the cross-linking agent can reduce recovery of enzyme activity as recently reported for the immobilization of urease (Das and others 1998). However, the most likely cause for this loss is a decrease in gel porosity at higher concentrations of crosslinking agent. (Baselga and others 1989; Pizarro and others 1997). As the gel porosity decreases, enzyme activity becomes reduced as a result of enzyme-network interactions (Pizarro and others 1997). It is also possible that enzyme molecules become more tightly entrapped in the gel matrix and become more difficult to recover for assay, or alternatively, diffusion of the substrate to the enzyme during assay may have been impeded because of the smaller pore size.

Enzyme diffusion from gel matrix

For development of a TTI, a slow diffusion rate of enzyme from the immobilization matrix into the food is necessary. Many enzymes have a low diffusion rate from polyacrylamide gel (Das and others 1998). In general, for both TTIs, enzyme activity decreased with incubation time until is apparently reached a stable point after 5 - 6 h of incubation. This observation can suggest an initial loss of enzyme activity due to reaction with polymerization agents following by leaching from the gel matrix (Figure 3, 4). There was no a significant effect of NaCl content and diffusion of enzyme from the gel following

immobilization. The residual activity of the immobilized enzymes decreased to around 40% of the original level in both cases decreased during 4 h of incubation.
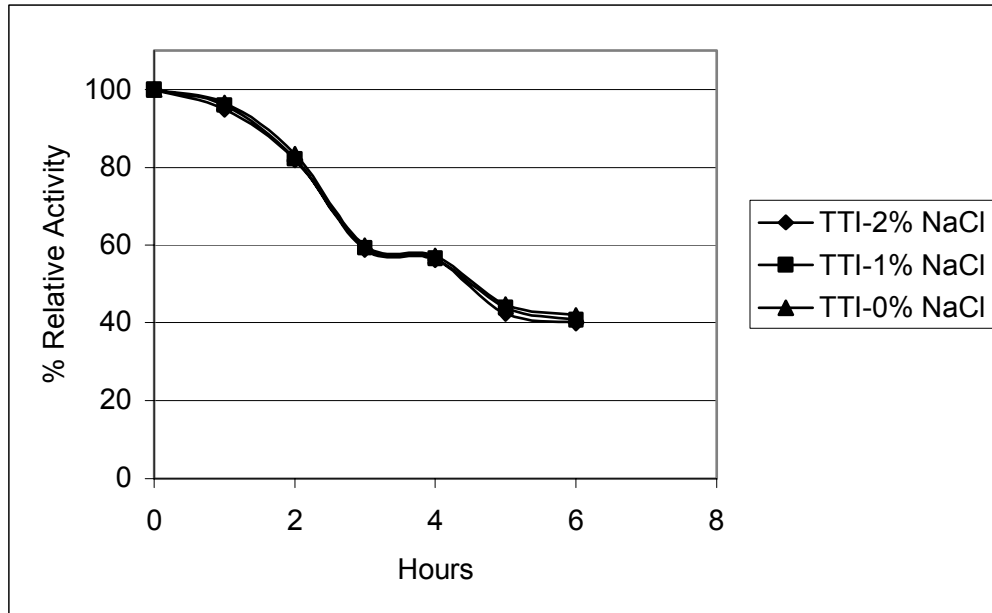
Fig. 3- α-Amylase diffusion from polyacrylamide gel, incubated in 0.05 M phosphate buffer, pH 7.1, 25 ^{o}C.
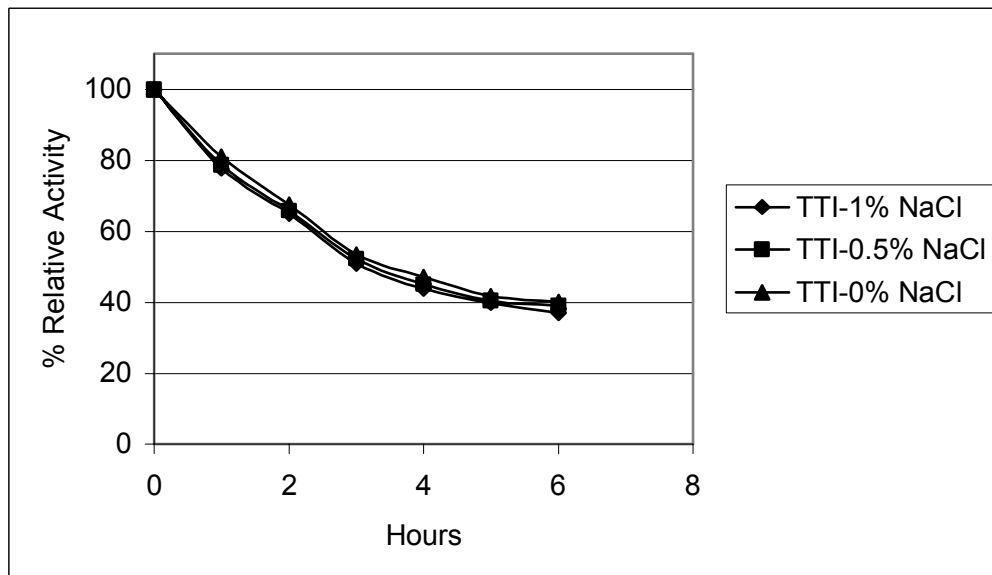
Fig. 4 – Phytase diffusion from polyacrylamide gel, incubated in 200 mM glycine buffer, pH 2.8, 25 ^{o}C.

These experiments were conducted by incubating the gel in a 1:40 (w/v) gel:buffer ratio for up to 6 h, a protocol that greatly exceeds what the TTI would be exposed to in a real food system. However, in food applications for this TTI, the loss of enzyme through diffusion from the gel is low enough to make the application feasible. TTI applications would be for solid foods, therefore substantially less diffusional loss would be expected to occur. Exposure of the TTI in a food during processing would be two hours or less, much shorter than the times used in these experiments.

Dielectric properties

The dielectric loss factor (ε'') of TTI and buffer system at 2,450 MHz increased with increasing NaCl concentration and temperature (Figure 5, 6, 7, 8). Dielectric loss is temperature sensitive. These findings support previous reports by Calay and others 1995 and Ohlsson and Bengtsson 1975. An increase in ε'' resulted from an increase in ionic conductivity as the concentration of dissolved salt increased (Mudgett 1986). This trend was observed with the three different food systems tested, and to a lesser degree than in buffer systems. Similar results were observed as an effect of temperature on ε'' of gel and buffer with varying salt content at 915 MHz (Figure 9, 10, 11, 12). At the same temperature, the ε'' at 915 was higher than at 2,450 MHz. Also, the rate of change in ε'' as a function of temperature was more pronounced at 915 MHz, particularly in buffer solutions, than at 2,450 MHz probably due to more heat conduction in liquid solutions compared to the polyacrylamide gel.

Electric conduction and various polarization mechanisms, including dipole, electronic, atomic and Maxwell-Wanger effects contribute to the dielectric loss factor (Kuang

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and Nelson 1998; Metaxas and Meredith 1993). The temperature dependence of the ε" at different NaCl concentrations can be explained by the relative effects of dipole rotation of free water and ionic conductivity (Mudgett 1986). Loss factor increases increasing temperature at both 915 and 2,450 MHz.

Fig. 5 – Dielectric loss factor (2,450 MHz) for α-amylase TTI at varying NaCl concentrations, and in shrimp, ground meat (GM) and mashed potatoes (MP).
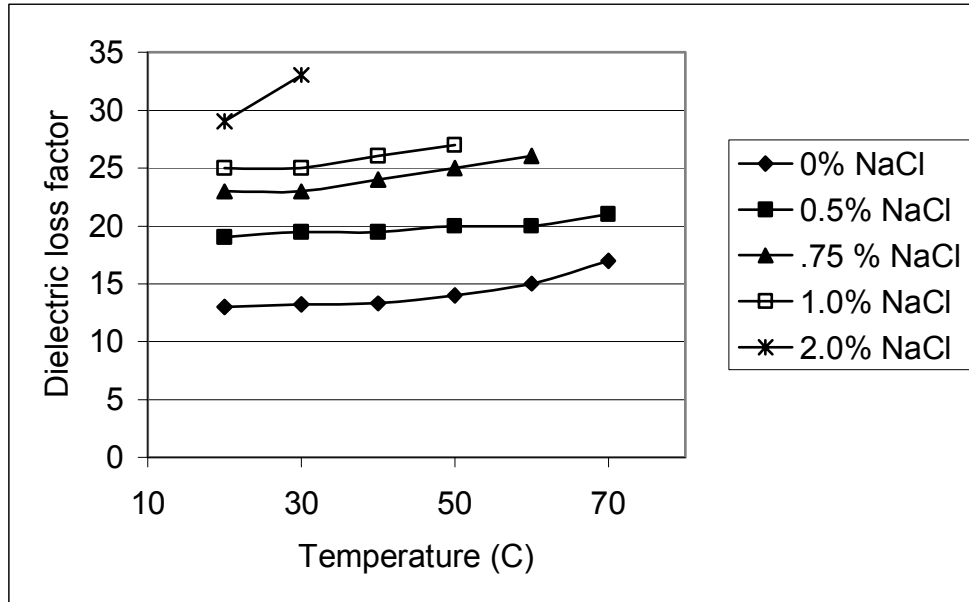
Fig. 6- Dielectric loss factor (2,450 MHz) of 0.05 M phosphate buffer, pH 7.1, at varying NaCl concentrations.
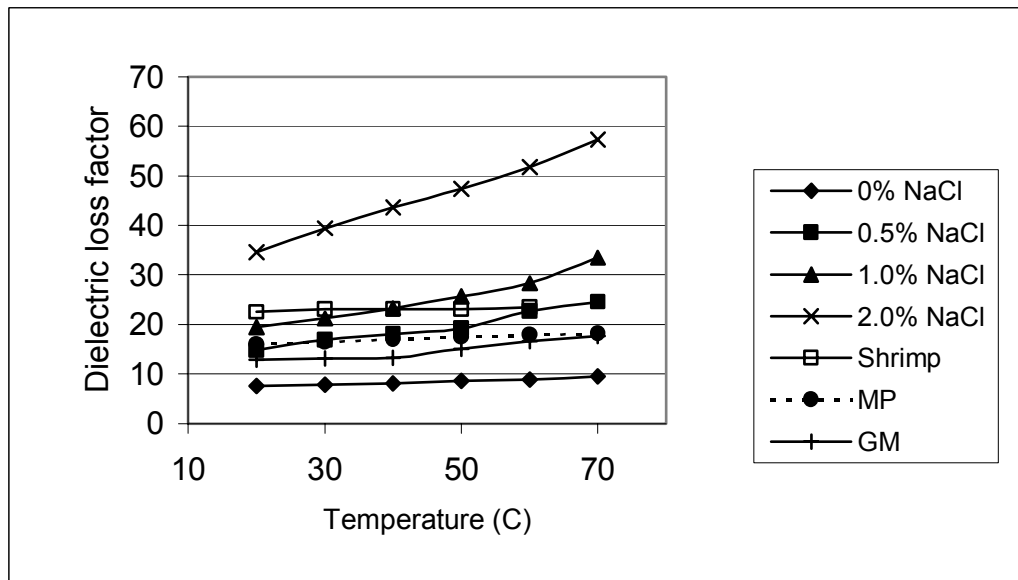
Fig. 7 – Dielectric loss factor (2,450 MHz) for phytase TTI at varying NaCl concentrations, and in shrimp, ground meat (GM) and mashed potatoes (MP).
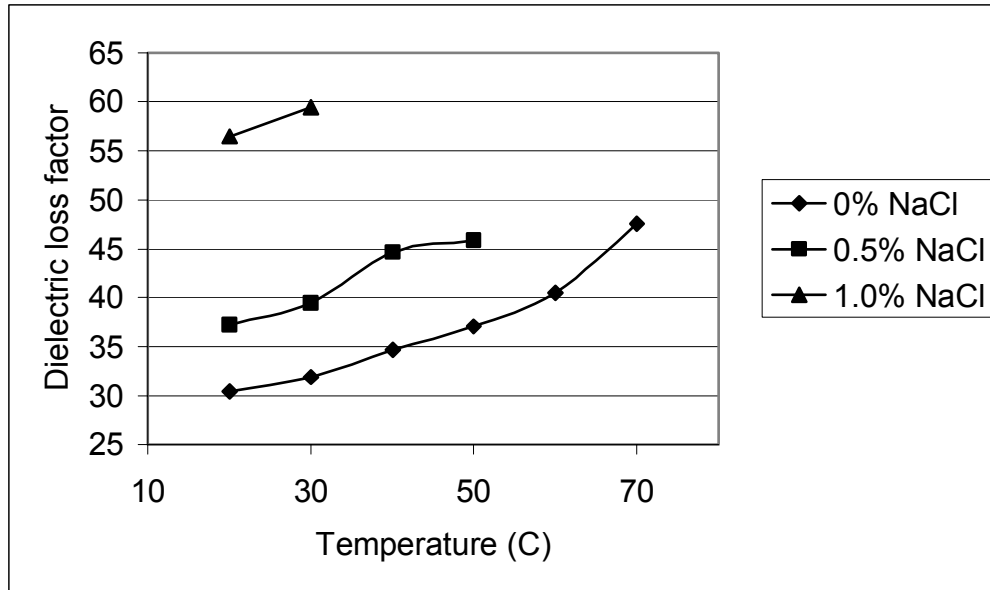
Fig. 8- Dielectric loss factor (2,450 MHz) of 200 mM glycine buffer, pH 2.8, at varying NaCl concentrations.
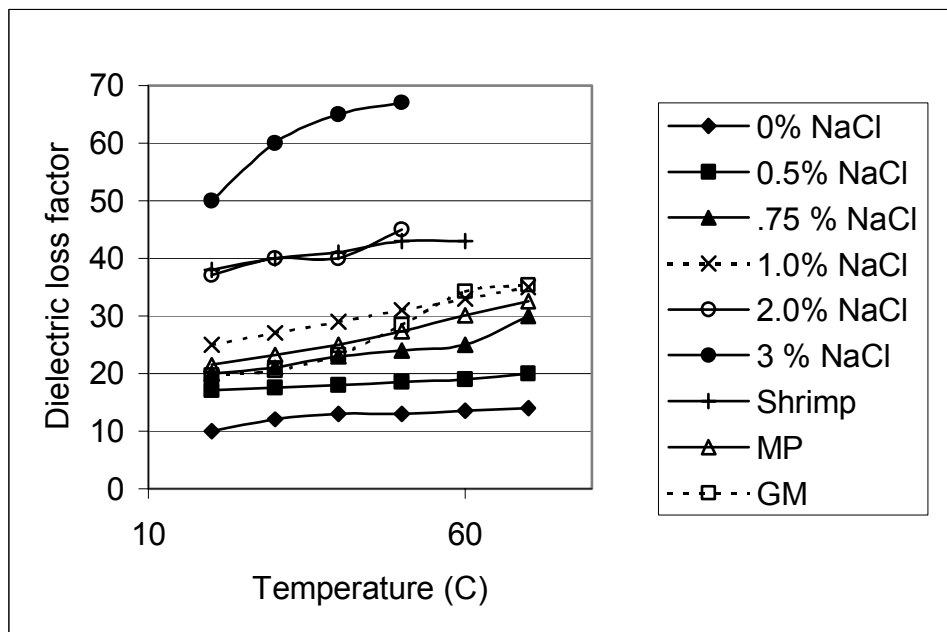
Fig. 9– Dielectric loss factor (915 MHz) for α-amylase TTI at varying NaCl concentrations, and in shrimp, ground meat (GM) and mashed potatoes (MP).
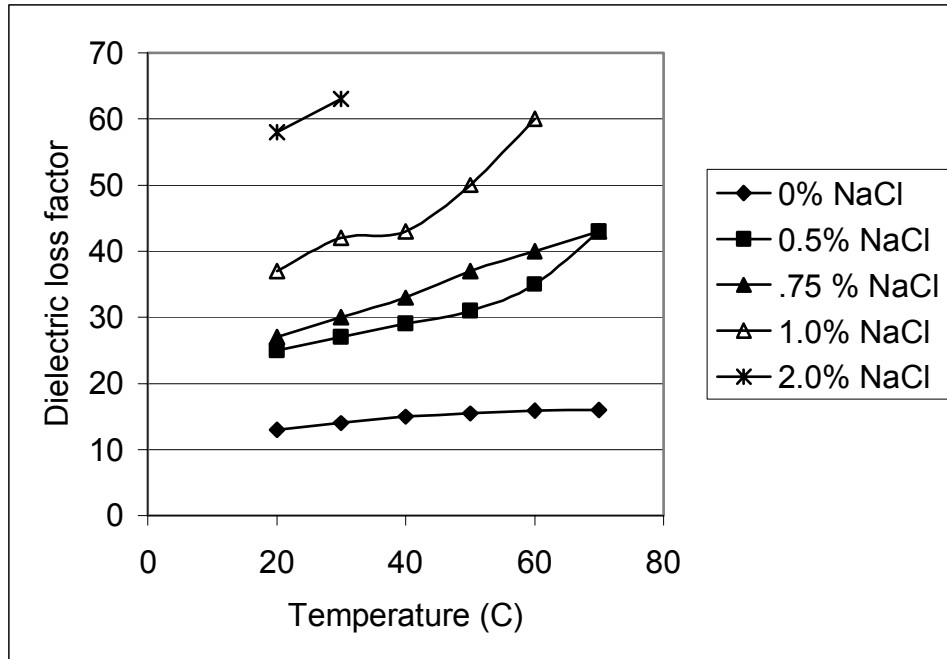
Fig. 10- Dielectric loss factor (915 MHz) of 0.05 M phosphate buffer, pH 7.1, at varying NaCl concentrations.
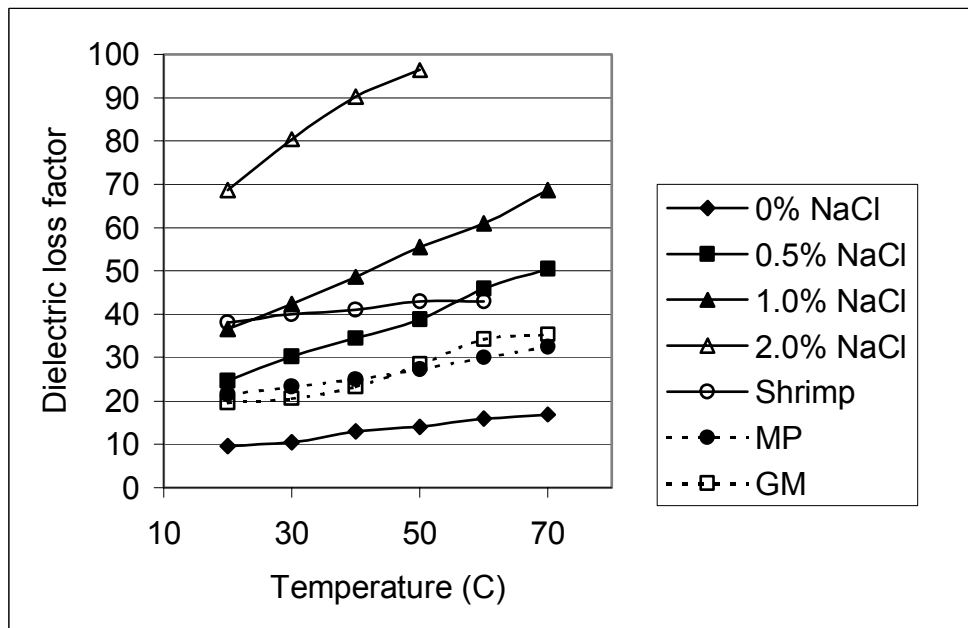
Fig. 11 – Dielectric loss factor (915 MHz) for phytase TTI at varying NaCl concentrations, and in shrimp, ground meat (GM) and mashed potatoes (MP).
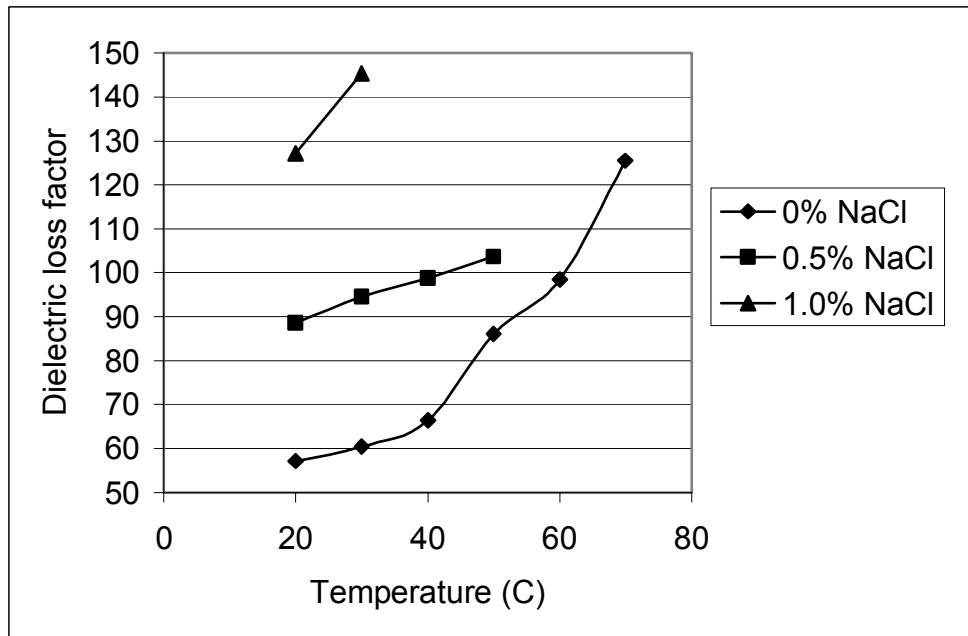
Fig. 12- Dielectric loss factor (915 MHz) of 200 mM glycine buffer, pH 2.8, at varying NaCl concentrations.

The dielectric constant (ε') of the immobilized enzyme gels (TTI) and buffer solutions with different NaCl concentrations measured at 2,450 MHz (Figure 13, 14, 15, 16, 17) and at 915 MHz (Figure 17, 18, 19, 20) decreased as salt content and temperature increased; however the trend at 2450 MHz was not as pronounced as that found for 915 MHz.

Some researchers have noticed that dielectric constant decreased with increasing temperature (Ohlsson and others 1974a; Ohlsson and Bengtsson 1975; Wang and others 2003) in foods with a moisture content < 70%. Because polar molecules, such as water, rotate and interact with other molecules in the material, they transfer kinetic energy generated by the electric field to the entire sample heating it. These intermolecular interactions also exert a retarding effect on the molecule itself. It is thus possible to visualize the polar molecules as if they existed in a highly viscous fluid similar to the dampening fluid in a shock absorber. If the temperature increases, the molecules continue to attempt to rotate within the electrical

field, but are impeded more and more by the viscous dampening. When the molecules can no longer fully rotate, the dipole moment (defined as the strength of the separate charge at the ends of the molecule) is less effective and the measured dielectric constant decreases (Buffler 1993).

As had been reported by other researchers, ε' is influenced by many factors including food composition, temperature and frequency. (Calay and others 1995; Ohlsson and Bengtsson 1975; Parkash and Armstrong 1970; Ryynanen 1995; Tong and Lund 1993; Hasted and others 1948; Bengtsson and Risman 1971).
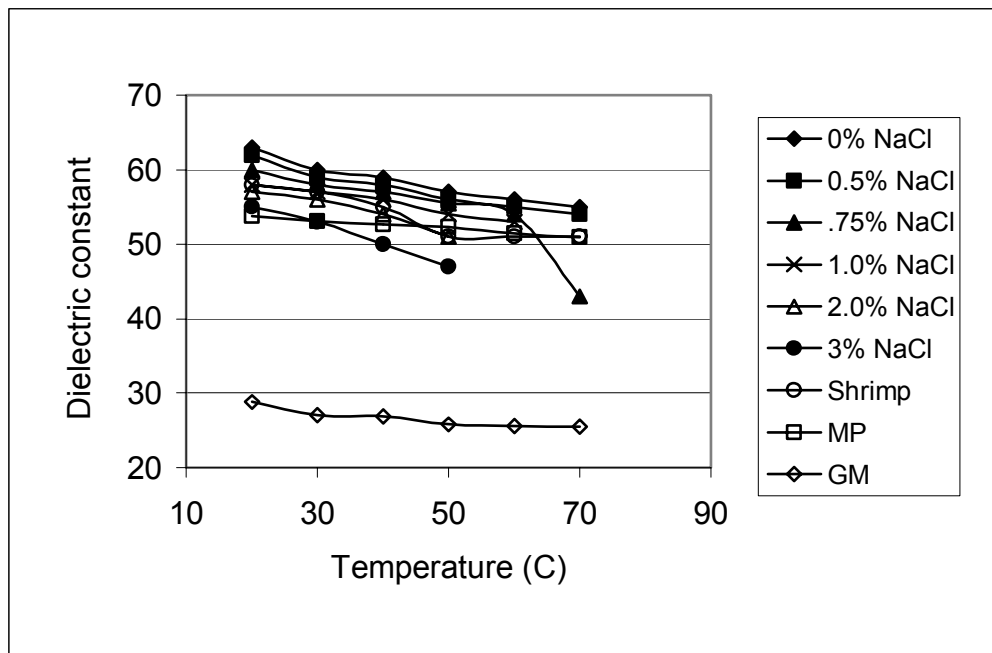
Fig. 13- Dielectric constant (2,450 MHz) for α-amylase TTI at varying NaCl concentrations, and in shrimp, ground meat (GM) and mashed potatoes (MP).
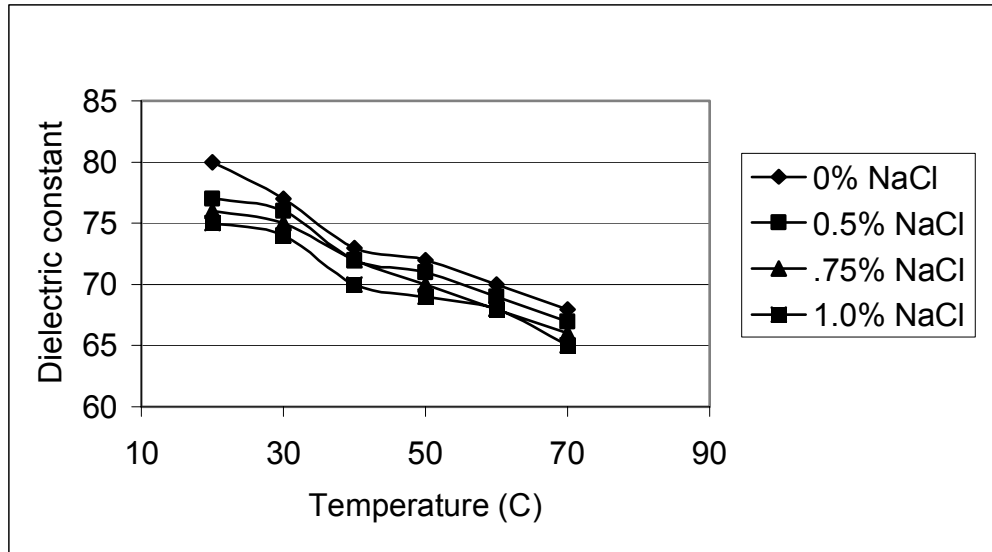
Fig. 14- Dielectric constant (2,450 MHz) of 0.05 M phosphate buffer, pH 7.1, at varying NaCl concentrations.
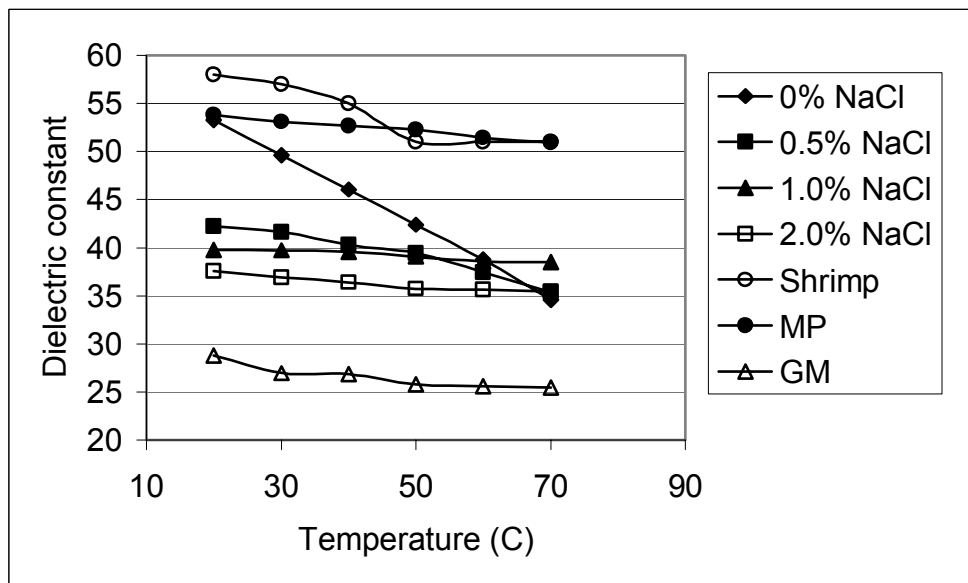
Fig. 15- Dielectric constant (2,450 MHz) for phytase TTI at varying NaCl concentrations, and in shrimp, ground meat (GM) and mashed potatoes (MP).
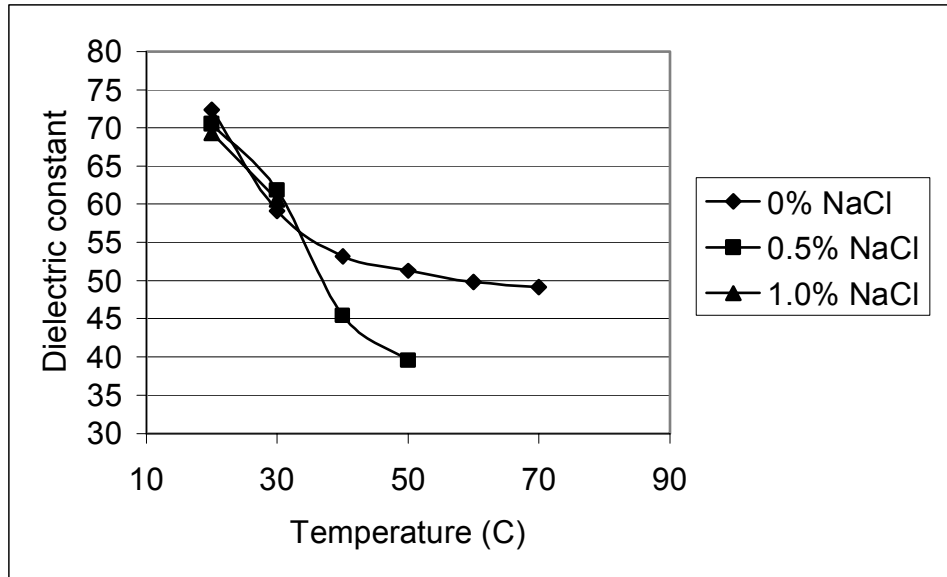
Fig. 16- Dielectric constant (2,450 MHz) of 200 mM glycine buffer, pH 2.8, at varying NaCl concentrations.
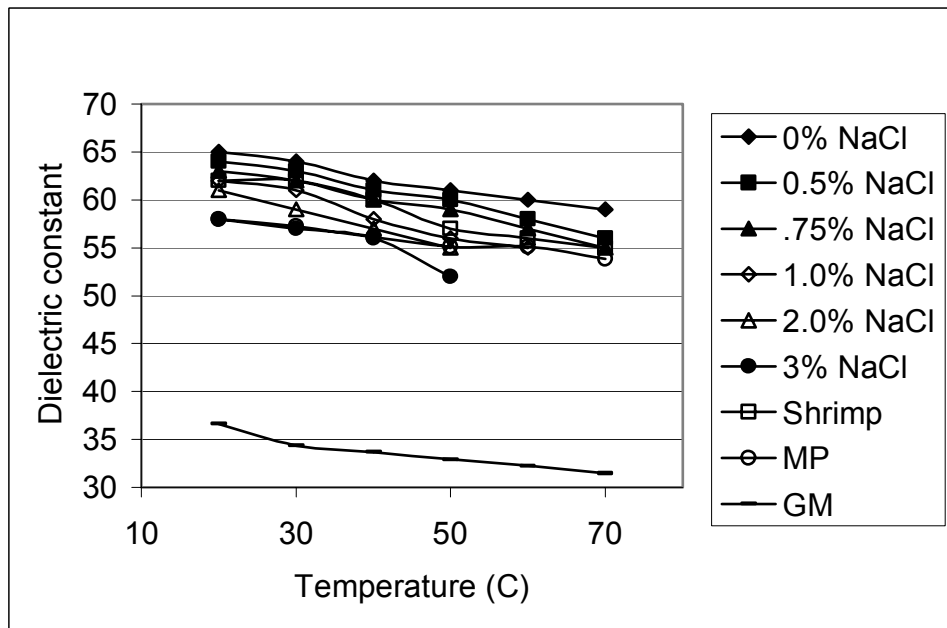
Fig. 17– Dielectric constant (915 MHz) for α-amylase TTI at varying NaCl concentrations, and in shrimp, ground meat (GM) and mashed potatoes (MP).
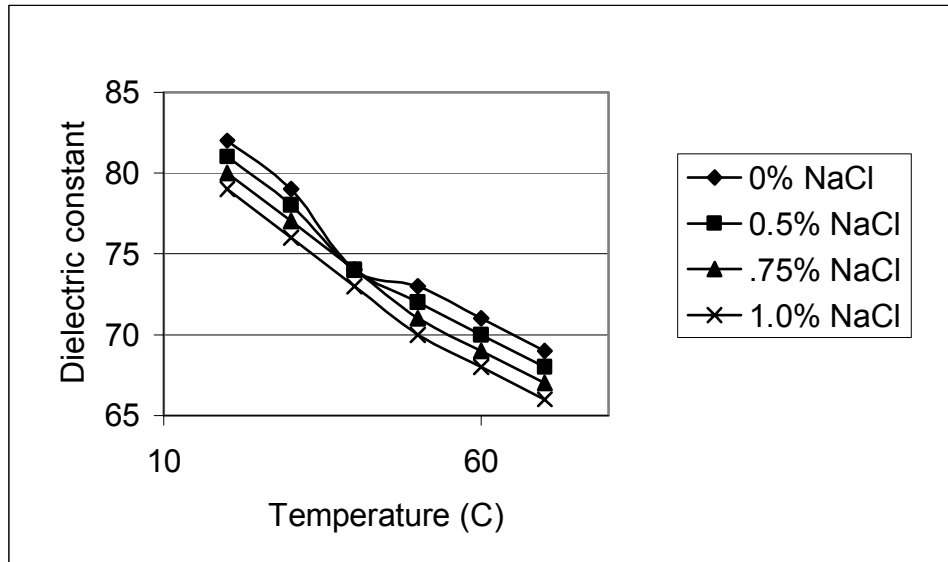
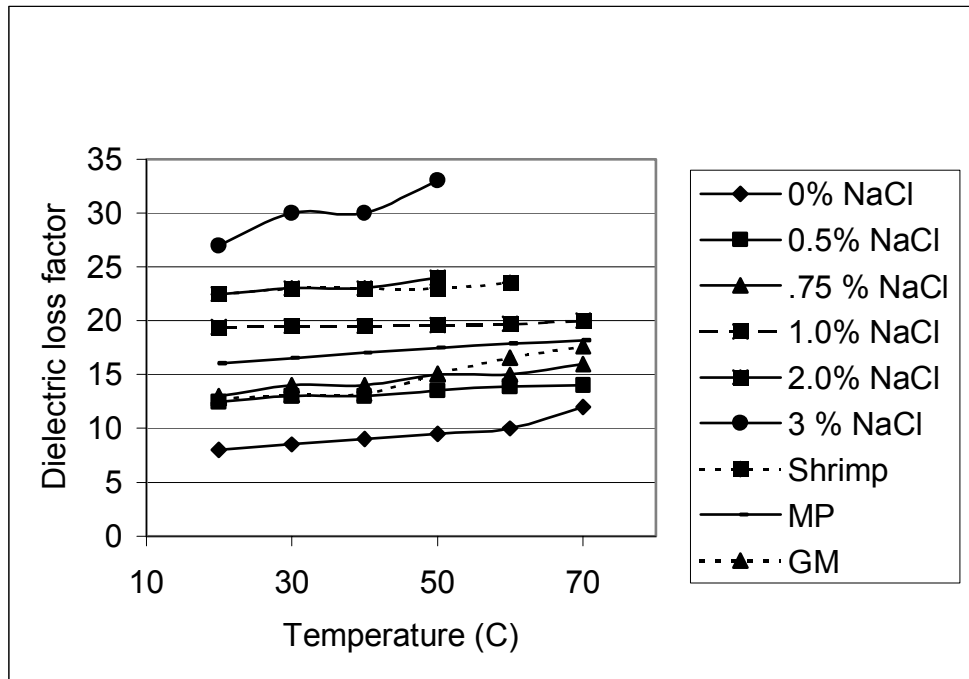**Fig. 18- Dielectric constant (915 MHz) of 0.05 M phosphate buffer, pH 7.1, at varying NaCl concentrations.**
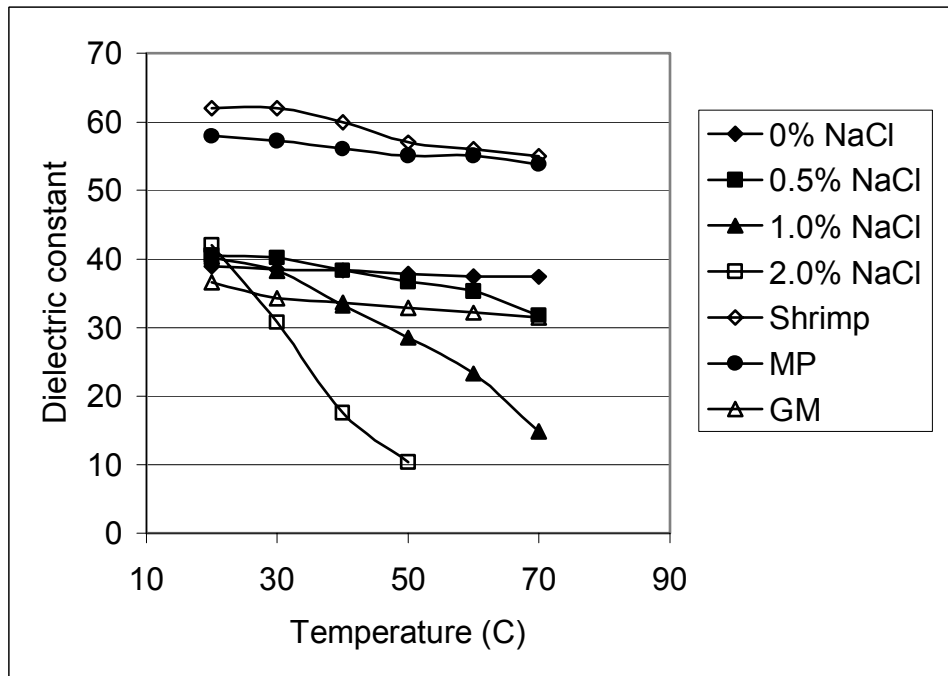
Fig. 19 – Dielectric constant (915 MHz) for phytase TTI at varying NaCl concentrations, and in shrimp, ground meat (GM) and mashed potatoes (MP).
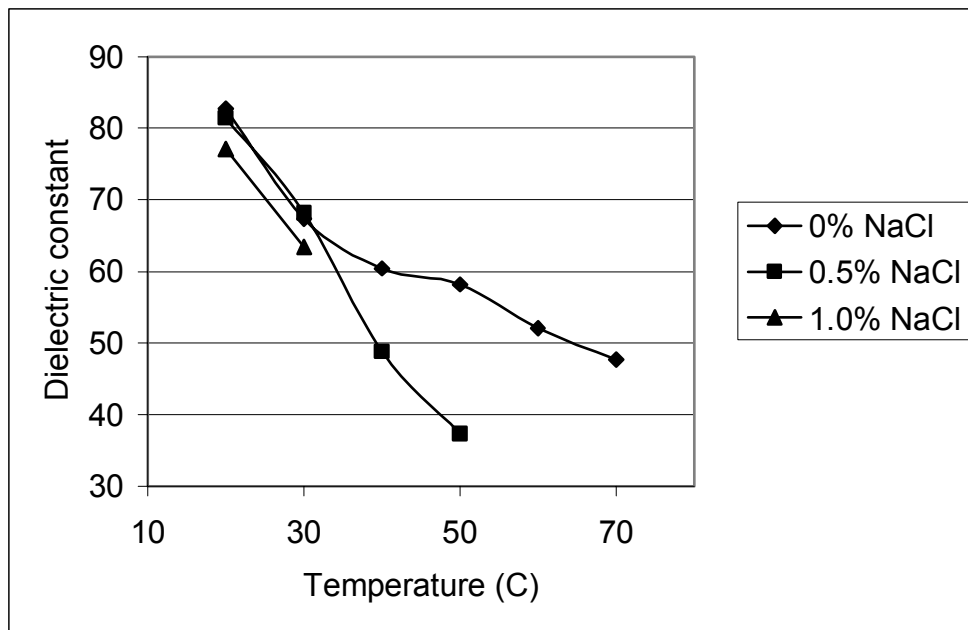
Fig. 20- Dielectric constant (915 MHz) of 200 mM glycine buffer, pH 2.8, at varying NaCl concentrations.

The ε' and ε'' yield information about how materials interact with electromagnetic radiation. Knowledge of dielectric properties of the target food sample is important for modifying a TTI system for microwave applications. The NaCl content required to produce a TTI with dielectric properties that match food products are shown in Figures 5, 7, 9, 11, 13, 15, 17 and 19. For example, if shrimp, mashed potatoes and ground meat are target food, dielectric properties at 2,450 MHz can be closely matched with an α-amylase gel containing 2.00, 1.00 and 0.75 NaCl%, respectively. In the case of phytase TTI a gel with 0.50% NaCl can be closely matched with all target foods. This study indicates that it is possible to produce TTIs with dielectric properties similar to food.

CONCLUSIONS

Enzyme based time-temperature indicators (TTI) that are physicochemically durable and suitable for the thermal processing of foods, particularly for microwave pasteurization

or RF applications are described here. After two months of storage at refrigerated conditions, the TTI showed relative high enzyme activity and enzymatic activity can be affected by NaCl concentration. The enzyme diffusion from the TTI was statistically significant, but the enzyme loss observed under the conditions studied did not limit utility of the TTI. These TTI could be used in a wide variety of food products and for selected pasteurized processes by chemically modifying the dielectric properties of the immobilization matrix to match the food by adding salt.

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ACKNOWLEDGEMENTS

CHAPTER 3

THERMOSTABILITY OF SOLUBLE AND IMMOBILIZED α-AMYLASE FROM *ASPERGILLUS ORYZAE* AND PHYTASE FROM *ASPERGILLUS FICUUM* IN POLYACRYLAMIDE GEL

ABSTRACT

Two enzyme based time-temperature indicators (TTI) suitable for pasteurization processes were developed with *Aspergillus oryzae* α-amylase and *Aspergillus ficuum* phytase physically immobilized in 20% polyacrylamide gel. Thermal inactivation in buffer systems, ground shrimp, ground meat (beef, 30% fat) and mashed potatoes was first order, and immobilization improved the thermal stability of the enzymes. The D- values for soluble and immobilized enzymes in buffer solutions ranged between 0.19 to 217.3 minutes with z values between 6.51 to 17.88 ºC. For immobilized enzymes, the D- values ranged between 0.43 to 555.00 min with z- values between 5.80 and 8.22 ºC depending upon the food matrices tested.

Key words: immobilized enzymes, α-amylase, phytase, pasteurization, and food.

INTRODUCTION

An important part of our food supply consists of foods preserved through thermal treatments (blanching, pasteurization or sterilization). Pasteurization of foods results in products with an extended shelf life by reducing the number of pathogenic and spoilage microorganisms. The heat treatment can be combined with other inhibitory factors (*e.g.,* pH reduction, low water content, refrigerated conditions) to ensure that any surviving organism is inhibited during the shelf life of the product (Van Loey and others 1996). Since many types of abuse could cause proliferation of microorganisms present in food, the heat treatment, generally coupled with refrigeration, is the major factor controlling vegetative pathogenic microorganisms to minimize any risk to product safety (Gaze 1992). Therefore an adequate quantitative process-impact evaluation in terms of safety is essential when designing control features for a thermal process.

To evaluate the impact of the thermal processes of food in terms of safety and quality, different methods are available: physical – mathematical and *in situ* methods (Hendrickx and others 1995). Limitations of both methods for the evaluation of new processing technologies have promoted the development of time-temperature integrators (TTI) as alternative process evaluation tools (Bruch 1973; Mulley and others 1975; Pflug and Smith 1977; Bunn and Sykes 1981; Sastry and others 1988; Rönner 1990; Weng and others 1991a; Whitaker 1991; De Cordt and others 1992a; Van Loey and others 1996; Guiavarc'h and others 2002).

A TTI is defined as a small, wireless, inexpensive device that shows a time and temperature dependence, easily measurable that can be correlated to the changes of a target

attribute of a food undergoing equivalent time temperature heat exposure (De Cordt and others 1992a, 1992b; Taoukis and Labuza 1989a, 1989b; Weng and others 1991a, 1991b; Van Loey and others 1995; Haentjens and others 1998). A target attribute can be any safety or other quality attribute of interest such as microorganism inactivation (safety), or loss of specific vitamin, texture or color property. The major advantage of TTI is the ability to quantify the impact of time-temperature exposure on the target attribute without having information on the actual time-temperature history of the product (Van Loey and others 1996). Hence TTI can be used as alternative process evaluation tool when *in situ* measurements are not feasible.

For a system to be used as a TTI several conditions must be fulfilled: theoretical determination of the kinetic properties of a single component TTI (Taoukis and Labuza 1989a; Tobback and others 1992), similar temperature sensitivity of the rate constant (z-value) of the TTI and the target attribute (Saraiva and others 1996; Van Loey and others 1995), and the decimal reduction time must be sufficiently high in the relevant range to avoid complete inactivation of the TTI system during thermal process.

The potentials of enzyme systems for developing TTI currently receive considerable interest (Hendrickx and others 1992; Tobback and others 1992; Weng and others 1991a, 1991b; De Cordt and others 1992a, 1992b, 1994; Saraiva and others 1993). An effective TTI comprised of an immobilized enzyme requires that the TTI possess the following physicochemical characteristics: 1) high recovery of enzyme activity, 2) high retention of enzyme activity over time, 3) slow diffusion of enzyme from the gel, 4) and enzymes with appropiate z- values slightly above that on the target pathogen (Raviyan and others 2003a Van Loey and others 1996).

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The enzymes α-amylase and phytase immobilized in polyacrylamide gel were used in this study for the reasons previously described in Chapter 1.

The objectives of this study were to: 1) develop two enzyme-based time-temperature indicators (TTI) employing α-amylase from *Aspergillus oryzae* and phytase from *Aspergillus ficuum* entrapped in 20% polyacrylamide gel, 2) determine thermal stability of the soluble and immobilized enzymes, and 3) determine inactivation kinetics of soluble and immobilized enzyme in buffer solutions, ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

MATERIALS AND METHODS

Enzymes

Aspergillus oryzae α-amylase (EC 3.2.1.1) and *Aspergillus ficuum* phytase (EC 3.1.3.8) from Sigma Aldrich Co. (St Louis, MO) were used as described in Chapter 2.

Preparation of enzymes solutions

For soluble enzyme measurement, both enzyme solutions were prepared as described in Chapter 2.

Immobilization procedure

The immobilized enzyme-containing gels for α-amylase and phytase were prepared as described in Chapter 2.

Substrate preparation for enzyme activity measurement

The maltodextrin and phytic acid used as a substrate for enzyme activity measurement were prepared as described in Chapter 2.

Assay of soluble enzymes

The enzymatic activity of α-amylase and phytase were measured spectrophotometrically as described in Chapter 2.

Assay of immobilized enzymes

The immobilized enzyme-containing gels for α-amylase and phytase were measured spectrophotometrically as described in Chapter 2.

Enzymatic activities

Enzymatic activities are expressed as described in Chapter 2.

Thermal inactivation experiments

Inactivation of soluble α-amylase in buffer solution

α-Amylase solution (2 ml) was added to 3 ml of phosphate buffer 0.05 M (pH 7.1) and heated in glass test tubes at temperatures between 55 and 70 ºC in a water bath (VWR Scientific Water Bath, Model 1245). Solution temperature was monitored using a thermocouple (Digital thermometer, model 600-1020, Barnet 115, Barrington, IL) inserted into the geometric center of the glass test tube. Zero time was defined as the time when the geometric center reached the target temperature. At predetermined time intervals, 0.6 ml of the heated solution was transferred to a glass containing 0.4 ml of cold phosphate buffer held on an ice bath. This solution was assayed as described in Chapter 2 for soluble enzyme. Three experiments were conducted in duplicate for each temperature. A similar set of experiments at 25 ºC was conducted as a control.

Inactivation of soluble phytase in buffer solution

Phytase solution (2 ml) was added to 3 ml of glycine buffer 200 mM (pH 2.8) and heated in glass test tubes at temperatures between 35 and 65 ºC in a water bath (VWR

Scientific Water Bath, Model 1245). Solution temperature was monitored using a thermocouple (Digital thermometer, model 600-1020, Barnet 115, Barrington, IL) inserted into the geometric center of the glass test tube. Zero time was defined as the time when the geometric center reached the target temperature. At predetermined time intervals 0.6 ml of the heated solution was transferred to a glass containing 0.4 ml of cold glycine buffer held on an ice bath. This solution was assayed as describe in Chapter 2 for soluble enzyme. Three experiments were conducted in duplicate for each temperature. A similar set of experiments at 25 ºC was conducted as a control.

Immobilized enzyme in buffer solution

Three pieces of immobilized enzyme gel were transferred to a glass test tube containing 1 ml of phosphate buffer 0.05 M (pH 7.1) for α-amylase or glycine buffer 200 mM (pH 2.8) for phytase and heated in glass test tubes at temperatures between 55-70 ºC in a water bath (VWR Scientific Water Bath, Model 1245) for α-amylase or 35-65 ºC for phytase. Solution temperature was monitored as previously described. After the desired heating time, 2 ml of cold phosphate buffer was immediately added to stop inactivation. The glass test tubes were quickly transferred to the ice bath and assayed as described previously. Three experiments were conducted in duplicate for each temperature. A similar set of experiments at 25 ºC was conducted as a control.

Inactivation of immobilized enzymes in food systems

Frozen shrimp (*Penaeus monodon)*, mashed potatoes and ground meat (beef, 30% fat) were obtained from a local supermarket (Pullman, WA) and kept at −35 °C. Twenty hours before the experiment, shrimp and ground meat (beef, 30% fat) were thawed under refrigeration conditions. The frozen shrimp, headless with intact exoskeleton, were peeled,

deveined, ground in a food processor, placed in Ziplock® plastic bag and kept in a refrigerator at 4 °C until used. Mashed potatoes were prepared by mixing ½ cup of instant potato flakes (100% Idaho Russet mashed potatoes, Safeway Inc., Oakland CA) with ½ cup of boiling water and kept in a refrigerator at 4 °C until used.

One gram of the food system was placed into 12 X 75 mm glass test tubes (VWR Scientific Product). Three pieces of the gel (0.5 X 0.5 X 0.5 cm) were placed into the geometric center of the food system. A thermocouple (Digital thermometer, model 600-1020, Barnet 115, Barrington, IL) was used to monitor the temperature at the geometric center of the sample. The gels remained in the food matrix no longer than 15 min prior to heat treatment. The glass test tubes were submerged in a water bath (VWR Scientific Water Bath, Model 1245) at 55, 60, 65 and 70 ℃ for α-amylase TTI and 53, 58 and 63 ℃ for phytase TTI for different time intervals. Zero time was defined as the time when the geometric center reached the target temperature. After the desired heating time, the glass test tubes were quickly transferred to an ice bath and kept cooled until assayed as described previously. Three experiments were conducted in duplicate for each temperature.

Kinetic data analysis

The following equations can be used to model enzyme inactivation (Tajchakavita and others 1997; Raviyan and others 2003b):

$$D A/\mathrm{d}t = -kA \tag{1}$$

The integrated form of equation 1 is:

$$A - A_0 = kt^z/z \tag{2}$$

where A represents the final enzyme activity (mg maltose/min or μmole of inorganic P/min), A_0 represents the initial enzyme activity (mg maltose/min or μmole of inorganic P/min), and t

and k represents time (min) and reaction rate constant (min^{-1}) at a particular temperature, respectively.

Reactions rate (k) increases with temperature, normally following an Arrhenius relationship

$$k = k_0 e^{-Ea/RT} \tag{3}$$

where k_0 is the rate constant of the TTI at a reference temperature (T_0), E_a is the activation energy, T is absolute temperature (273.1 + ºC), and R is the universal gas constant (8.314 J/mol·K or 1.987 cal/mol·K). Alternatively, k can be calculated from D- values (Ulbrich and Schellenberger 1986). The D- values is the heating time require to inactivate 90% of the enzyme, measured as a decrease from initial activity, at a given temperature. The D- value is defined as the negative reciprocal slope of the regression log (A/A_0) versus t.

$$k = 2.303/D \tag{4}$$

The z- value is defines as the change in temperature required to yield a 10-fold change in D- value and was calculated by determining the negative reciprocal slope from semi logarithmic plots of D- values as a function of temperature (ºC).

Statistical analysis

All experiments in this study were repeated at least three times and results are reported as means. General Linear Model procedures for analysis of variance and regression (Proc GLM) were determined using Statistical Analysis System version 8 (SAS Institute, Inc., Cary, N.C, 1999). Arithmetic means were compared by the Fisher LSD grouping test at the 95% confidence level (p ≤ 0.05). Interaction effects were analyzed by the least square means model.

RESULTS AND DISCUSSION

Thermal inactivation of soluble and immobilized enzymes in buffer solution

Similar shapes of the α-amylase activity profiles for the soluble and immobilized enzyme following immobilization were found over the entire range of the temperature tested (Figure 1). Figure 2 shows the results for soluble and immobilized phytase.

Together, these results indicate that the immobilization process had a minor effect on temperature optima and suggested that under these experimental conditions, the immobilization protocol may provide a microenvironment similar to that of the bulk solution (Raviyan and others 2003b). The temperature optimum of entrapped α-amylase was between 55-60 °C, this result was consistent with that of prior studies (Kundu and Das 1970). The temperature optima of entrapped phytase was observed between 35-45 °C. At temperatures between 50-55 °C phytase undergoes an irreversible conformational rearrangement that is associated with losses in enzymatic activity of 70 to 80% (Wyss and others 1998).
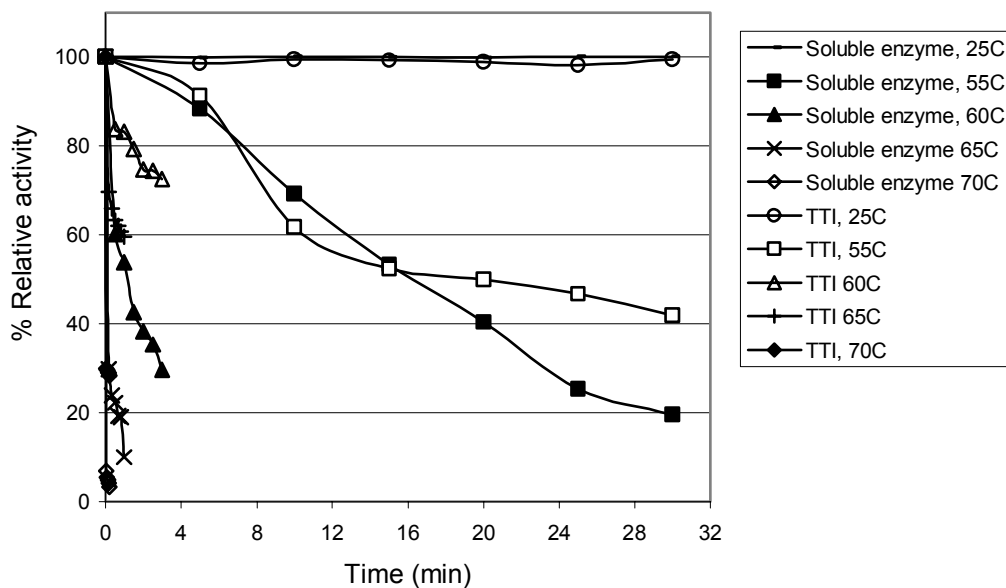
Fig 1. Thermostability of α-amylase in phosphate buffer 0.05 M (pH 7.1).
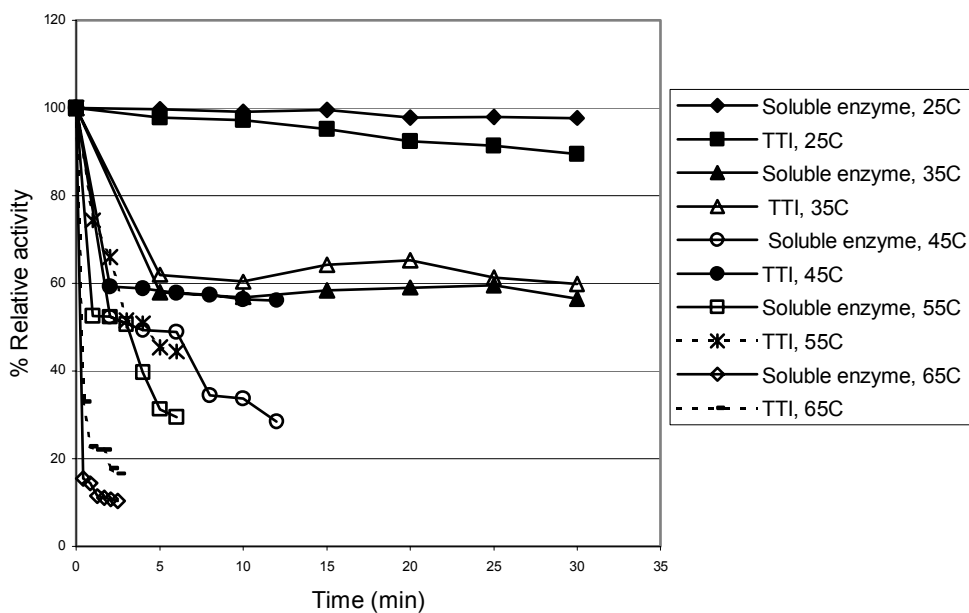
Fig 2. Thermostability of phytase in glycine buffer 200 mM (pH 2.8).

The thermal inactivation of free and immobilized enzymes in both cases was first order (Figure 3, Figure 4) (De Cordt and others 1992b; Saraiva and others 1996; Van Loey and others 1996; Haentjens and others 1998; Guivarc'h and others 2002; Raviyan and other 2003b). The z values were significantly different in both cases. The k and D- values for the free and immobilized enzymes in buffer solutions are listed in Table 1. In general, greater denaturation was observed for the free enzymes compared with the immobilized counterparts. Increased thermal stability for immobilized enzymes compared to the free enzyme has been reported (Ulbrich and Schellenberger 1986; De Cordt and other 1992b), and in this study, resistance to thermal inactivation increased following the immobilization by polyacrylamide gel entrapment. The increased thermostability may result from an increased electrostatic binds that may form when an enzyme is held within the polyacrylamide porous network. The effect of entrapment on enzyme stability depends upon many factors. An important factor is changing the enzyme conformation, which can occur as the result of micro environmental binding effects. Localization within the cell for an enzyme in its native state is another important factor. Enzymes that are located within the cell wall or bind directly to the gel matrix tend to exhibit greater thermostability than the intracellular soluble form of the same enzyme (Wasserman 1984). Even though discrepancies may result from the different experimental conditions, these results clearly indicates that thermal stability experiments must be conducted for each newly developed immobilized enzyme system for which thermal inactivation kinetics would be important.
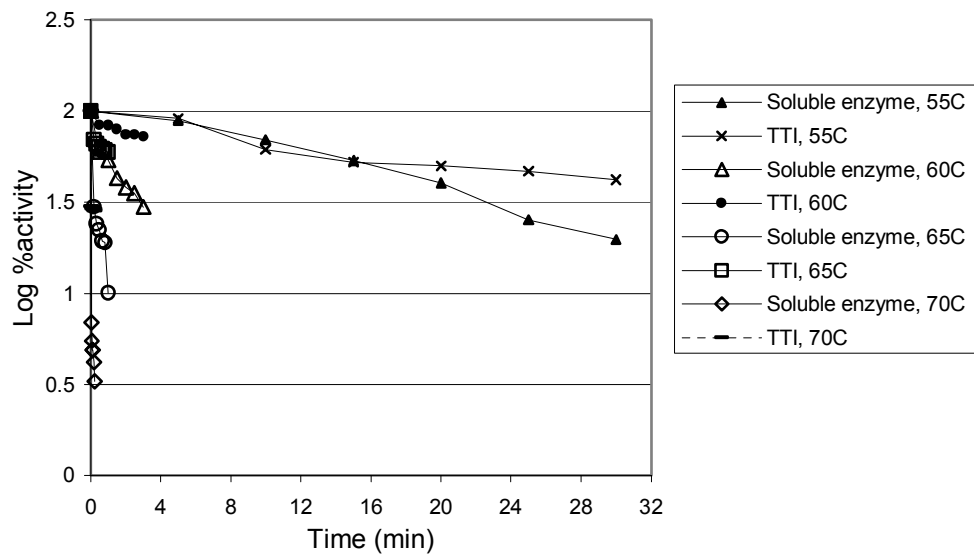
Fig 3. Inactivation of soluble and immobilized α-amylase in phosphate buffer 0.05 M (pH 7.1).
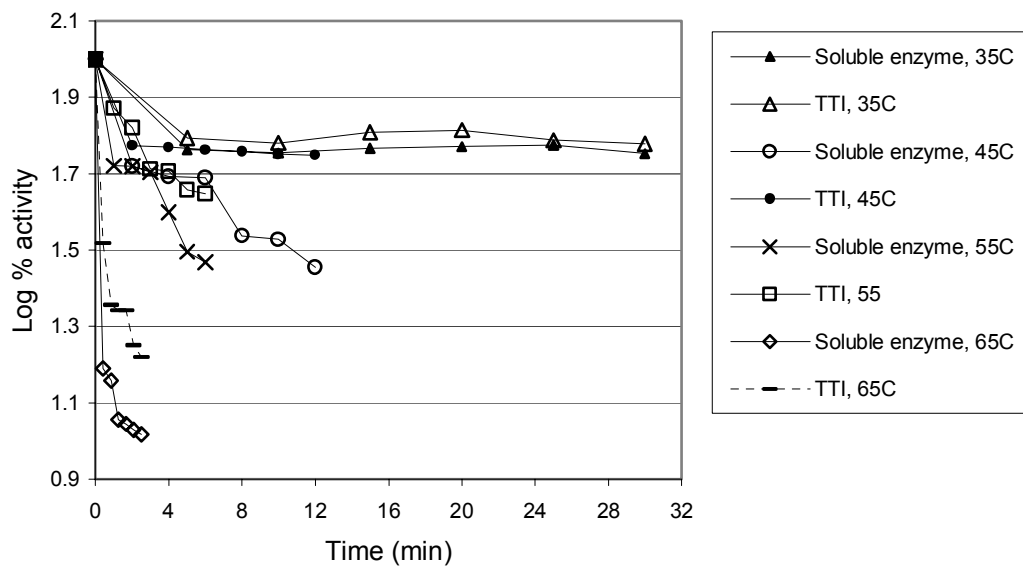
Fig 4. Inactivation of soluble and immobilized phytase in glycine buffer 200 mM (pH 2.8).

Enzyme/Buffer	Temp (ºC)	D value (min)	k (min^{-1})	z value (ºC)
Soluble α-amylase/buffer	55	40.81	0.06	6.45
	60	6.39	0.36	
	65	1.34	1.72	
	70	0.19	12.12	
Immobilized α-amylase/buffer	55	77.50	0.03	7.11
	60	24.60	0.10	
	65	5.67	0.41	
	70	0.56	4.11	
Soluble phytase/buffer	35	200.00	0.01	17.87
	45	25.77	0.09	
	55	12.90	0.18	
	65	3.44	0.67	
Immobilized phytase/buffer	35	217.30	0.01	17.25
	45	68.90	0.03	
	55	17.50	0.13	
	65	4.03	0.57	

Table 1. Kinetic parameters for free and immobilized α-amylase in phosphate buffer 0.05 M (pH 7.1), and free and immobilized phytase in glycine buffer 200 mM (pH 2.8).

Thermal inactivation of immobilized enzymes in food systems

The thermal inactivation of the immobilized enzyme was affected by the food matrix used as indicated by different z- values (Figure 5, Figure 6; Table 2). Although α-amylase stability improved in the presence of substrate and in products with low moisture content (Haentjens and others 1998), the same was not true for phytase. The tested ranged for phytase was outside the enzyme range for optimal pH stability and this could have masked any matrix effects on thermal stability for particular foods. Because different food matrices may interact differently with the TTI, matrices should be individually tested if optimal temperature sensitivity and accuracy are required.
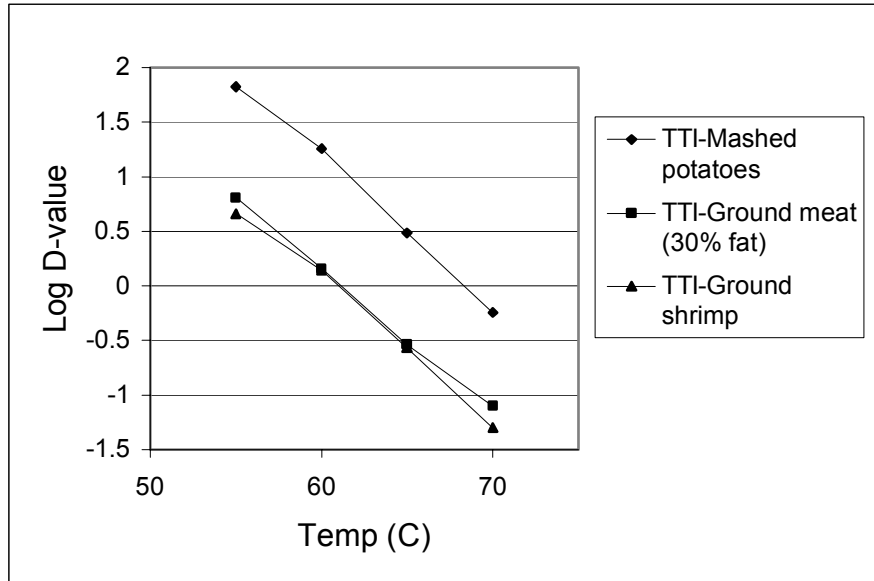
Fig 5. Thermal inactivation curves for immobilized α-amylase in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

Fig 6. Thermal inactivation curves for immobilized phytase in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

Enzyme/Food system	Temp (˚C)	D value (min)	k (min-1)	z value (˚C)
TTI α-amylase/Ground shrimp	55	33.89	0.07	8.22
	60	10.42	0.22	
	65	2.04	1.13	
	70	0.55	4.19	
TTI α-amylase/Mashed potatoes	55	66.60	0.03	7.16
	60	18.08	0.13	
	65	3.05	0.76	
	70	0.57	4.04	
TTI α-amylase/Ground meat (beef, 30% fat)	55	66.23	0.03	6.82
	60	11.90	0.19	
	65	2.04	1.13	
	70	0.43	5.36	
TTI phytase/Ground shrimp	53	222.00	0.01	6.19
	58	33.55	0.07	
	63	5.41	0.43	
TTI phytase/Mashed potatoes	53	312.50	0.01	5.96
	58	45.66	0.05	
	63	6.55	0.35	
TTI phytase/Ground meat (beef, 30% fat)	53	555.00	0.00	5.8
	58	58.47	0.04	
	63	10.40	0.22	

Table 2. Kinetic parameters for immobilized α-amylase and immobilized phytase in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

CONCLUSIONS

The thermostability of enzymes changed depending of on the environmental conditions and/or its state (soluble or immobilized). Immobilization caused changes in the thermal inactivation of the enzymes. First, it gives rise to a globally higher thermostability (higher D- values and lower k values) and a lower temperature sensitivity of the inactivation rate constant (higher z- values). In the frame work of the development of TTI to monitor safety of pasteurization processes, the high D- values together with z- values between 5.8 and 8.22 ºC make the polyacrylamide immobilized enzymes interesting candidates for the

development of enzyme based time temperature indicators for monitoring the thermal destruction of bacterial food pathogens, particularly in systems where control of dielectric parameters is important.

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CHAPTER 4

PREDICTING THERMAL INACTIVATION OF *Listeria monocytogenes* USING AN α-AMYLASE-BASED TIME-TEMPERATURE INDICATOR

ABSTRACT

An enzyme based time-temperature indicator (TTI) was developed with *Aspergillus oryzae* α-amylase immobilized in 20% polyacrylamide gel. The thermal inactivation kinetics of the TTI and *Listeria monocytogenes* in ground shrimp, ground meat (beef, 30% fat) and mashed potatoes were first order. During thermal processing at 55, 60, 65 and 70 ºC, D-values (min) ranged between 66.22 to 0.43 in ground meat (beef, 30% fat), 66.60 to 0.57 in mashed potatoes and 33.89 to 0.55 in ground shrimp were obtained for the immobilized α-amylase. D- values ranged between 6.42 to 0.08 in ground meat (beef, 30% fat), 5.13 to 0.06 in mashed potatoes and 4.60 to 0.05 in ground shrimp were obtained for *Listeria monocytogenes*.

Key words: thermal inactivation, α-amylase, immobilized enzyme, food, *Listeria monocytogenes*.

INTRODUCTION

Food-borne diseases continue to be a problem of global concern (Bellara and others 1999). Active surveillance of infections caused by bacterial food-borne pathogens in the USA revealed 130,000 culture-confirmed cases in the population in 1997. Confirmed cases of food-borne diseases represent only a fraction of the total, and estimates of the true incidence was likely to have been approximately eight million cases (Angulo and others 1988). Poultry, eggs and red meat products are frequently identified as vehicles of food poisoning, with the most common factors contributing to outbreaks include inappropriate heat treatment, cross-contamination, and inadequate cooking or reheating by consumers (Bellara and others 1999).

Listeria monocytogenes is a psychotropic human pathogen, which is widely found in the environment. The organism can be readily isolated from water, vegetation, sewage and a variety of warm-blooded animals (Jay 2000). *Listeria monocytogenes* can also be isolated from various raw foods such as milk, poultry, red meat and seafoods (Johnson and others 1990; Pearson and Marth 1990). The ability of *Listeria monocytogenes* to tolerate heat, growth at refrigeration temperatures, and endure high NaCl and acidic environments makes microbial destruction more crucial (Beuchat and others 1986; Golden and others 1988; Petran and Zottola 1989; Jay 2000; Mackey and Bratchell 1989).

In the past few decades, *Listeria monocytogenes* has emerged as a food-borne pathogen of major significance (Chhabra and others 1999). Listeriosis, the disease caused by *L. monocytogenes*, typically afflicts immunocompromised individials, but cases have also been associated with disease in healthy individuals (Donelly 1994). *Listeria*

monocytogenes has become a serious public health concern because of the high fatality rate (20 to 30%) and a long incubation period associated with listeriosis (Donelly 1994; Rocourt and Cossart 1997). In 1983, consumption of pasteurized whole and 2% milk in Massachusetts was implicated in 49 cases of listeriosis (Fleming and others 1985). This outbreak caused an increase in research of the adequacy of the pasteurization processes (Bradshaw and others 1987; Bunting and others 1988; Donelly and others 1987; Mackey and Bratchell 1989). In 1985, a Mexican-style cheese product was implicated in an outbreak involving 142 cases of listeriosis in California resulting in 49 deaths (Linnan and others 1988). More recently, an outbreak in France resulted in 33 cases of listeriosis after consumption of a soft cheese (Goulet and others 1995). The products at highest risk are ready-to-eat (RTE) foods that are stored at refrigeration temperatures for long periods of time (Rocourt and Cossart 1997).

An important part of our food supply consists of foods preserved through thermal treatments (blanching, pasteurization or sterilization). Pasteurization of foods results in products with an extended shelf life by reducing the number of pathogenic and spoilage microorganisms. Many extrinsic factors of foods affect thermal death of microbial cells. Heat resistance of cells is often dependent upon water activity, pH, NaCl content, protein and fat percentages of the food product (Stumbo 1965). In this context, an ability to predict the safety margins of inactivation or preservation processes becomes particularly important.

To evaluate the impact of the thermal processes of food in terms of safety and quality, different methods are available: physical – mathematical and *in situ* methods (Hendrickx and others 1995). Limitations of both methods for the evaluation of new processing technologies have promoted the development of time-temperature indicators

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(TTI) as alternative process evaluation tools (Bruch 1973; Mulley and others 1975; Pflug and Smith 1977; Bunn and Sykes 1981; Sastry and others 1988; Rönner 1990; Weng and others 1991a; Whitaker 1991; De Cordt and others 1992a; Van Loey and others 1996; Guiavarc'h and others 2002; Ulbrich and Schellenberger 1986).

The potential of enzyme systems as the basis for TTI has received considerable interest (Hendrickx and others 1992; Tobback and others 1992; Weng and others 1991a, 1991b; De Cordt and others 1992a, 1992b, 1994; Saraiva and others 1993; Taoukis and Labuza, 1989a, 1989b; Van Loey and others 1995; Raviyan and others 2003a; 2003b). α-Amylases (α-1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) are endo-enzymes, which catalyzed the hydrolysis of α-(1,4)-glycosidic bonds of amylose and amylopectin to a range of maltooligosaccharides (Adams 1991). The enzyme also hydrolyzes α-(1,4)-glycosidic bonds in smaller polyglucans, such as maltodextrin. This enzyme is of particular interest for TTI applications: 1) it is inexpensive and commercially available, 2) and the assay is fast and simple (Van Loey and others 1997; Yamane and Maruo 1974; Borgia and Campbell 1978).

Enzymes have been immobilized in polyacrylamide gel (Pinto and Macias, 1996; Pundle and others 1988; Bihari and others 1984; Das and others 1998; Kashchenko and others 1992; Khare and others 1994); polyacrylamide immobilization was chosen here because we could easily alter the physical and chemical properties of the gel to make them compatible with food matrices. Also, it is possible to immobilize enzymes in polyacrylamide with minimal diffusional losses (Pizarro and others 1997).

The objectives of this study were to: 1) develop an enzyme based time temperature indicators (TTI) employing α-amylase from *Aspergillus oryzae* entrapped in 20% polyacrylamide gel, 2) determine inactivation kinetics parameters for TTI and *Listeria monocytogenes* in ground shrimp, mashed potatoes and ground meat (beef, 30% fat), 3) develop prediction equations that correlate thermal inactivation of the TTI with *Listeria monocytogenes* inactivation, 4) and validate prediction of microbial inactivation using experimental data obtained from parallel thermal inactivation studies of *Listeria monocytogenes* with the TTI in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

MATERIALS AND METHODS

Enzyme

Aspergillus oryzae α-amylase (EC 3.2.1.1) from Sigma Aldrich Co. was used as described in Chapter 2.

Immobilization procedure

The immobilized enzyme containing gel for α-amylase was prepared as described in Chapter 2.

Maltodextrin preparation for α-amylase activity measurement

The maltrodextrin used as a substrate was MALTIN® M040 (Grain Processing Corporation (Muscatine, WI 52761, USA). The substrate was prepared as described in Chapter 2.

Assay of immobilized α-amylase (TTI)

The immobilized enzyme-containing gel was measured spectrophotometrically as described in Chapter 2.

Enzymatic activity

Enzymatic activity is expressed as described in Chapter 2.

Thermal inactivation experiments

Food products

Frozen shrimp (*Penaeus monodon)*, mashed potatoes and ground meat (beef, 30% fat) were obtained from a local supermarket (Pullman, WA) and kept at -35 °C. All food samples were prepared as described in Chapter 3.

Inactivation of immobilized α-amlylase (TTI) in food systems

The inactivation experiments for α-amylase TTI were performed as described in Chapter 3.

Inactivation of *Listeria monocytogenes* in food systems

Microbial cultures

Listeria monocytogenes ATCC # 7644, # 19114 and # 19113 cells (obtained from Dr. Don-Hyun Kang, Washington State University, Pullman, WA) were incubated at 37 ºC for 24 hr in tryptic soy broth supplemented with 0.60% yeast extract (Difco Laboratories, Detroit, Michigan). Since the stationary phase is the most resistant stage in the bacterial cell life span, stationary phase cells were used for the thermal inactivation experiments (Heddlenson and others 1991; Jay 2000). After the incubation period, one ml of equal volumes of the three strains of *Listeria monocytogenes* were combined in a sterile flask to obtain a cocktail that was used to inoculate about 10.00 g of each food.

Thermal inactivation experiments

One gram of the inoculated food system was placed inside 12 X 75 mm glass test tubes (VWR Scientific Product) and capped with rubber sleeves. The glass test tubes were submerged in a water bath (VWR Scientific Water Bath, Model 1245) at 55, 60, 65 and 70 ºC for different time intervals. A sample was removed immediately after the "come up time" (time when the geometric center reached the target temperature) was reached and was designated as time zero. After the desired heating time, the glass test tubes were quickly transferred to an ice bath and kept cooled until assayed. Three experiments were conducted in duplicate for each temperature.

Listeria monocytogenes **enumeration**

The survivors following thermal treatment were enumerated by diluting each 1.00 g heated treated sample into 9 ml of sterile 0.1% peptone in stomacher bags for 2 min. The homogeneous dilution was serially diluted into 9 ml of 0.1% peptone (10^{-1}-10^{-9}) and then plated in duplicate using an overlay method (Lee and Kang 2001) to determine the number of the survivors. This overlay method was designed specifically to improve the recovery of heat-injured cells. Tryptic soy agar (TSA) (Difco Laboratories, Detroit, Michigan) plates (non-selective agar medium) supplemented with 0.60% yeast extract agar were incubated at 37 °C for 2 hr to allow the injured cells to repair and resuscitate. Then ~ 7 ml of the *Listeria* selective Palcam agar (Difco Laboratories, Detroit, Michigan) containing the antimicrobial supplement was overlaid onto the TSA. The plates were incubated for an additional 46 h at 37 °C and determined viable cells.

Calculation of D- and z- values

All D, z and k values calculations were calculated as described in Chapter 3.

Statistical analysis

All experiments in this study were repeated at least three times and results are reported as means. General Linear Model procedures for analysis of variance and regression (Proc GLM) were determined using Statistical Analysis System version 8 (SAS Institute, Inc., Cary, N.C, 1999). Arithmetic means were compared by the Fisher LSD grouping test at the 95% confidence level ($p \leq 0.05$). Interaction effects were analyzed by the least square means model.

Mean absolute percentage error (MAPE) was used to evaluate the accuracy of the models for predicting the experimental values (1).

$$MAPE = \frac{\sum_{i=1}^{n} \left| \left[\frac{X - \overline{X}}{X} \right](100) \right|}{n} \qquad (1)$$

where, X = experimental value, $\overline{X}$ = predicted value and n = number of samples

RESULTS AND DISCUSSION

Thermal inactivation of immobilized α-amylase (TTI) in food systems

The thermal inactivation of the immobilized α-amylase in the food samples tested followed first order inactivation kinetics (Figure 1) (De Cordt and others 1992b; Saraiva and others 1996; Van Loey and others 1996; Haentjens and others 1998; Guivarc'h and others 2002; Raviyan and others 2003b). The D- values, z- values and k values for the α-amylase enzyme based TTI in all food systems tested are listed in Table 1. Regression parameters are listed in Table 2.

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Fig 1. Thermal inactivation curves for immobilized α-amylase in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

Enzyme/Food system	Temp (ºC)	D value (min)	k (min^{-1})	z value (ºC)
TTI α-amylase/Mashed potatoes	55	66.60	0.03	7.16
	60	18.08	0.13	
	65	3.05	0.76	
	70	0.57	4.04	
TTI α-amylase/Ground meat (beef, 30% fat)	55	66.23	0.03	6.82
	60	11.90	0.19	
	65	2.04	1.13	
	70	0.43	5.36	
TTI α-amylase/Ground shrimp	55	33.89	0.07	8.22
	60	10.42	0.22	
	65	2.04	1.13	
	70	0.55	4.19	

Table 1. Kinetic parameters for immobilized α-amylase in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

TTI/Food system	Temp (ºC)	y-intercept (b)	slope (m)	R^2
TTI α-amylase /Mashed potatoes	55	1.99	-0.02	0.95
	60	1.94	-0.06	0.99
	65	1.94	-0.33	0.97
	70	1.86	-1.74	0.95
TTI α-amylase /Ground meat (beef, 30% fat)	55	1.99	-0.02	0.99
	60	1.93	-0.08	0.99
	65	1.90	-0.49	0.96
	70	1.80	-2.34	0.94
TTI α-amylase /Ground shrimp	55	2.00	-0.03	0.99
	60	1.99	-0.10	0.95
	65	1.95	-0.49	0.98
	70	1.78	-1.81	0.97

Table 2. Regression parameters for immobilized α-amylase in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

The thermal inactivation of the immobilized enzyme was affected by the food matrix used, indicated by statistically different z- values. The immobilized α-amylase may be more stable in the presence of substrate such as potato starch. Potato starch contains relatively high amounts of low molecular weight carbohydrates, and diffusion of these moieties into the gel during inactivation experiments may be improved α-amylase stability. Covalently bound carbohydrate stabilizes hydrolase enzymes (Kennedy 1987; Pazur and others 1970). Another possible explanation can be the moisture content difference among the food systems. As the moisture content of the medium decreases the thermostability of the enzymes tend to increase (Haentjens and others 1998). Because different food matrices may interact differently with the TTI, matrices should be individually tested if optimal temperature sensitivity and accuracy are required.

Thermal inactivation of *Listeria monocytogenes* in food systems

A log linear decrease of *Listeria monocytogenes* survivors as a function of heating time was observed (Figure 2). Thermal inactivation kinetic parameters for *Listeria monocytogenes* in food matrices used were determined based upon the relative numbers of survivors after thermal treatments at 55 to 70 ºC (Table 3). Regression parameters are listed in Table 4. No *Listeria monocytogenes* (< 25 CFU/g by direct plating) was detected before inoculation in any of the food matrices used in these studies.
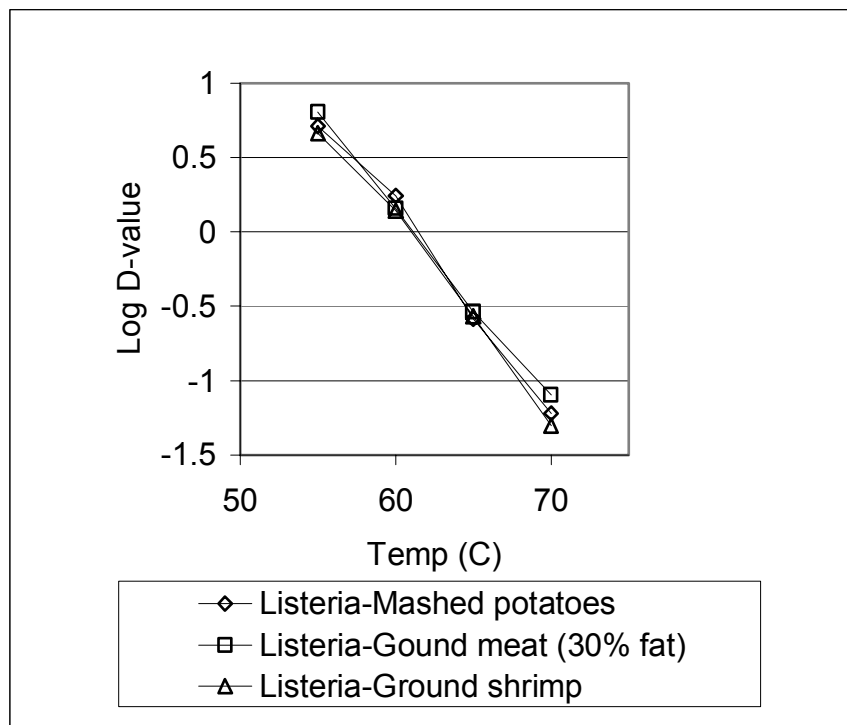
Fig 2. Thermal inactivation curves for *Listeria monocytogenes* in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

Listeria monocytogenes/Food system	Temp (°C)	D value (min)	k (min-1)	z value (°C)
L. monocytogenes/Mashed potatoes	55	5.13	0.45	7.55
	60	1.75	1.32	
	65	0.26	8.86	
	70	0.06	38.38	
L. monocytogenes/Ground meat (beef, 30% fat)	55	6.42	0.36	7.8
	60	1.44	1.60	
	65	0.29	7.94	
	70	0.08	28.79	
L. monocytogenes/Ground shrimp	55	4.60	0.50	7.57
	60	1.39	1.66	
	65	0.27	8.53	
	70	0.05	46.06	

Table 3. Kinetic parameters for *Listeria monocytogenes* in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

Listeria monocytogenes/Food system	Temp (°C)	y-intercept (b)	slope (m)	R^2
L. monocytogenes/Mashed potatoes	55	9.13	-0.20	0.97
	60	7.77	-0.57	0.90
	65	7.30	-3.83	0.99
	70	5.86	-14.48	0.95
L. monocytogenes/Ground meat (beef, 30% fat)	55	8.45	-0.16	0.97
	60	8.14	-0.69	0.99
	65	7.25	-3.45	0.99
	70	5.82	-12.00	0.90
L. monocytogenes/Ground shrimp	55	8.04	-0.22	0.98
	60	8.13	-0.72	0.99
	65	7.16	-3.65	0.99
	70	7.03	-17.97	0.99

Table 4. Regression parameters for *Listeria monocytogenes* in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

The z- values for *Listeria monocytogenes* in ground shrimp and mashed potatoes were slightly lower than in ground meat (beef, 30% fat). Food composition has a marked effect on the heat resistance of *Listeria* spp. The protective effect of fatty materials in the heating

medium on the heat resistance of microorganisms is well documented (Ahmed and Conner 1995). Theories behind increased heat resistance in foods with higher fat contents relate to reduce water activity and poorer heat penetration (lower heat conductivities) in the fat portion (Juneja and Eblen 2000). The D- and z- values found in this study in beef were similar to those reported by Fain and others (1991) and Schoeni and others (1991). Previous reports have shown altered heat resistance of *Listeria* with different strains, food composition, enumeration medium and enumeration techniques (Heddleson and others 1996; Juneja and others 1998). Differences in heating profile resulting from differences in heating technique significantly affect the microbial thermal resistance (Coote and others 1991).

Validation of the TTI (Prediction equation in food products)

The thermal inactivation for TTI and *Listeria monocytogenes* studied here were first order reactions, the plot of the logarithm of A/A_0 vs. t therefore results in a straight line defined as (2):

$$\text{Log } A/A_0 = mt + b \tag{2}$$

The following equations 3 and 4 were used to represent the inactivation for TTI and *Listeria monocytogenes,* respectively.

$$\text{Log } A'/A'_0 = m't + b' \tag{3}$$

where:

A' represent residual α-amylase activity (mg maltose/min)

A'_0 represent initial α-amylase activity (mg maltose/min)

m', t and b' represent slope, time (min) and y-intercept at a particular temperature, respectively for the TTI.

$$\text{Log } A''/A''_0 = m''t + b'' \tag{4}$$

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where:

A" represent *Listeria monocytogenes* population after heat treatment (CFU/g)

A"$_0$ represent initial *Listeria monocytogenes* population (CFU/g)

m", t and b" represent slope, time (min) and y-intercept at a particular temperature, respectively for the *Listeria monocytogenes*.

Rearranging equation 3 results in (5):

$$t = [(\text{Log } A'/A'_0)/m'] - b' / m' \qquad (5)$$

Rearranging equation 4 results in (6):

$$t = [(\text{Log } A''/A''_0)/m''] - b'' / m'' \qquad (6)$$

At a given time, t in equation 5 = t in equation 6, equation 4 – equation 5 can obtained (7):

$$[(\text{Log } A'/A'_0)/m'] - [(\text{Log } A''/A''_0)/m''] = [b' / m'] - [b'' / m''] \qquad (7)$$

Rearranging equation 7, the relationship between *Listeria monocytogenes* (Log A"/A"$_0$) and TTI enzyme reduction (Log A'/A'$_0$) can be expressed as (8):

$$(\text{Log } A''/A''_0) = [(\text{Log } A'/A'_0) \, m''/m'] - (b'm''/m') + b'' \qquad (8)$$

Based upon equation 8, inactivation of *Listeria monocytogenes* at a particular heat treatment in a tested food can be determined from the inactivation of the α-amylase in the TTI using predetermined slope (m" and m') and y-intercept (b' and b").

The inactivation of *Listeria monocytogenes* at other temperatures can be obtained by constructing new equations for both TTIs and *Listeria monocytogenes*. The prediction equations developed by conducting parallel experiments in which both TTIs and *Listeria monocytogenes* were incorporated into the different food products and subjected to heat treatments at 55-70 ºC (Figure 3, Figure 4 and Figure 5) are presented in Table 5. The predicted equation was evaluated by plotting predicted log reductions of *Listeria*

monocytogenes vs. the experimental determined values (Figure 6, Figure 7 and Figure 8). The coefficient of determination (R^2) between predicted and experimental values was high: 0.90-0.99 in mashed potatoes, 0.90-0.99 in ground meat (beef, 30%fat) and 0.95-0.99 in shrimp. The mean absolute percentage error (MAPE) was relatively low: 2.21-6.66% in mashed potatoes, 0.96-11.36% in ground meat (beef, 30% fat), and 2.60-5.53% in ground shrimp; indicating that an error of prediction of *Listeria monocytogenes* inactivation based upon the residual α-amylase TTI activity was within one log cycle.
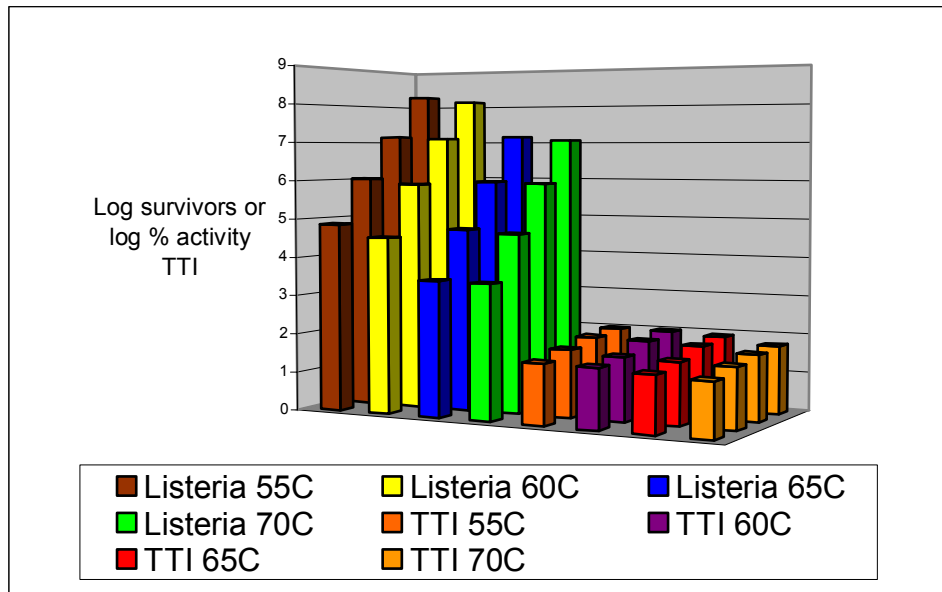
Fig 3. Inactivation of *Listeria monocytogenes* and TTI in ground shrimp at 55-70 ºC.
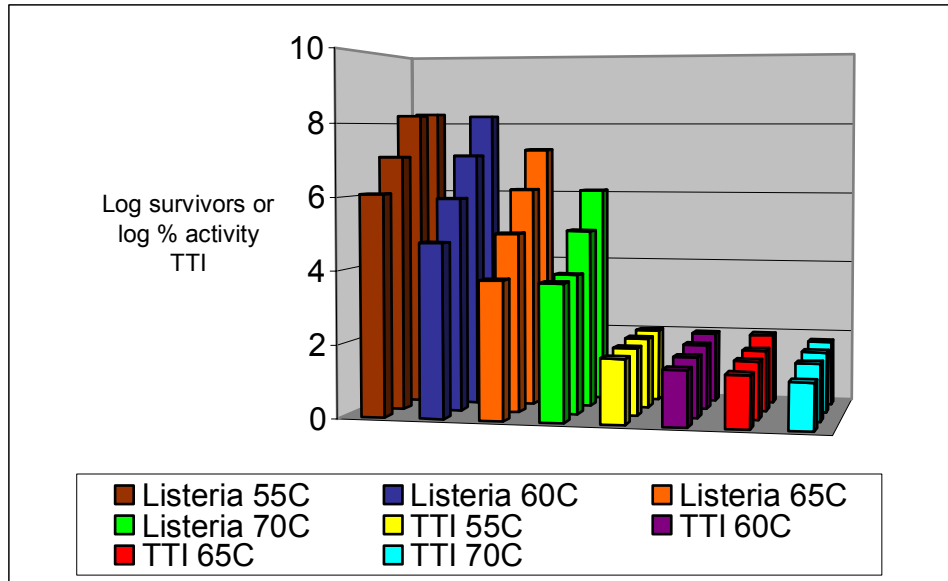
Fig 4. Inactivation of *Listeria monocytogenes* and TTI in ground meat (beef, 30% fat) at 55-70 ºC.
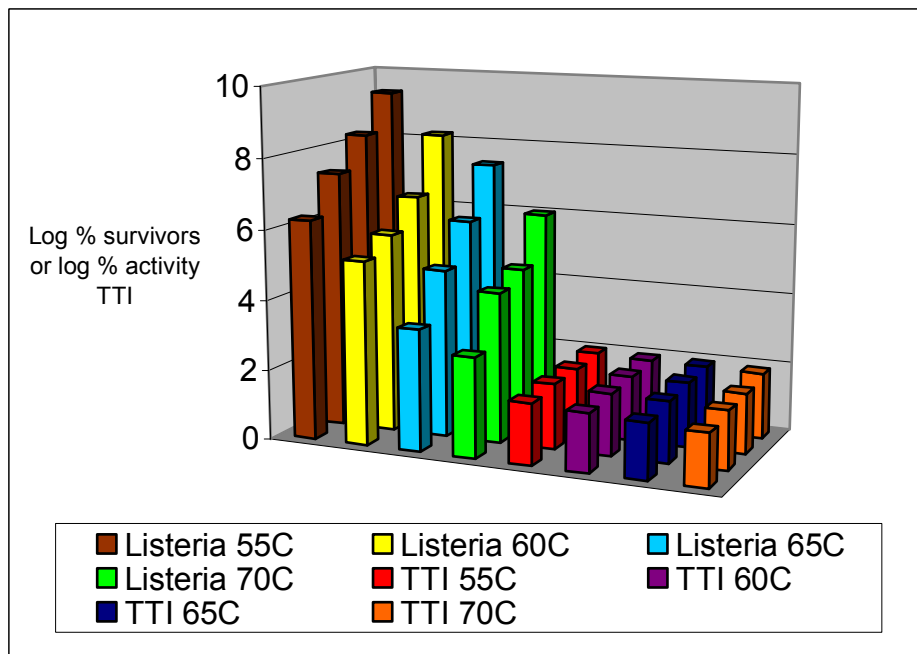
Fig 5. Inactivation of *Listeria monocytogenes* and TTI in mashed potatoes at 55–70 ºC.

Listeria monocytogenes/Food system	Temp (ºC)	Prediction equation	MAPE (%)
L. monocytogenes/Mashed potatoes	55	log A"/Ao" = 12.92 (log A'/Ao') + -16.53	2.21
	60	log A"/Ao" = 10.31 (log A'/Ao') + -12.25	6.66
	65	log A"/Ao" = 11.68 (log A'/Ao') + -15.39	4.97
	70	log A"/Ao" = 8.32 (log A'/Ao') + -9.61	1.18
L. monocytogenes/Ground meat (beef, 30% fat)	55	log A"/Ao" = 10.31 (log A'/Ao') + -12.04	4.55
	60	log A"/Ao" = 8.26 (log A'/Ao') + -7.78	0.96
	65	log A"/Ao" = 7.07 (log A'/Ao') + -6.21	4.22
	70	log A"/Ao" = 5.11 (log A'/Ao') + -3.48	11.36
L. monocytogenes/Ground shrimp	55	log A"/Ao" = 7.37 (log A'/Ao') + -6.71	2.60
	60	log A"/Ao" = 7.51 (log A'/Ao') + -6.82	3.41
	65	log A"/Ao" = 7.47 (log A'/Ao') + -7.42	3.16
	70	log A"/Ao" = 9.93 (log A'/Ao') + -10.66	5.53

Table 5. Prediction equations for *Listeria monocytogenes* inactivation and MAPE results in mashed potatoes, ground meat (beef, 30% fat), and ground shrimp based upon TTI results at various temperatures.
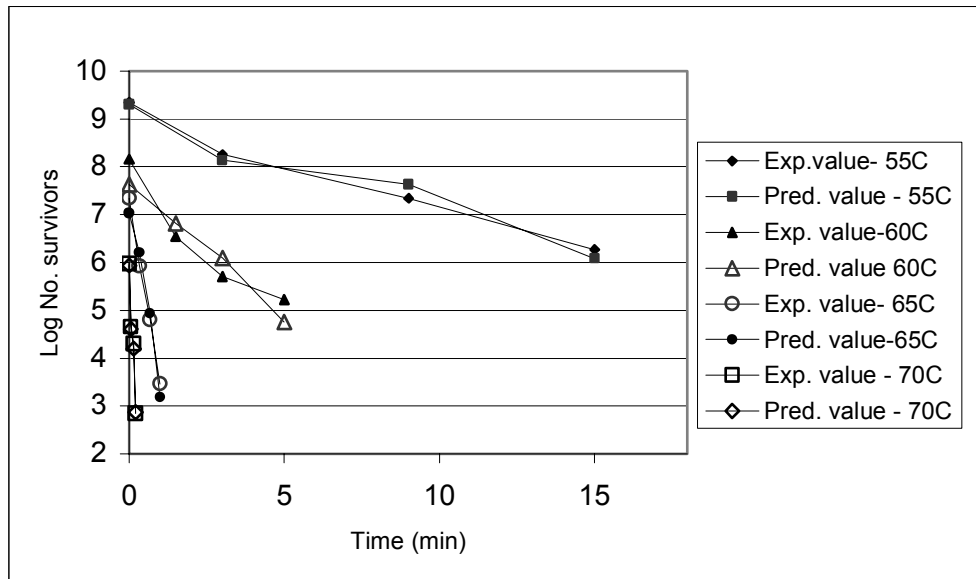
Fig. 6 Prediction values for *Listeria monocytogenes* inactivation in mashed potatoes based upon TTI results at various temperatures (Exp.= experimental, Pred.= predicted).
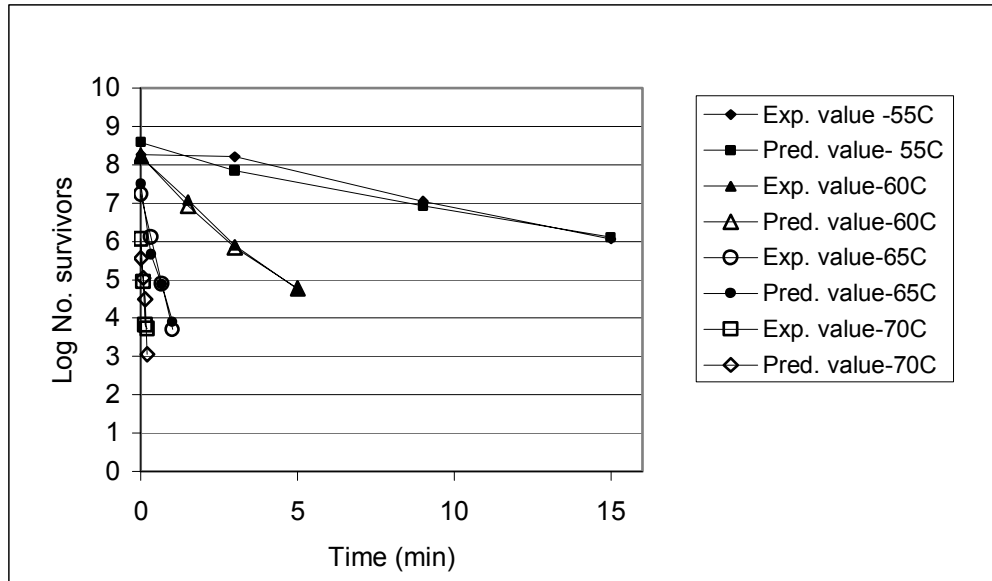
Fig. 7 Prediction values for *Listeria monocytogenes* inactivation in ground meat (beef, 30% fat) based upon TTI results at various temperatures (Exp.= experimental, Pred.= predicted).
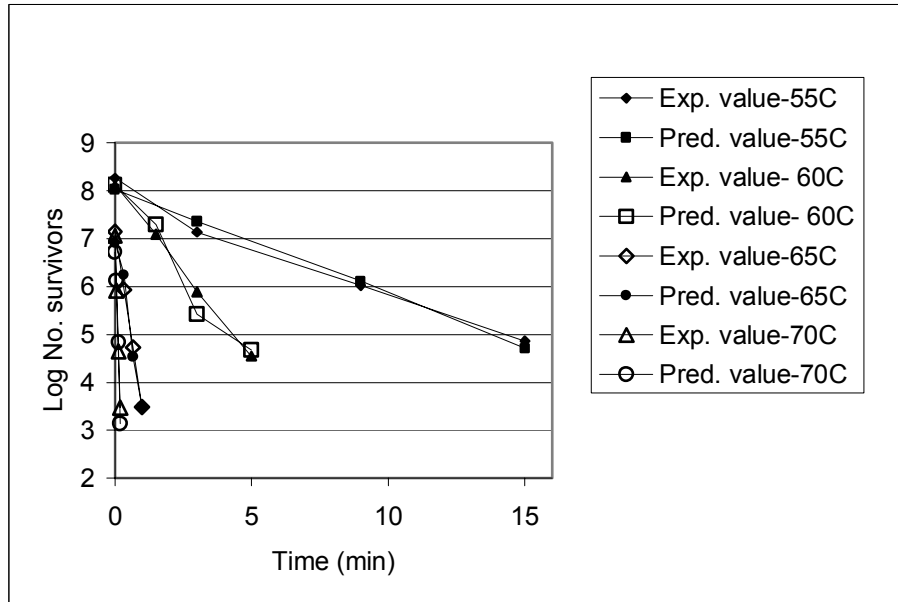
Fig. 8 Prediction values for *Listeria monocytogenes* inactivation in ground shrimp based upon TTI results at various temperatures (Exp.= experimental, Pred.= predicted).

Developing a generalized prediction equation for *Listeria* spp. inactivation in foods may not be feasible since food composition, growth temperature, medium composition, stage of growth, etc., can alter the microbial susceptibility to heat treatment (Juneja and others 1998; Tomlins and Ordal 1976). Also, thermal inactivation of enzymes can be affected by the food matrices (Pazur and others 1970). Therefore, as shown in this research, establishing separate TTI prediction equations for different foods is necessary for a reliable prediction of the adequacy of a pasteurization process using enzyme based TTI methods.

CONCLUSIONS

Two major factors need to be considered in developing a pasteurization process. First, the target pathogen or spoilage microorganism and secondly the numbers of log cycle reductions for that particular microorganisms that should comply with regulations or safety requirements for that particular food (Van Loey and others 1997). Using a α-amylase based TTI system as the validation tool for pasteurization is feasible as long as the kinetics of inactivation for the TTI and the target microorganism in a particular food is known.

The thermal inactivation kinetics of *Listeria monocytogenes* and α-amylase TTI were first order. The D- values for the TTI, in all cases, were higher than the D- values for *Listeria monocytogenes*. The higher D- value for the TTI is desirable because it makes possible the development of a sensitive quantitative assay based upon residual α-amylase activity. The z-values were within the recommended range for pasteurization (Van Loey and others 1997).

A simple assay of α-amylase immobilized in polyacrylamide gel is one possible way for predicting process lethality at pasteurization temperatures. The TTI provides a fast, relatively accurate, inexpensive and simple approach, which could be implemented in

industrial settings. The primary advantage of a TTI is a reduction in routine microbial testing for process validation.

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CHAPTER 5

PREDICTING THERMAL INACTIVATION OF *Salmonella typhimurium* AND *Escherichia coli* O157:H7 USING AN ENZYME-BASED TIME TEMPERATURE INDICATOR, PHYTASE FROM *Aspergillus ficuum*

ABSTRACT

An enzyme based time-temperature indicator (TTI) was developed with *Aspergillus ficuum* phytase immobilized in 20% polyacrylamide gel. The thermal inactivation kinetics of the TTI, *Salmonella typhimurium* and *Escherichia coli* O157:H7 in ground shrimp, ground meat (beef, 30% fat) and mashed potatoes were first order. During thermal processing at 53, 58, and 63 ºC, TTI D- values (min) ranged from 555.00 to 10.40 in ground meat (beef, 30% fat), 312.50 to 6.54 in mashed potatoes and 222.00 to 5.41 in ground shrimp. *Salmonella typhimurium* D- values ranged from 27.02 to 0.79 in ground meat (beef, 30% fat), 24.57 to 0.67 in mashed potatoes and 20.37 to 0.54 in ground shrimp. *Escherichia coli* O157:H7 D-values ranged from 33.00 to 0.79 in ground meat (beef, 30% fat), 26.59 to 0.71 in mashed potatoes and 21.05 to 0.55 in ground shrimp.

Key words: thermal inactivation, immobilized enzyme, food, *Salmonella typhimurium* and *Escherichia coli* O157:H7.

INTRODUCTION

Food-borne diseases continue to be a problem of global concern (Bellara and others 1999) with *Salmonella* spp. and pathogenic *Escherichia coli* being important causes of food-borne illness in insufficiently heated foods.

Salmonella is a genus of the Enterobacteriaceae familiy. These are Gram negative, non-sporeforming, facultative anaerobic rods that cause food-borne gastroenteritis. The majority of *Salmonella* spp. are motile with peritrichous flagella. They are widely distributed in nature, with humans and animals being their primary reservoirs. *Salmonella enteritica* serovar *typhimurium* is a food-borne pathogen, which regularly causes large outbreaks of food poisoning. The *Salmonella* food-poisoning syndrome (Salmonellosis) is caused by the ingestion of food that contains significant numbers of the microorganim. Salmonellosis can be fatal and the cost associated with the infection can be very high. Most outbreaks of salmonellosis have result from the consumption of contaminated meat, eggs, or dairy products (Mattick and others 2001; Carramiñana and others 1997; Hendberg and others 1996; Hennessy and others 1996; Lammerding 1988). From the time of ingestion of food, symptoms usually develop in 12-14 h, although shorter or longer times have been reported. The symptoms consist of nausea, vomiting, abdominal pain (not as severe as with staphylococcal food poisoning), headache, chills and diarrhea. These symptoms are usually accompanied by prostration, muscular weakness, faintness, restlessness, and drowsiness. Symptoms usually persist for 2-3 days. The average mortality rate is 4.1%. Although these organisms generally disappear from the intestinal tract, up to 5% of patients may become carriers of the organisms upon recovery from this disease (Jay 2000).

Escherichia coli was established as a food-borne pathogen in 1971 when imported cheeses that were contaminated with an *E. coli* enteroinvasive strain, turned up in 14 American states; in total, nearly 400 individuals became ill. Prior to 1971, at least five food-borne outbreaks were reported in other countries, with the earliest being from England in 1947. As a human pathogen, evidence suggested that it was recognized as a cause of infant diarrhea as early as the 1700s (Wilson and others 1983).

Like *Salmonella*, *Escherichia coli* are also members of the Enterobacteriaceae family. As such, the organisms are Gram negative, catalase-positive, oxidase-negative, facultative anaerobic short rods. *Escherichia coli* O157:H7 is an etiological agent of haemorrhagic colitis, the life-threatening post-diarrhoeal complications of haemolytic uraemic syndrome and thrombic thrombocytopenic purpura (Tarr 1994; Swerdlow and others 1992).

Escherichia coli O157:H7 is an emerging cause of foodborne illness. In recent years, severe outbreaks of disease caused by *Escherichia coli* O157:H7 have become a real concern for consumers, meat processors, and government regulatory agencies (Orta-Ramirez and others 1997). An estimated 73,000 cases of infection and 61 deaths occur in the USA each year. Cattle appear to be the main reservoir of *E. coli* O157:H7. Transmission to humans is principally through the consumption of contaminated foods, such as raw or undercooked meat products and raw milk. Freshly pressed apple juice or cider, yogurt, cheese, salad vegetables and cooked maize have also been implicated. Fecal contamination of water and other foods, as well as cross-contamination during food preparation, are also implicated as transmission routes (Oteiza and others 2003).

The inactivation of infectious non-spore-forming pathogens using a heat treatment is a critical control point in the safe preparation of many foods. Because of reliability and,

generally, economical use (compared to other methods) and relatively low post-process storage cost, thermal processing has been widely used in the food industries for many years (Cerf and others 1996). The knowledge of thermal inactivation kinetics of potential contaminating microorganisms is therefore essential to proper sterilizer design and operation. Establishing safe thermal processes is a key task, which should be undertaken by those who are fully aware of the potential risks and the margins of error (Tucker 1997). Insufficient processing, insufficient cooking or reheating are often contributing factors in food-poisoning outbreaks (Roberts 1991).

To evaluate the impact of the thermal processes of food in terms of safety and quality, different methods are available: physical – mathematical and *in situ* methods (Hendrickx and others 1995). However, limitations of both methods have promoted the development of time-temperature integrators (TTI) as alternative evaluation tool to evaluate the effectiveness of thermal processes (Bruch 1973; Mulley and others 1975; Pflug and Smith 1977; Bunn and Sykes 1981; Sastry and others 1988; Rönner 1990; Weng and others 1991; Whitaker 1991; De Cordt and others 1992a; Van Loey and others 1996; Guiavarc'h and others 2002; Raviyan and others 2003a, 2003b).

A TTI based upon *Aspergillus ficuum* phytase immobilization in polyacrylamide gel provides a number of advantages over current designs including: ease of preparation, physical durability, and a fast, simple and inexpensive assay.

In Chapter 4, α-amylase immobilized in 20% polyacrylamide gel was used to predict *Listeria monocytogenes* inactivation, in this part of the research, phytase immobilized in 20% polyacrylamide gel was used instead for several reasons. The

temperature sensitivity of phytase is similar to both *Escherichia coli* O157:H7 and *Salmonella typhimurium,* suggesting that this enzyme could be used as a time-temperature indicator in food products where inactivation of these microbes is of particular concern. In addition α-amylase appears to be too heat stable for applications involving these microbes, making its use impractical due to poor sensitivity and accuracy.

The objectives of this study were to: 1) develop an enzyme-based time-temperature indicators (TTI) employing phytase from *Aspergillus ficuum* entrapped in 20% polyacrylamide gel, 2) determine inactivation kinetics parameters for the TTI, *Salmonella typhimurium* and *Escherichia coli* O157:H7 in ground shrimp, mashed potatoes and ground meat (beef, 30% fat), 3) develop prediction equations that correlate thermal inactivation of the TTI with *Salmonella typhimurium* and *Escherichia coli* O157:H7 inactivation, 4) and validate prediction of microbial inactivation with the experimental data obtained from parallel thermal inactivation studies of *Salmonella typhimurium* and *Escherichia coli* O157:H7 and the TTI in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

MATERIALS AND METHODS

Enzymes

Aspergillus ficuum phytase (EC 3.1.3.8) from Sigma Aldrich Co. was used as described in Chapter 2.

Immobilization procedure

The immobilized enzyme containing gel for phytase was prepared as described in Chapter 2.

Assay of immobilized enzyme

The immobilized enzyme-containing gel was measured spectrophotometrically as described in Chapter 2.

Enzymatic activity

Enzymatic activity is expressed as described in Chapter 2.

Thermal inactivation experiments

Food products

Frozen shrimp (*Penaeus monodon)*, mashed potatoes and ground meat (beef, 30% fat) were obtained from a local supermarket (Pullman, WA) and kept at -35 °C. All food samples were prepared as previously in Chapter 3.

Inactivation of immobilized phytase (TTI) in food systems

The inactivation experiments for phytase TTI were followed as described in Chapter 3.

Inactivation of *Salmonella typhimurium and E.coli O157:H7* in food systems

Microbial cultures

Salmonella typhimurium ATCC# 19585, # 363755 and # 46174 cells and *Escherichia coli* O157:H7 ATCC# 35150, # 43889 and # 43890 (obtained from Dr. Don-Hyun Kang, Washington State University, Pullman, WA) were incubated at 37 ºC for 24 hr in tryptic soy broth supplemented with 0.6% yeast extract (Difco Laboratories, Detroit, Michigan). Since the stationary phase is the most resistant stage in the bacterial cell life span, stationary phase cells were used for the thermal inactivation experiments (Heddleson and others 1991; Jay 2000). After the incubation period, one ml of equal volumes of the six strains of bacteria

were combined in a sterile flask to obtain a cocktail that was used to inoculate about 10.00 g of the food system.

Thermal inactivation experiments

Thermal inactivation experiments at 53, 58, and 63 ºC for *Salmonella typhimurium* and *Escherichia coli* O157:H7 were followed as described in Chapter 4 for *Listeria monocytogenes*.

***Salmonella typhimurium and Escherichia coli O157:H7* enumeration**

The survivors following thermal treatments were enumerated by diluting each 1.00 g heated treated sample into 9 ml of sterile 0.1% peptone in stomacher bags for 2 min. The homogeneous dilution was serially diluted into 9 ml of 0.1% peptone (10^{-1}-10^{-9}) and then plated on Xylose Lysine Desoxycholate agar (Difco Laboratories, Detroit, Michigan) and McConkey Sorbitol Agar (Difco Laboratories, Detroit, Michigan) for *Salmonella typhimurim* and *Escherichia coli* O157:H7, respectively. The plates were incubated for 18-24 hr at 37 °C.

Calculation of D- and z- values

All D-values and z values calculations were made as described in Chapter 3.

Statistical analysis

All experiments in this study were repeated at least three times and results are reported as means. General Linear Model procedures for analysis of variance and regression (Proc GLM) were determined using Statistical Analysis System version 8 (SAS Institute, Inc., Cary, N.C, 1999). Arithmetic means were compared by the Fisher LSD grouping test at the 95% confidence level ($p \leq 0.05$). Interaction effects were analyzed by the least square means model.

Mean absolute percentage error (MAPE) was used to evaluate the accuracy of the models for predicting the experimental values as described in Chapter 4.

RESULTS AND DISCUSSION

Thermal inactivation of immobilized enzymes (TTI) in food systems

The thermal inactivation of the immobilized phytase in the food sample tested was first order (Figure 1). The D- values, z- values and k values for the immobilized phytase in all food systems are listed in Table 1. All regression parameters are listed in Table 2. The thermal inactivation of the immobilized enzyme was affected by food matrices as indicated by different z- values.
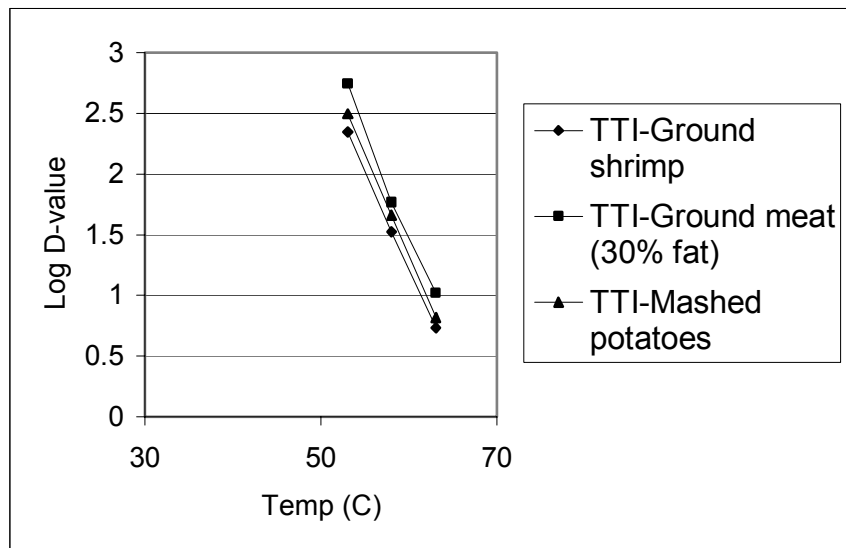
Fig 1. Thermal inactivation curves for immobilized phytase in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

Enzyme/Food system	Temp (°C)	D value (min)	k (min^{-1})	z value (°C)
TTI phytase/Mashed potatoes	53	312.50	0.01	5.96
	58	45.66	0.05	
	63	6.55	0.35	
TTI phytase/Ground meat (beef, 30% fat)	53	555.00	0.00	5.80
	58	58.47	0.04	
	63	10.40	0.22	
TTI phytase/Ground shrimp	53	222.00	0.01	6.19
	58	33.55	0.07	
	63	5.41	0.43	

Table 1. Kinetic parameters for immobilized phytase in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

TTI/Food system	Temp (°C)	y-intercept (b)	slope (m)	R^2
TTI phytase/Mashed potatoes	53	1.98	-0.00	0.96
	58	1.96	-0.02	0.89
	63	1.90	-0.15	0.98
TTI phytase/Ground meat (beef, 30% fat)	53	2.00	-0.00	0.99
	58	1.99	-0.02	0.89
	63	1.95	-0.10	0.94
TTI phytase/Ground shrimp	53	1.98	-0.00	0.95
	58	1.94	-0.03	0.92
	63	1.85	-0.18	0.96

Table 2. Regression parameters for immobilized phytase in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

Thermal inactivation of *Salmonella typhimurium* and *Escherichia coli* O157:H7 in food systems

A log linear decrease of *Salmonella typhimurium* and *Escherichia coli* O157:H7 survivors was observed as a function of heating time (Figure 2). Thermal inactivation kinetic parameters for *Salmonella typhimurium* and *Escherichia coli* O157:H7 in all food systems were determined based upon the relative numbers of survivals after thermal treatments at 53, 58 and 63 ºC (Table 3). Regression parameters are listed in Table 4. No *Salmonella typhimurium* or *Escherichia coli* O157:H7 (< 25 CFU/g by direct plating) were detected before inoculation in any of the food systems used in these studies.
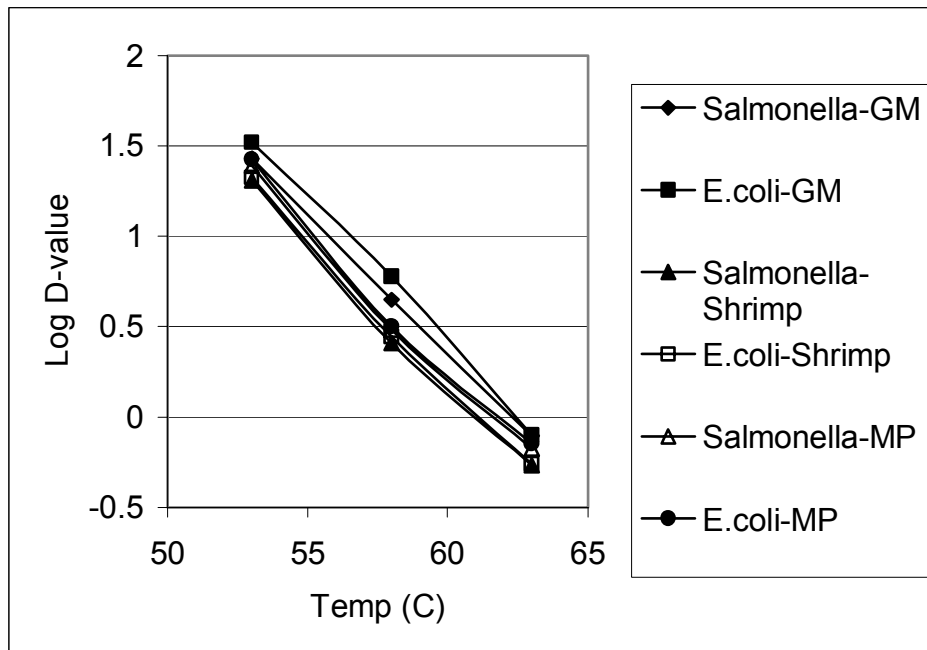
Fig 2. Thermal inactivation curves for *Salmonella typhimurium* and *Escherichia coli* O157:H7 in shrimp, mashed potatoes (MP) and ground meat (beef, 30% fat) (GM).

Pathogen/Food system	Temp (˚C)	D value (min)	k (min-1)	z value (˚C)
Salmonella typhimurium/Mashed potatoes	53	24.57	0.09	6.39
	58	3.03	0.76	
	63	0.67	3.44	
Salmonella typhimurium /Ground meat (beef, 30% fat)	53	27.02	0.09	6.51
	58	4.45	0.52	
	63	0.79	2.92	
Salmonella typhimurium /Ground shrimp	53	20.37	0.11	6.34
	58	2.56	0.90	
	63	0.54	4.25	
Escherichia coli O157:H7/Mashed potatoes	53	26.59	0.09	
	58	3.17	0.73	6.35
	63	0.71	3.24	
Escherichia coli O157:H7/Ground meat (beef, 30% fat)	53	33.00	0.07	6.17
	58	5.99	0.38	
	63	0.79	2.92	
Escherichia coli O157:H7/Ground shrimp	53	21.05	0.11	6.31
	58	2.79	0.83	
	63	0.55	4.19	

Table 3. Kinetic parameters for *Salmonella typhimurium* and *Escherichia coli* O157:H7 in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

Pathogen/Food system	Temp (˚C)	D value (min)	k (min-1)	z value (˚C)
Salmonella typhimurium /Ground meat (beef, 30% fat)	53	27.02	0.09	6.51
	58	4.45	0.52	
	63	0.79	2.92	
Salmonella typhimurium/Mashed potatoes	53	24.57	0.09	6.39
	58	3.03	0.76	
	63	0.67	3.44	
Salmonella typhimurium /Ground shrimp	53	20.37	0.11	6.34
	58	2.56	0.90	
	63	0.54	4.26	
Escherichia coli O157:H7/Mashed potatoes	53	26.59	0.09	
	58	3.17	0.73	6.35
	63	0.71	3.24	
Escherichia coli O157:H7/Ground shrimp	53	21.05	0.11	6.31
	58	2.79	0.83	
	63	0.55	4.19	
Escherichia coli O157:H7/Ground meat (beef, 30% fat)	53	33.00	0.07	6.17
	58	5.99	0.38	
	63	0.79	2.92	

Table 4. Regression parameters for *Salmonella typhimurium* and *Escherichia coli* O157:H7 in ground shrimp, mashed potatoes and ground meat (beef, 30% fat).

Salmonella typhimurium was more heat resistant (higher z- values) than *Escherichia coli* O157:H7 in all food tested. In general, the heat resistance of bacteria increased with decreasing water activity. For *Salmonella typhimurium* and *Escherichia coli* O157:H7, higher D- values were observed in ground meat (beef, 30% fat) than in others food tested.

The protective effect of fatty materials in the heating medium on the heat resistance of microorganisms is well documented (Ahmed and Conner 1995). Increased heat resistance in foods with higher fat contents is related in part to a reduce water activity and to poorer heat penetration (lower heat conductivities) in the fat portion (Juneja and Eblen 2000).

Bacteria entrained in high starch food may be less susceptible to thermal inactivation because the foods contained lower available moisture (Oteiza and others 2003).

Several authors have reported D- and z- values for *Escherichia coli* O157:H7 and *Salmonella typhimurium* in ground beef, turkey, lamb, pork and poultry (Amhed and others 1995; Goodfellow and Brown 1987; Juneja and Marmer 1999; Juneja and others 1997). The D- and z- values found in this study for beef were similar to those reported by Doyle and Schoeni (1984), Line and others (1991), Goodfellow and Brown (1987) and Juneja and Eblen (2000). However, slight differences in D- and z- values among studies may be attributed to different strains or isolates (Heddleson and others 1996; Juneja and others 1998), physiological conditions of the cells or the use of cultures in different growth phases, fat content or pH of the meat, methodology used for recovery of survivors (Juneja and Marmer 1999), or differences in heating technique (Coote and others 1991).

For an enzyme based TTI to be an ideal indicator of thermal processing, it should have similar temperature dependence and temperature sensitivity during thermal inactivation as the microorganisms used to established the process design (Hendrickx and others 1995; Van Loey and others 1996b; Orta-Ramirez and others 1997). Then, regardless of the time-temperature combination used for processing, a residual concentration of the indicator can be established that indicates adequacy of processing without the need for extensive engineering modelling. Our results suggest that *Aspergillus ficuum* phytase immobilized in polyacrylamide might serve as a useful exogenous indicator for the adequacy of thermal processing.

Validation of the TTI (Prediction equations in food products)

The thermal inactivation for the TTI, *Escherichia coli* O157:H7 and *Salmonella typhimurium* studied here were first order reactions. The predictive equations for each bacteria were developed as described in Chapter 4.

The prediction equations developed by conducting parallel experiments in which both TTI and *Escherichia coli* O157:H7 or *Salmonella typhimurium* were incorporated into the different food products and subjected to heat treatments at 53-68 ºC (Figure 3, Figure 4 and Figure 5) are presented in Table 5. The efficiency of the predicted equation was evaluated by plotting predicted log reductions of *Escherichia coli* O157:H7 or *Salmonella typhimurium* vs. experimentally determined values (Figure 6, Figure 7, Figure 8, Figure 9, Figure 10 and Figure 11). The coefficient of determination (R^2) between predicted and experimental values was within 0.90 to 0.99 in mashed potatoes, 0.92 to 0.99 in ground meat (beef, 30%fat) and 0.91 ato 0.99 in ground shrimp for both microorganisms. The mean absolute percentage error (MAPE) was very low in all cases tested, indicating that an error of prediction for *Escherichia coli* O157:H7 or *Salmonella typhimurium* inactivation based upon the residual phytase TTI activity was within one log cycle.

Developing a generalized prediction equation for microbial inactivation for foods may not be feasible since food composition, growth temperature, medium composition, stage of growth, etc., can alter the microbial susceptibility to heat treatment (Juneja and others 1998; Tomlins and Ordal 1976). Also, thermal inactivation of enzymes can be affected by the food matrices (Pazur and others 1970). Therefore, as showed in this research, establishing separate TTI prediction equations for different foods will be necessary for a reliable prediction of the adequacy of a pasteurization process.

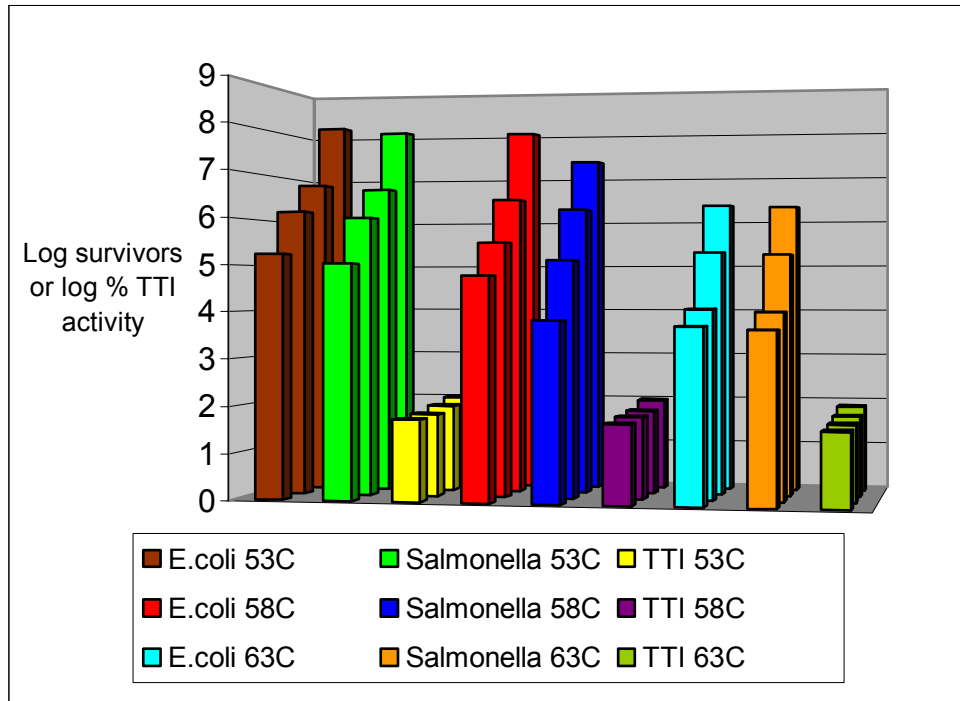**Fig 3. Inactivation of *Salmonella typhimurium*, *Escherichia coli* O157:H7 and TTI in ground shrimp *Listeria monocytogenes* at 53-63 ºC.**
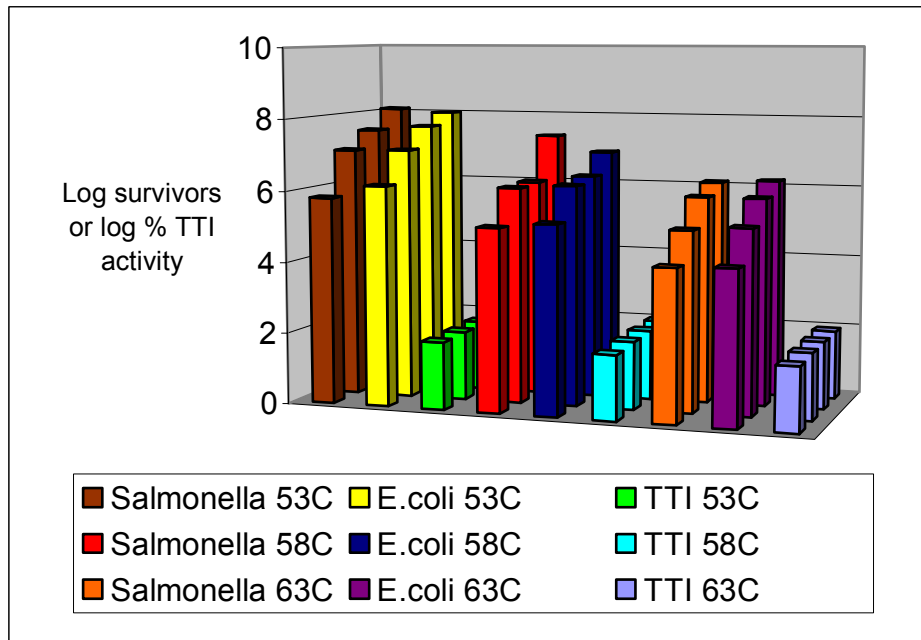

Fig 4. Inactivation of *Salmonella typhimurium*, *Escherichia coli* O157:H7 and TTI in ground meat (beef, 30% fat) at 53-63 ºC.
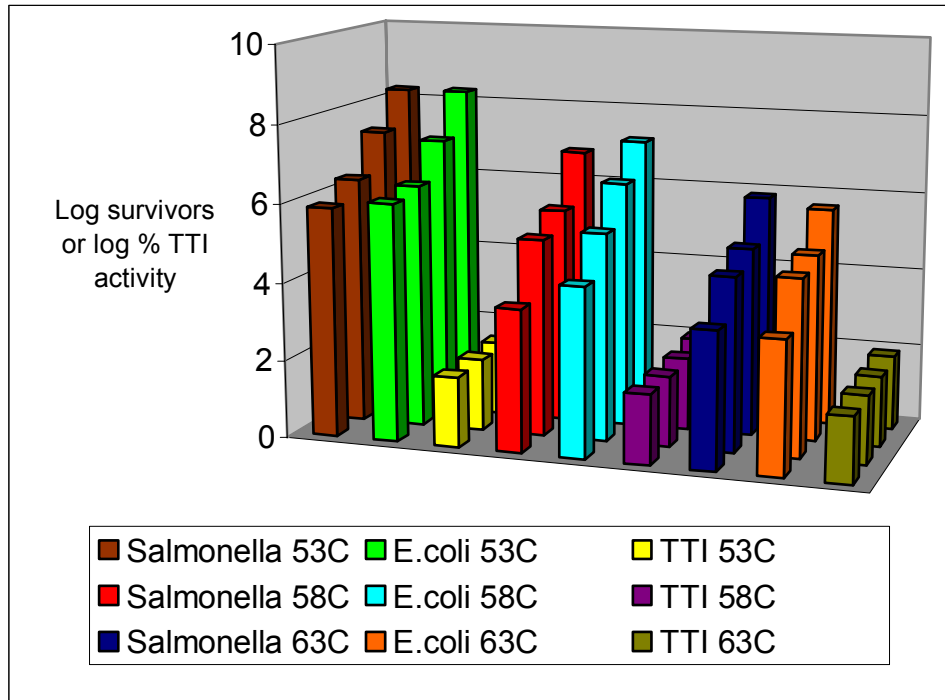
Fig 5. Inactivation of *Salmonella typhimurium*, *Escherichia coli* O157:H7 and TTI in mashed potatoes at 53-63 ºC.

Pathogen/Food system	Temp (ºC)	Prediction equation	MAPE (%)
Salmonella typhimurium/Mashed potatoes	53	log A"/Ao" = 12.70 (log A'/Ao') + -17.03	2.80
	58	log A"/Ao" = 15.06 (log A'/Ao') + -22.78	8.38
	63	log A"/Ao" = 9.73 (log A'/Ao') + -12.72	1.30
Salmonella typhimurium /Ground meat (beef, 30% fat)	53	log A"/Ao" = 20.38 (log A'/Ao') + -32.60	2.64
	58	log A"/Ao" = 13.13 (log A'/Ao') + -18.95	5.55
	63	log A"/Ao" = 13.19 (log A'/Ao') + -19.43	1.48
Salmonella typhimurium /Ground shrimp	53	log A"/Ao" = 10.91(log A'/Ao') + -13.67	2.96
	58	log A"/Ao" = 13.12(log A'/Ao') + -18.07	6.96
	63	log A"/Ao" = 10.01 (log A'/Ao') + -12.30	1.96
Escherichia coli O157:H7/Mashed potatoes	53	log A"/Ao" = 11.75 (log A'/Ao') + -15.21	2.80
	58	log A"/Ao" = 14.40 (log A'/Ao') + -21.02	7.00
	63	log A"/Ao" = 9.11 (log A'/Ao') + -11.68	3.25
Escherichia coli O157:H7/Ground meat (beef, 30% fat)	53	log A"/Ao" = 16.83 (log A'/Ao') + -25.56	1.96
	58	log A"/Ao" = 9.76 (log A'/Ao') + -12.50	2.60
	63	log A"/Ao" = 13.12 (log A'/Ao') + -19.24	2.55
Escherichia coli O157:H7/Ground shrimp	53	log A"/Ao" = 10.55(log A'/Ao') + -12.87	2.22
	58	log A"/Ao" = 12.04(log A'/Ao') + -15.55	2.14
	63	log A"/Ao" = 9.87(log A'/Ao') + -12.01	1.94

Table 5. Prediction equations and MAPE results for *Salmonella typhimurium* and *Escherichia coli* O157:H7 based upon TTI results at various temperatures.

Fig. 6 Prediction values for *Salmonella typhimurium* inactivation in ground shrimp based upon TTI results at various temperatures (Exp.= experimental, Pred.= predicted).

Fig. 7 Prediction values for *Escherichia coli* O157:H7 inactivation in ground shrimp based upon TTI results at various temperatures (Exp.= experimental, Pred.= predicted).

Fig. 8 Prediction values for *Salmonella typhimurium* inactivation in ground meat (beef, 30% fat) based upon TTI results at various temperatures (Exp.= experimental, Pred.= predicted).

Fig. 9 Prediction values for *Escherichia coli* O157:H7 inactivation in ground meat (beef, 30% fat) based upon TTI results at various temperatures (Exp.= experimental, Pred.= predicted).
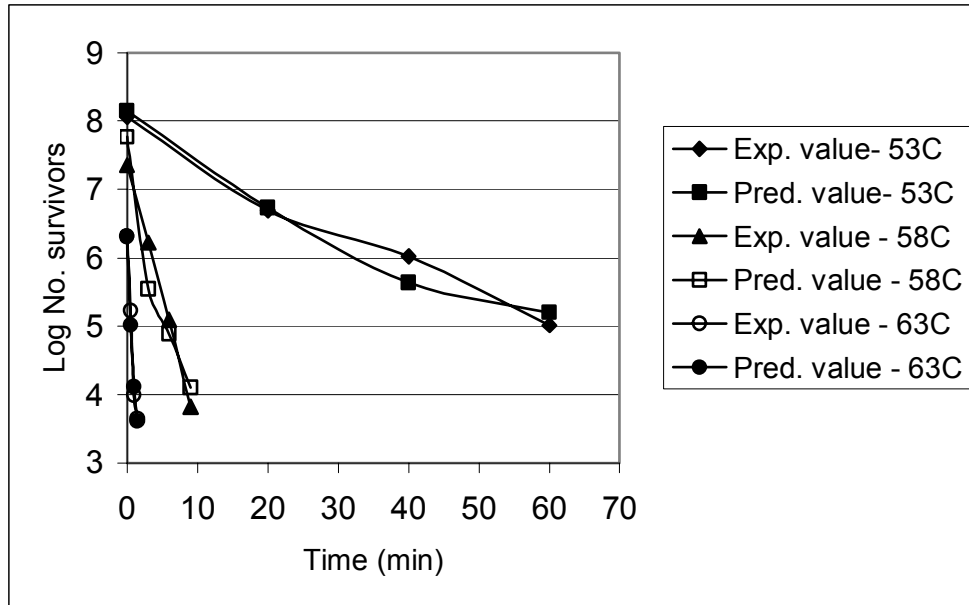
159

Fig. 10 Prediction values for *Salmonella typhimurium* inactivation in mashed potatoes based upon TTI results at various temperatures (Exp.= experimental, Pred.= predicted).
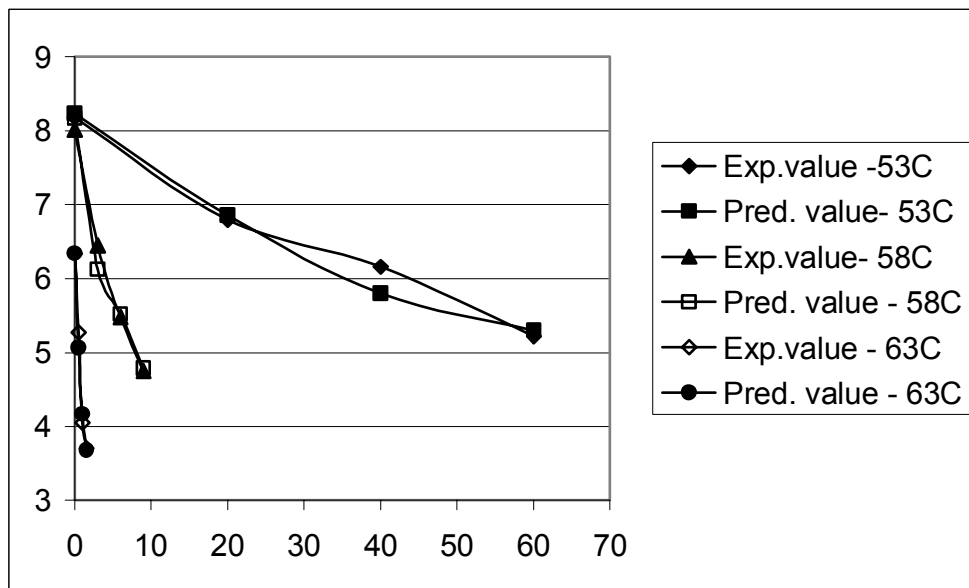

Fig. 11 Prediction values for *Escherichia coli* O157:H7 inactivation in mashed potatoes based upon TTI results at various temperatures (Exp.= experimental, Pred.= predicted).
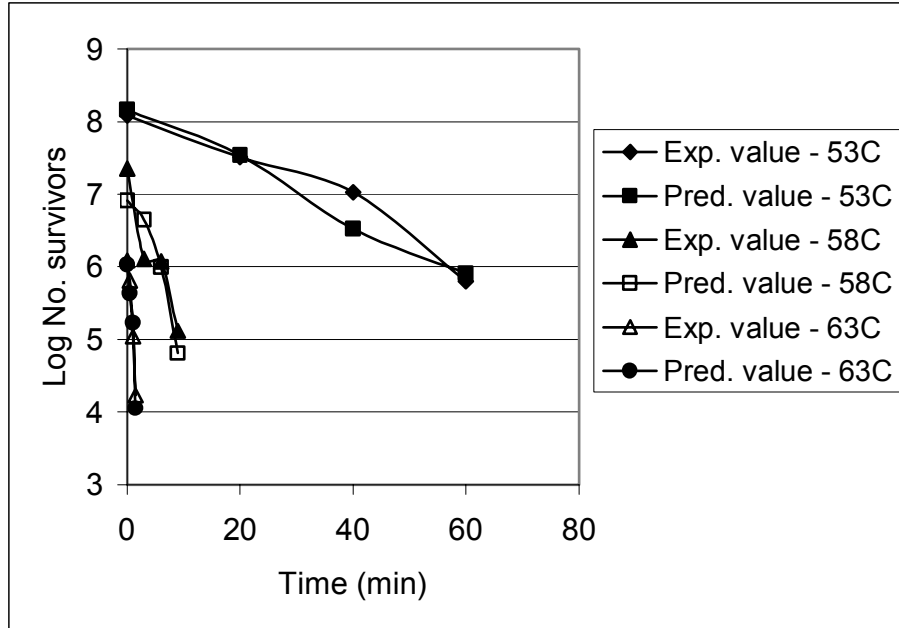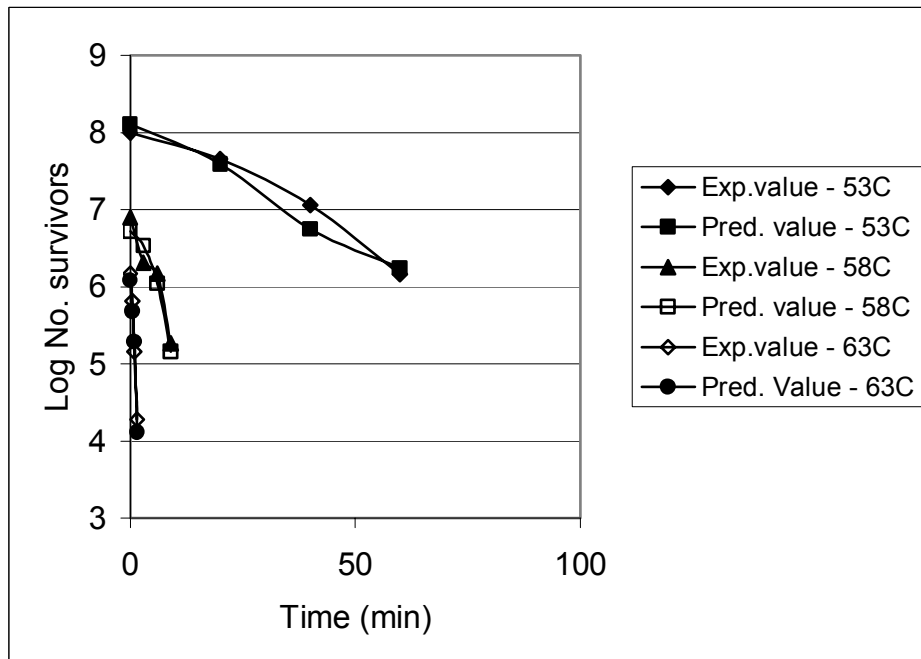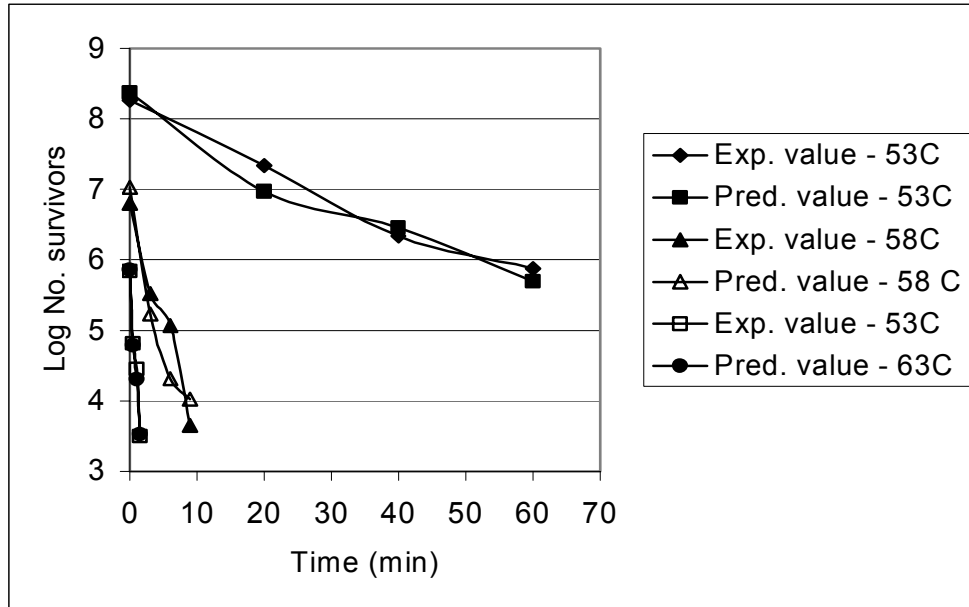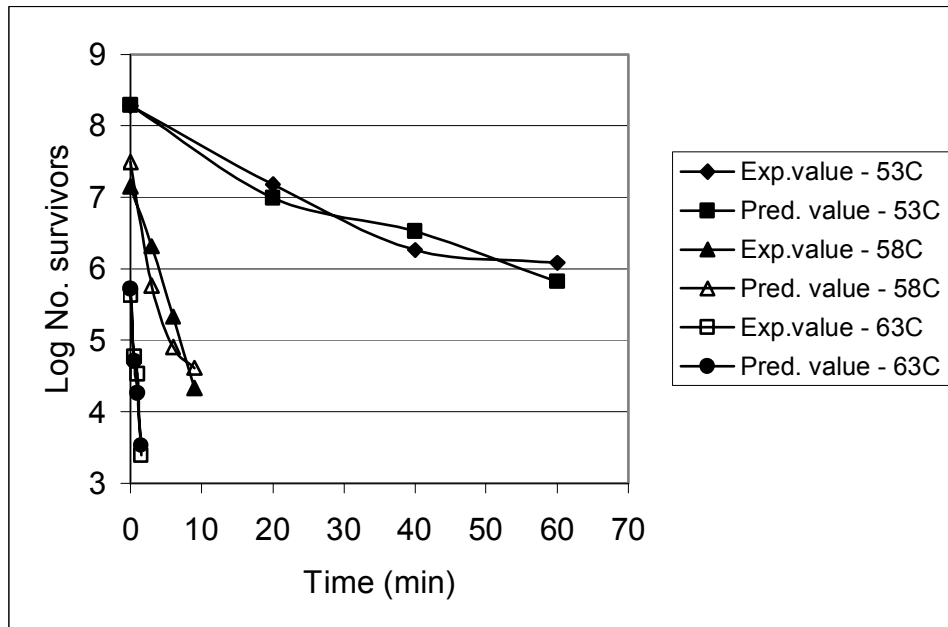
CONCLUSIONS

The thermal inactivation kinetics of *Escherichia coli* O157:H7, *Salmonella typhimurium* and phytase TTI were first order. The D- values for the TTI, in all cases, were higher than the D- values of the microorganisms. The higher D- value for the TTI is desirable because it makes possible the development of a sensitive quantitative assay based upon residual phytase activity. The z- values were within the recommended range for pasteurization processes (Van Loey and others 1997).

Using a phytase based TTI system, as the validation tool for pasteurization is feasible as long as the kinetics of inactivation for the TTI and the target microorganism in a particular food is known.

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CHAPTER 6

PREDICTING HEAT EXPOSURE DURING MICROWAVE HEATING OF FOODS USING ENZYME-BASED TIME TEMPERATURE INDICATORS BASED UPON IMMOBILIZATION OF α-AMYLASE FROM *Aspergillus oryzae* AND PHYTASE FROM *Aspergillus ficuum* IN POLYACRYLAMIDE GEL

ABSTRACT

Two enzyme based time-temperature indicator (TTI) were developed using *Aspergillus oryzae* α-amylase and *Aspergillus ficuum* phytase immobilized in 20% polyacrylamide gel. Both TTI were used to map heat distribution during the processing of ground meat (beef, 30% fat), ground shrimp and mashed potatoes using a 915 MHz Microwave Circulated Water Combination (MCWC) heating system. Temperature profiles obtained with optical fiber probes could be correlated with the residual TTI activity in all food tested. Both time-temperature indicators can be used to determine heat distribution during microwave heating when the direct measurement is impractical or costly.

Key words: time-temperature-indicators, food, microwave heating, phytase and α-amylase.

INTRODUCTION

Thermal sterilization has been widely used in the food industries for many years (Cerf and others 1996). In conventional thermal processing operations, slow heat conduction from the heating medium to the "cold-spot" often results in treatment of the material that is far more severe than that required to achieve commercial sterilization (Meredith 1998). Dielectric heating, which includes radio frequency (RF) and microwave heating, has the potential to replace conventional retort processes. Retorting can be greatly improved upon by eliminating the excessive heating from conventional retort processing with a more rapid heating from a direct interaction between microwave or RF energy and the food (Wang and others 2003). The volumetric heat generated by microwaves can significantly reduce the total heating time at the elevated temperatures needed for commercial sterilization (Decareau 1985) and similar effects are seen for dielectric pasteurization processes, conducted at temperatures of 75 ºC or less (Raviyan 2000; Al-Holy, 2003).

Microwave heating ("cold pasteurization") refers to the use of electromagnetic waves of frequencies between 300 MHz and 300 GHz to generate heat in a material (Metaxas and Meredith 1993). Polar molecules such as water have negatively and positive charged ends. In the presence of a microwave electric field, the water molecule attempts to line up with the field. Since the microwave field is reversing polarity millions of times per second, the water molecules move first in one direction and then reverse to move in the opposite direction. The friction caused by constantly oscillating charged particles produce heat. Most foods are composed of 50-90% water (Ohlsson 1983), and it is the unbound water that contributes to the heating effect. In addition, ions, because of their electric charge, flow in one direction, then in the opposite direction in an electromagnetic field. This effect leads to a higher

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temperature at the surface of water or foods containing salt. Therefore, production of heat occurs during microwave heating is primarily through the dipolar rotation and ionic polarization caused by these field oscillations (Chipley 1980).

Microwave pasteurization is a promising technology because the irradiation passes through many packaging material with little energy losses. Treating a food in a hermetically sealed package eliminates the possibility of post processing contamination. However there are several disadvantages associated with microwave heating. One disadvantage is that microwave heating is often non-uniform. Temperature distribution in microwave heating is controlled by a number of interrelated factors such as microwave field pattern, composition, density, size, shape, thickness of food and food packaging. The poor control of heat uniformity leads to the survival of microorganisms in the processed food and results in a poor quality product, possibly one that is unsafe as well. Another disadvantage is the difficulty of monitoring and predicting microwave heating patterns in the microwave cavity as well within the tested food product.

Validating microwave processes is critical. Before microwave sterilization or pasteurization processes can be accepted as a scheduled processes by the US Food and Drug Administration (FDA), and then accepted and adopted by the US food industry, two issues need to be addressed: the development of a system that can deliver predictable and uniform heating to food systems, and the development of a reliable monitoring procedure to ensure the safety of microwave processed foods (US Department of Health and Human Services 2000; Lau and others 2003).

To design an effective thermal process to ensure adequacy sterility for thermally processed foods, it is necessary to determine the location of the "cold-spot" in packaged

foods (21 CFR, Part 113). For conventional thermal processes, heat transferred from the heating medium (steam or water) to food interior via conduction (solid foods) or convention (liquid foods), and the coldest location in the package is well defined, e.g., normally at the geometrical center in solid foods or about 1/5 from the bottom of the cans for liquid foods (Holdsworth 1997). However, the "cold spot" in a product subjected to microwave heating is different from conventional heating since the heating patterns, being dependent upon the interaction between microwave energy and food, are difficult to predict (Decareau 1985). Thus, although assessment of temperature distribution within the packaged foods during microwave sterilization is essential, it cannot be determined with a single-point or even various point temperature measurements (Ohlsson 1972). Consequently, one alternative method for assessment of temperature distribution within the packaged foods during microwave processing is by assessing cumulative heating on time temperature indicators (TTI).

When materials get hot they radiate energy in the visible as well as in the infrared region of the electromagnetic spectrum, and the "color" of the material is a fair indicator of its temperature. Although conventional methods of temperature measurement using thermometers and thermocouples are still commonly used for many applications, IR sensors have become less expensive and more reliable. Therefore, non-contact measurement using infrared sensors has become an increasingly desirable alternative to conventional temperature measurement methods. Instruments using infrared detectors and optics capable of measuring target surface temperatures are available with sensitivities at 0.1 °C and with response times in the microsecond range. Infrared thermal sensing and imaging instruments allow us to measure and map surface temperature and thermal distribution passively and

non-intrusively; where the brightness intensity or color hue of any spot on the map is representative of the temperature of the surface at that point (Kaplan 1999; Holst 2000). The four most common stated advantages of non-contact thermal infrared measurement over contact measurement are that it is non-intrusive, remote, much faster than conventional methods and that it measures the temperature (or radiant thermal distribution) at the surface of the object of interest, not the surrounding air. However readings within the mass of the product are still not possible using infrared devices, therefore having TTI to complement these readings becomes an important factor for validation of pasteurization processes.

The objectives of this study were to: 1) use enzyme based time temperature indicators employing α-amylase from *Aspergillus oryzae* and phytase from *Aspergillus ficuum* entrapped in 20% polyacrylamide gel for assessment of temperature distribution within the packaged ground shrimp, mashed potatoes and ground meat (beef, 30% fat) during microwave pasteurization (~80 ºC) using a 915 MHz Microwave Circulated Water Combination (MCWC) heating system, and 2) map heat distribution in the packaged foods using infrared thermal imaging.

MATERIALS AND METHODS

Enzymes

Aspergillus oryzae α-amylase (EC 3.2.1.1) and *Aspergillus ficuum* phytase (EC 3.1.3.8) from Sigma Aldrich Co. were used as described in Chapter 2.

Immobilization procedure

The immobilized enzyme-containing gels for α-amylase and phytase were prepared as described in Chapter 2. The dielectric properties of the gels were matched with the dielectric properties of the food under study by NaCl addition as described in Chapter 2.

Maltodextrin preparation for α-amylase activity measurement

The maltrodextrin used as a substrate was MALTIN$^{®}$ M040 (Grain Processing Corporation (Muscatine, WI 52761, USA). The substrate was prepared as described in Chapter 2.

Phytic acid solution preparation for phytase activity measurement

A 44.1 mM phytic acid solution (pH 2.5) was prepared using phytic acid, magnesium-potassium salt from Sigma Aldrich Co. as described in Chapter 2.

Assay of immobilized enzymes

The immobilized enzyme-containing gels for α-amylase and phytase were measured spectrophotometrically, as described in Chapter 2.

Enzymatic activity

Enzymatic activities are expressed as described previously in Chapter 2.

Food products

Frozen shrimp (*Penaeus monodon)*, mashed potatoes and ground meat (beef, 30% fat) were obtained from a local supermarket (Pullman, WA) and kept at -35 °C. All food samples were prepared as described in Chapter 3.

Microwave Circulated Water Combination (MCWC) heating system

The 915 MHz MCWC heating system consisted of three major components: 1) a 5 kW 915 microwave generating system (Microdry Model IV-5 Industrial Microwave Generator, Microdry Incorporated, Crestwood, KY) and a multimode cavity (121.3 cm wide

X 121.3 cm long X 151.1 cm high), 2) a pressurized microwave heating vessel, and 3) a water circulation heating and cooling system.

The 915 MHz microwave system was equipped with a circulator to protect the microwave generator from heat damage caused by reflected power. A directional coupler with appropriate sensors was used to measure forward and reflected powers. The output microwave power was calibrated and stabilized at 1.0 kW by regulating anode current to the magnetron.

The pressurized microwave-heating vessel allows for the thermal treatment of trays with over-pressure. The chamber sidewalls consist of a cylindrical aluminium tube (23.0 cm in diameter and 5.0 cm in height). The top and bottom plates are Tempalux (Ultem Polyetherimide Resin, Lennin, PA, USA) wich has a high melting temperature (above 150 ºC) and is transparent to microwaves. Over-pressure is provided by compressed air in a surge tank and used within the vessel to maintain the integrity of the food package during microwave processing. Fittings were designed to permit temperature measurements and for concurrent circulation of pressurized water (Guan 2003).

In the circulated water control system, circulation water was maintained at the desired temperature by two plate heat exchangers and used to heat (~80 ˚C for the heating period) and cool the food packaged during MCWC processing. The exchangers were heated and cooled with steam and tap water, respectively. A Think & Do™ computer program (Entivity, Ann Arbor, MI) was used to control the modulating valves of the exchangers. The flow rate of the circulated water was 9.5 L/min.

Temperature mapping during MCWC processing

To measure the sample temperature three optical fiber sensors were inserted through holes in the side of the microwave tray in the middle of the layer (10.0 cm wide X 14.0 cm long X 2.5 cm deep X 0.3 cm thickness, Polypropylene and EVOH trays, Rexam ™ Union, MO) as showed in Figure 1 and Figure 2 through a polyimide tubing (OD: 0.1905 cm; ID: 0.18034 cm; thickness: 0.00508 cm, Cole-Parmer, IL, USA) sealed at one end using silicone sealant (Dow Corning®, Dow Corning Corp., Midland, MI, USA). Two pieces of rubber (diameter: ~1 cm, thickness: 0.7938 cm, McMaster-CARR Supply Company, CA, USA) adhered the tubing to both sides of the tray wall using silicone sealant, keeping it from shifting in the sample.
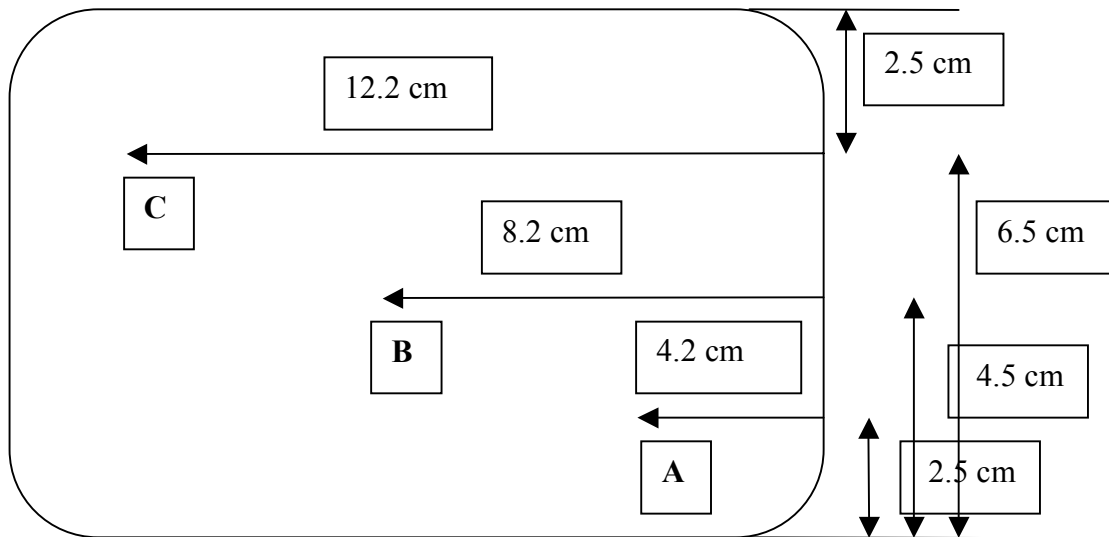

Figure 1. Location of TTI and fiber optics sensors in the microwave trays
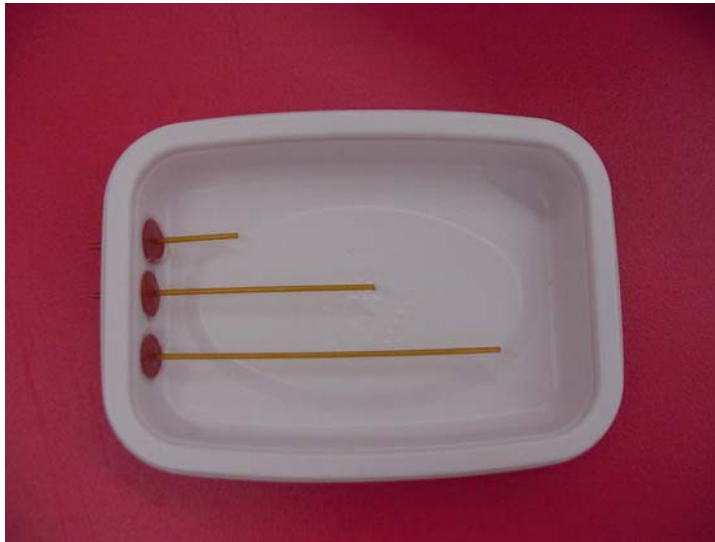
Figure 2. Typical microwave tray for MCWC processing

Package integrity and sealing of products

Package integrity, critical to product stability, was visually observed before and after processing. The products were sealed under vacuum to make rapid microwave heating and cooling possible. Nitrogen flush was applied during sealing and the overpressure was regulated throughout the process.

The sealing prototype unit, customize built by Rexam Containers (Model No.1, Rexam™, Union, MO), consisted of a heating mechanism in an enclosed chamber. A pump and a nitrogen tank were connected to the chamber for vacuum seal and subsequent gas flushing. A metal "nest" holder secured trays containing the product in the sealing chamber. The holder was aligned with a thermostatically controlled heat-sealing head driven by a pneumatic cylinder. A control panel displayed the operation parameters including seal pressure (psig), sealing head temperature (ºF), chamber vacuum (inches of mercury) and seal duration (seconds).

For each treatment, 100 g of the food system was placed into the tray, three pieces of immobilized gel were embedded at each location (Figure 1) and covered with 100 more grams of the food, before sealing the product container. The tray filled with the immobilized enzyme TTI was flushed with nitrogen and heat sealed with a 0.1 mm lid stock (Polypropylene/EVOH laminated) under vacuum (58.69 kPa).

MCWC heating process procedures

The trays in the vessel of the MCWC were held stationary at the center of the heating tunnel. The experiment was terminated when any fiber optic reached ~ 80 ºC. The operating parameters of the MCWC were deliberatedly altered to generate a relatively non-uniform heating process for these experiments. The purpose being to obtain as wide a temperature range within the treated product as possible. After heating, the trays were quickly cooled in ice water and the gel pieces removed from the food products. The gel pieces were transferred into a Ziplock® plastic bag and kept in cooler at a 10 ºC or less until residual TTI activity was assayed. Each experiment was repeated three times.

Power calculations

The quantity of energy (Q) needed to change the absolute temperature (ºK) of any material with a known specific heat (c), is given by (1):

$$Q = m \, c \, \Delta T \qquad (1)$$

where:

m = mass (kg)

ΔT = temperature change (ºK)

assuming:

m = .001 kg

c_{Shrimp} = 3,480 Joules/kg·°K (Polly and others 1980)

$c_{Beef\ mincemeat}$ = 3,520 Joules/kg·°K (Polly and others 1980)

$c_{Potatoes}$ = 3,520 Joules/kg·°K (Polly and others 1980)

No energy dissipation

The total heat energy (P) or power put into the material was calculated as (2) (Watts= Jolues/seconds) :

$$P = Q \text{ (Joules) / Processing time (seconds)} \qquad (2)$$

Process lethality calculations (Improved General and Ball's Method)

Process lethality, F_0 (3), was calculated by integrating the lethality value, L (4), using time-temperature data for the process and z- values for each pathogen as determined in Chapter 4 and 5.

$$F_0 = \Sigma\ L_T\ \Delta t \qquad (3)$$

$$L = [10]^{\ T - To\ /\ z} \qquad (4)$$

where:

T_0 = 80 ˚C

T = final temperature reached during microwave process

Δt = time increments

Infrared thermal imaging

Two hundred grams of the food tested were placed and sealed in microwave trays as described previously. After microwave heating treatment, the seal was removed and photographed with ThermaCAM™ SC 300 (Flir Systems, Danderyd, Sweden) infrared camera equipped with 24º lenses.

Statistical analysis

All experiments in this study were repeated at least three times and results are reported as means. General Linear Model procedures for analysis of variance and regression (Proc GLM) were determined using Statistical Analysis System version 8 (SAS Institute, Inc., Cary, N.C, 1999). Arithmetic means were compared by the Fisher LSD grouping test at the 95% confidence level ($p \leq 0.05$). Interaction effects were analyzed by the least square means model.

RESULTS AND DISCUSSION

MCWC processing

Figures 3, 4, and 5 show the MCWC time temperature heating history of ground meat (beef, 30% fat), ground shrimp and mashed potatoes, respectively. The temperature history of each product was obtained through fiber optical sensors inserted at different locations in the tray and is indicated by different letters.
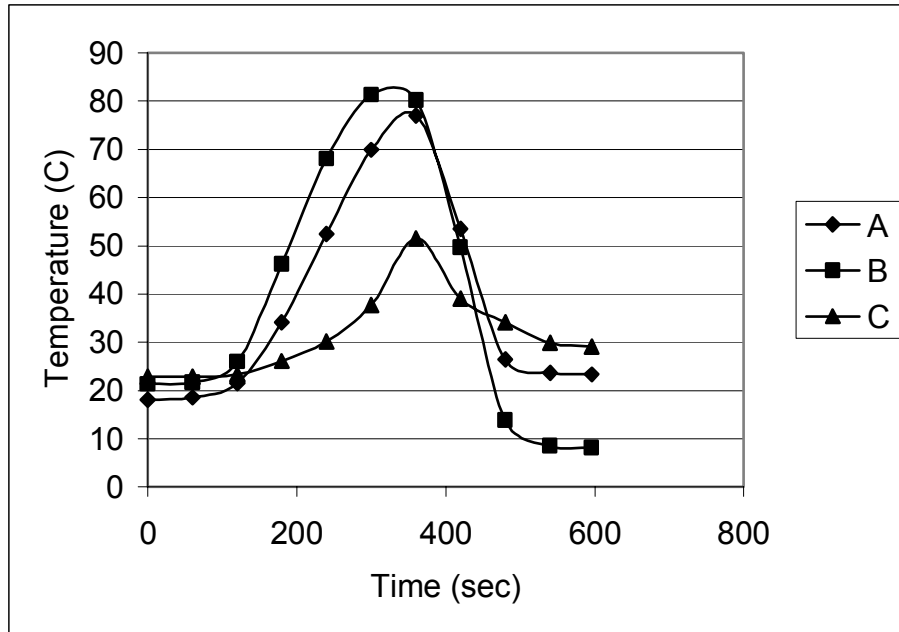
Fig 3. Temperature-time heating history of ground meat (beef, 30% fat) during MCWC heating. For sensor placement, refer to Figure 1.
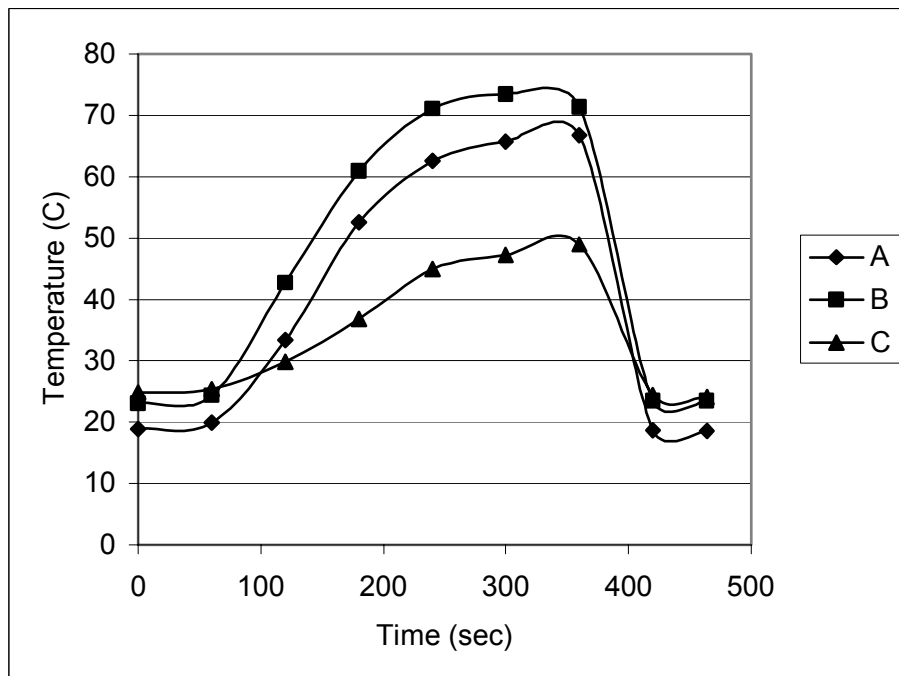

Fig 4. Temperature-time heating history of ground shrimp during MCWC heating. For sensor placement, refer to Figure 1.
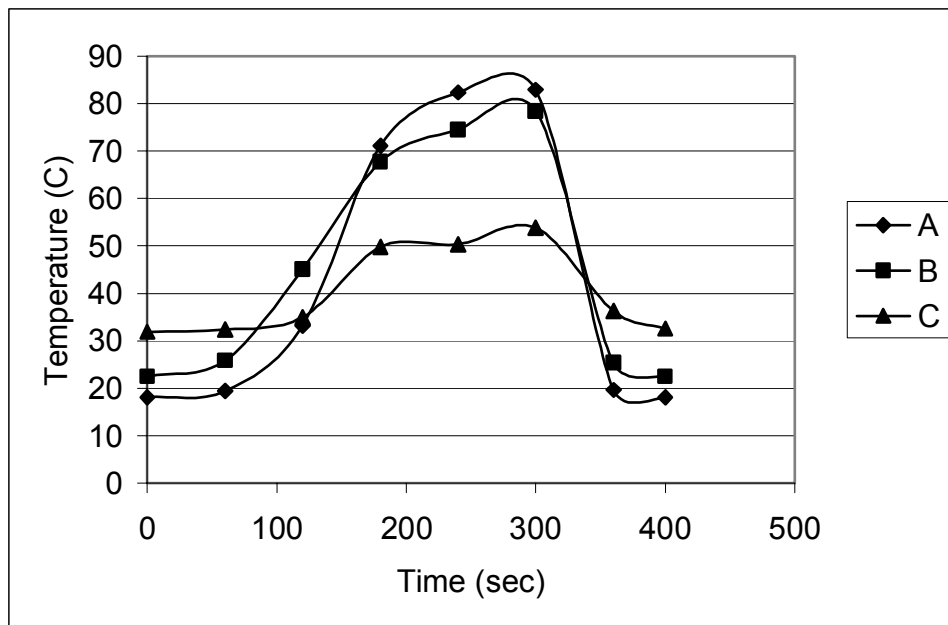
Fig 5. Temperature-time heating history of mashed potatoes during MCWC heating. For sensor placement, refer to Figure 1.

Package integrity was visually examined after the MCWC processing. The tray wall was slightly softened upon removal from the process vessel after processing. The package expanded slightly, with stretching of the lid stock material, but the package integrity was maintained during the microwave heating. All food products had an appropriate odor and appearance after the MCWC processing.

As expected, temperature was uneven throughout the different food tested with the hottest temperature at point B for ground meat (beef, 30% fat) and ground shrimp, but at point A for mashed potatoes. In all cases, the lowest temperature was found around point C. The lowest and highest residual enzyme activity, for both immobilized gel systems, after MCWC processing was found around point B and around point C, respectively, suggest the practicability of using TTI to monitor the microwave processes for this 915 MHz heating test system (Table 1 and Table 2). Figure 9, Figure 10 and Figure 11 show infrared thermal

images of all food tested after MCWC processing. Computer analysis (ThermaCAM™ Researcher 2001, Flir Systems, Danderyd, Sweden) of the infrared pictures found similar temperature lectures compared with the lectures of the fiber optics sensors.

Food system	Dielectric constant (2450 MHz)	Position	% Relative activity	Range % relative activity	Power (Watts)
Ground meat	20 C = 28.80	A	53.30	47.28-57.85	3.30
(beef, 30% fat)	70 C = 25.50	B	14.43	10.71-17.03	3.36
		C	70.19	67.84-72.83	2.95
Ground shrimp	20 C = 58.00	A	12.92	6.31-17.18	3.71
	70 C = 51.09	B	4.91	4.99 - 6.23	3.75
		C	26.67	24.81-29.80	3.45
Mashed potatoes	20 C = 53.82	A	27.89	22.32-32.15	3.96
	70 C = 50.94	B	29.22	24.08-34.36	3.86
		C	52.47	51.83-53.17	3.46

Table 1. Percent of residual immobilized α-amylase activity after MCWC processing (N=3).

Food system	Dielectric constant (2450 MHz)	Position	% Relative activity	Range % relative activity	Power (Watts)
Ground meat	20 C = 28.80	A	88.23	89.97 - 86.71	3.30
(beef, 30% fat)	70 C = 25.50	B	68.92	69.79 - 68.48	3.36
		C	93.92	90.62 - 96.35	2.95
Ground shrimp	20 C = 58.00	A	55.63	45.96 - 57.42	3.71
	70 C = 51.09	B	49.60	45. 57- 51.04	3.75
		C	62.48	64.19 - 60.67	3.45
Mashed potatoes	20 C = 53.82	A	62.02	59.24 - 66.27	3.96
	70 C = 50.94	B	63.75	58.98 - 66.92	3.86
		C	84.59	82.03 - 92.83	3.46

Table 2. Percent of residual immobilized phytase activity after MCWC processing (N=3).
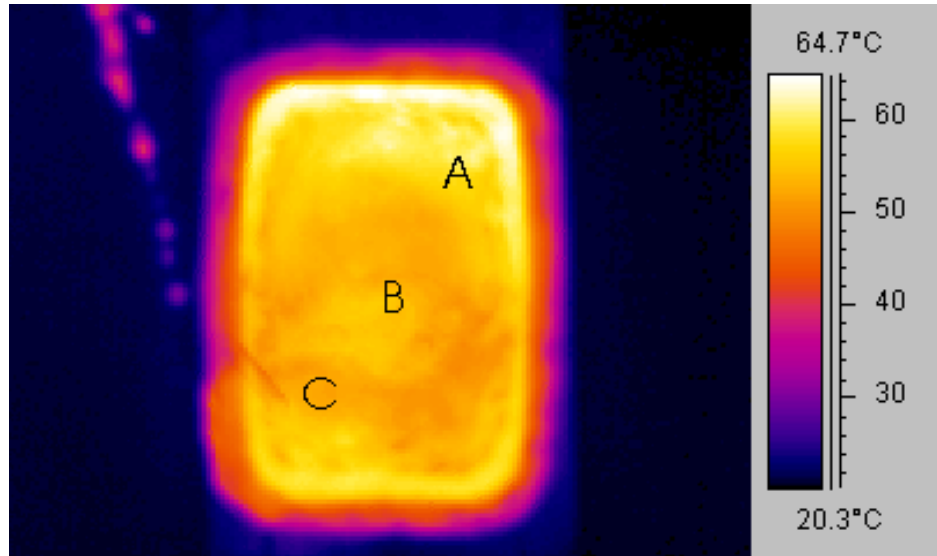
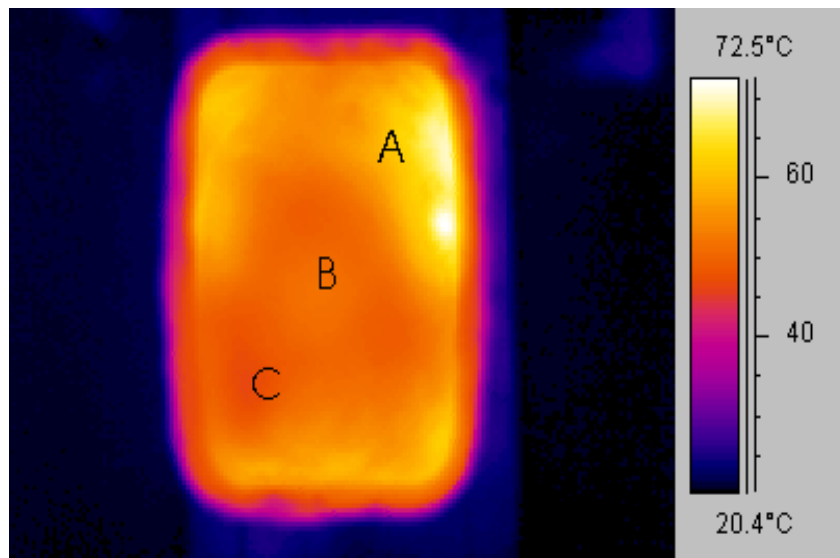**Figure 6. Infrared thermal image of ground meat (beef, 30% fat) after MCWC processing.**

Figure 7. Infrared thermal image of ground shrimp after MCWC processing.
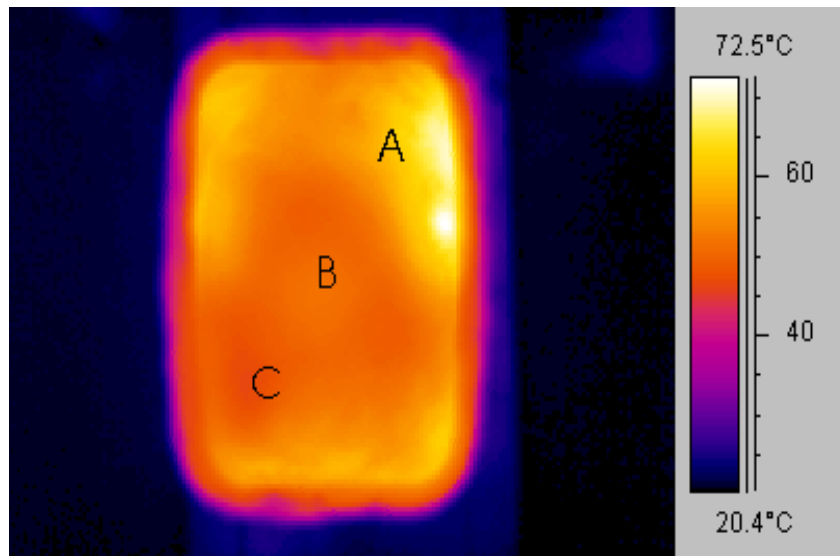
Figure 8. Infrared thermal image of mashed potatoes after MCWC processing.

This research demonstrated that the predict temperature in microwave heating using α-amylase TTI or phytase TTI is sensitive and can be used to determine the location of "cold and hot spots" in microwave heated foods. The method could be applied for predicting a temperature distribution in other foods with similar dielectric properties and containers with similar dimensions. With this inexpensive and simple TTI method it is possible to achieve multi-point measurement, without the use of optical fiber optics which are expensive (US $200.00 each) and can be broken during processing.

Process lethality

Table 3 showed process lethality values for each pathogen under all conditions tested. Higher process lethality was found for *S. typhimurium* and *E. coli* O157:H7 compared to *L. monocytogenes* under all conditions tested. The Ball's equation could not be used to calculated lethality at point C, because these temperatures were less than T_0, however TTIs

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inactivation suggest that some microbial inactivation occurs at point C, although it is difficult to calculate the amount based on the available data. The highest calculated process lethality was at point B for all pathogens in ground meat (beef, 30% fat) ($T > T_0$). More experiments will be needed in order to adequately evaluate process lethality nd to correlate microbial inactivation with residual acticity in the TTIs.

Food system	Position	F_0 (min)		
		E. coli O157:H7	*S. typhimurium*	*L. monocytogenes*
Mashed potatoes	A	5.08	5.05	4.39
	B	0.60	0.86	0.79
	C	0.00	0.00	0.00
Ground meat (beef, 30% fat)	A	3.08	2.92	2.47
	B	89.60	71.13	35.92
	C	0.00	0.00	0.00
Ground shrimp	A	0.06	0.06	0.04
	B	0.35	0.36	0.25
	C	0.00	0.00	0.00

Table 3. Process lethality (F_0) for *Salmonella typhimurium*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* after MCWC processing.

CONCLUSIONS

A simple assay of immobilized enzymes in polyacrylamide gel is one possible way for mapping heat distribution. *Aspergillus oryzae* α-amylase and *Aspergillus ficuum* phytase immobilized in polyacrylamide gel are two effective and applicable time temperature indicators for mapping heat distribution during MCWC processing. Both time-temperature indicators can be used to determine heat distribution during microwave heating when the direct measurement is impractical or costly. The assays for these particular TTI provides a fast, relatively accurate, inexpensive and simple approach, which could be implemented in industrial settings.

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CHAPTER 7

OVERALL CONCLUSIONS AND FUTURE WORK

This research was conducted to evaluate the feasibility of using immobilized enzymes as time-temperature indicators (TTI) for validating pasteurization processes and mapping the cumulative effect of time and temperature during the processing of ground meat (beef, 30% fat), ground shrimp and mashed potatoes using a 915 MHz Microwave Circulated Water Combination (MCWC) heating system. Two enzyme based time-temperature indicator (TTI) were developed using *Aspergillus oryzae* α-amylase and *Aspergillus ficuum* phytase immobilized in 20% polyacrylamide gel. The entrapment of both enzymes in polyacrylamide gel enhanced enzyme thermal stability.

Both enzyme based time temperature indicators maintained high recovery of enzyme activity, long term of storage stability, and compatibility with microwave and RF pasteurization. The dielectric properties of the TTI can be altered to match the food by adding salt.

Thermal inactivation of both TTI in buffer system, ground meat (beef, 30% fat), ground shrimp and mashed potatoes were first order with z- values falling within the recommended range for pathogen inactivation. D- values ranged greatly depending largely upon characteristics of the food matrices. The thermal inactivation of the TTI and reduction of pathogens in tests foods could be correlated and predictive equations can be established; therefore TTI can provide a simple, fast and relatively precise method for validating pasteurization processes. One of the most important outcomes is the potential to reduce routine microbial testing.

Temperature distribution profiles obtained with direct optical fiber temperature measurements within all foods tested subjected to microwave heating could be correlated with residual enzyme activity. Therefore, both time-temperature indicators can be used to map heat distribution during microwave heating when the direct measurement is impractical or costly. However, it is worthwhile to explore the thermal inactivation kinetics of the enzymes during microwave heating and try to compare them with the inactivation kinetics of the microbes under similar treatments.

Under certain conditions, RF imparts more uniform heating compared to microwave dielectric heating. Therefore, the use of time-temperature indicators during RF heating could be examined to map heat distribution and evaluate the effectiveness of RF processes for microbial control in foods.

The assays for these enzymes are straight forward and simple, but it would be worth examining whether it is possible to simplify the assays further, or to develop a way to incorporate color change features into the TTI itself.