Inactivation of bacterial endospores by application of Pulsed Electric Fields (PEF) in combination with thermal energy

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Zusammenfassung

Die Anwendung von gepulsten elektrischen Feldern (PEF) ist im Bereich Inaktivierung vegetativer Mikroorganismen zur schonenden Haltbarmachung von hitzeempfindlichen Produkten wissenschaftlich validiert. Eine deutlich intensivere Behandlung ist bei Produkten, die mit bakteriellen Endosporen kontaminiert sind, nötig. Das Ziel dieser Studie ist eine Inaktivierung von bakteriellen Endosporen mit geringerer thermischer Produktbelastung durch den Einsatz von PEF in Kombination mit thermischer Energie zu erreichen. Der Einfluss von verschiedenen Prozess (elektrische Feldstärke, Temperatur und spezifischer Energieeintrag) und Produkt (pH, Zucker-, Ionen- und Fettkonzentration) Parametern auf die Inaktivierung von *B. subtilis*, *A. acidoterrestris* und *G. stearothermophilus* Sporen wurde untersucht. Es wurde ein mathematisches Modell basierend auf einer Schulterformation mit anschließendem linearen Inaktivierungsverlauf angewendet, um den Einfluss der PEF Bedingungen und Produktparameter bewerten zu können. Im Bereich Prozessparameter zeigte die Eingangstemperatur den größten Einfluss auf die zur Inaktivierung benötigte Energie. Die Zucker- und Fettgehalte von maximal 10 °Bx bzw. 10 % Fett zeigten keinen Effekt auf die Inaktivierung. Geringere Energieeinträge konnten bei geringen Salzkonzentrationen (1 mS/cm, pH 4) eingesetzt werden. Der Einfluss der Temperatur auf die Inaktivierung wurde durch eine Kombination aus sporenspezifischen thermischen Inaktivierungskinetiken und des Temperatur Zeit Profils des PEF Prozesses modelliert. Das Modell ermöglichte die Trennung der Gesamtinaktivierung in thermische und PEF induzierte Inaktivierung. Es zeigte sich ein Anteil thermische Inaktivierung von *B. subtilis* und *A. acidoterrestris* Sporen, jedoch hatte die PEF induzierte Inaktivierung den größeren Anteil. Aufgrund der hohen thermischen Resistenz der *G. stearothermophilus* Sporen, ist die Gesamtinaktivierung größtenteils durch PEF induziert.

Der Wirkmechanismus der PEF induzierten Inaktivierung konnte nicht eindeutig geklärt werden. Mikroskopische Aufnahmen und DPA Messungen nach der PEF Behandlung zeigten einen sich zur thermischen Behandlung unterscheidenden Mechanismus. Durch den schnellen Temperaturanstieg und das elektrische Feld wurden Strukturveränderungen induziert, welche ein Auslaufen der Sporen bewirken könnten.

Die Validierung des entwickelten PEF Prozesses wurde durch Fallstudien analysiert. Der Karottensaft und das Hefeextrakt zeigten eine nach einer thermischen Behandlung vergleichbare Haltbarkeit mit höherer Produktqualität.

Die Ergebnisse dieser Studie zeigen die Anwendung von PEF zur Inaktivierung bakterieller Endosporen bei geringeren Temperaturen. Somit können qualitativ hochwertige und mikrobiologisch sichere Produkte hergestellt werden.

Abstract

Pulsed Electric Fields (PEF) technology is widely studied in literature for the inactivation of vegetative microorganisms and offers a gentle preservation for heat sensitive products. Some products might be contaminated with bacterial endospores and require a more severe treatment for inactivation. The objective of this study was the inactivation of bacterial endospores by application of PEF with a lower heat load compared to thermal sterilization. Therefore, the PEF process was combined with thermal energy. The influence of different process (electric field strength, temperature and specific energy) and product (pH, sugar-, ion- and fat concentration) parameters on the inactivation of *B. subtilis*, *A. acidoterrestris* and *G. stearothermophilus* spores were analyzed. From experimental data, a model based on shoulder followed by linear distribution was applied. This model was used for evaluating the effect of PEF conditions and product parameters on the resistance of spores. As a result, the inlet temperature was identified as the most influencing process parameter. No effect of sugar and fat on the energy required for inactivation was obtained in the studied range from a maximum of 10 °Bx and 10 % fat. Low specific energy values were obtained at low salt concentrations (1 mS/cm) and pH 4. The impact of temperature on the inactivation by the developed process was modeled by combination of thermal inactivation kinetics of each spore type and the related temperature time profile of the PEF process. The model allowed a separation of total inactivation in thermal and PEF induced inactivation. The separation indicated a thermal inactivation rate for *B. subtilis* and *A. acidoterrestris* spores, but the PEF induced inactivation had a higher part. Based on the high thermal resistance of *G. stearothermophilus* spores, the obtained total inactivation was mainly based on PEF.

The PEF induced inactivation of spores cannot be clearly explained as the mechanism of action is unclear. Microscopic studies and measurements of DPA after PEF treatment demonstrate a different mechanism compared to thermal inactivation. Due to the fast temperature increase and the electric field, changes in structure were induced, which might cause a leakage of spore content. The validation of the developed PEF process was analyzed by performance of case studies. Therefore, yeast extract and carrot juice were PEF treated and the microbial safety as well as quality were studied over shelf life. In both cases, the obtained shelf life was comparable to the thermally treated product, but a higher quality could be achieved.

The presented data in this study demonstrates the possibility of inactivation of bacterial endospores at lower temperature than thermal sterilization. Therefore, the developed process can be used to produce products with a higher quality and ensure microbial safety.

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List of Abbreviations

AAE	Ascorbic acid equivalence [mg/l]
A_f	Accuracy factor [-]
a_w	Water activity [-]
A_0	Pre factor [-]
B_f	Bias factor [-]
C	Capacity [F]
CLSM	Confocal laser scanning microscopy
DPA	Dipicolinic acid
E	Electric field strength [kV/cm]
E_a	Activation energy [kJ/mol]
E_c	Critical electric field strength [kV/cm]
ΔE	Color difference [-]
F	Fat concentration [%]
F_{PEF}	Thermal death time for PEF process [min]
$F_{thermal}$	Thermal death time for thermal process [min]
GAE	Gallic acid equivalence [mg/l]
I	Current [A]
N	Spore concentration after treatment [spo/ml]
N_0	Initial spore concentration [spo/ml]
PA	Peak area [-]
PEF	Pulsed Electric Fields
PME	Polymethylesterase
R	Relative gas constant (8.314 J/(mol K))
RT	Ambient temperature [°C]
S	Sugar concentration [%]
SEM	Scanning electron microscopy
Sl	Shoulder length [kJ/kg]
T	Temperature [°C]
T_{in}	Inlet temperature [°C]
T_{out}	Outlet temperature [°C]
T_R	Reference temperature [°C]
T(t)	Temperature time profile
TPC	Total plate count [cfu/ml]
U	Voltage [V]
W	Specific energy input [kJ/kg]
W_{pulse}	Energy per pulse [J/pulse]
c_p	Specific heat capacity [kJ/(kg K)]
k	Inactivation rate
k_{max}	Specific inactivation rate [$(kJ/kg)^{-1}$]
n	Proportionality factor
t	Time [s]
t_0	Heating up time [s]
x_1	Experimentally observed data
x_2	Predicted data
κ(T)	Conductivity [mS/cm]

1. Introduction

The safety of food products is essential for prevention of foodborne illness. Many worldwide outbreaks cause illness and deaths. The most recent outbreak in Germany was reported to be in 2011 caused by an enteroaggregative *Escherichia coli* O104:H4. A contamination of fresh vegetables was the source of the infection causing 3950 people to become ill of which 53 died (European Food Safety Authority 2011).

To guarantee and ensure the safety of food products, preservation techniques are required. Therefore, thermal processes are widely used. Depending on the desired shelf life and product conditions, such as pH or level of contamination, a temperature time regime is applied. Due to the heat load, health promoting ingredients, such as vitamins, are, depending on the temperature destroyed. Moreover, the taste changes, which is mostly described by a cooked flavor.

A gentle preservation could be achieved by application of novel technologies. Pulsed electric fields (PEF) technology has been known since the beginning of the past century. The first applications of PEF have been described by Doevenspeck (1960) and Flaumenbaum (1968) for disintegration of biological material and first studies on inactivation of microorganisms were published in 1968 by Sale and Hamilton. Since then, the research on application of PEF increases and more than 25 research groups are working worldwide in this field (Barbosa-Cánovas et al. 1999). The first commercial applications were found for preservation of premium juices and smoothies (Clark 2006, Irving 2012), for disintegration of fruit mash prior to pressing (Jaeger et al. 2012) and of potatoes prior to fries production (Toepfl 2012).

Many studies focused on inactivation of vegetative microorganisms in order to achieve a certain shelf life of the product without affecting or minimally affecting the food quality (Barbosa-Cánovas et al. 1999). Only a few studies have been published demonstrating the ability of PEF for inactivation of bacterial endospores. Moreover, controversial results were obtained indicating no spore inactivation by PEF (Hamilton et al. 1968; Knorr et al. 1994; Pagán et al. 1998; Pol et al. 2001; Cserhalmi et al. 2002; Shin et al. 2008) and spore reduction by PEF (Yonemoto et al. 1993; Jin 2001; Qin 2001; Uemura et al. 2003; Marquez et al. 2007; Bermúdez-Aguirre et al. 2012; Reineke et al. 2013). The main reason for the different results is based on the different PEF equipment and different applied processing parameters. The inactivation of spores is relevant for low acid products and products containing ingredients, which have been in contact with soil. Examples of these kinds of food products are vegetable juices or soups. Because of potential pathogenicity and the related risk to human health, an inactivation of spores is essential. Spores are formed at conditions, which are unfavorable for the multiplication and proliferation of the microorganism (Christie 2012). After the sporulation process, the spores can survive for decades without any nutrients (Nicholson

et al. 2000) and are highly resistant to extreme environmental conditions, such as extreme pH or heat (Setlow et al. 2006). For inactivation of spores, the high resistance plays a key role in order to require a more severe treatment than vegetative microorganisms. Spores show a high resistance to chemical, radiation or heat treatment (Setlow et al. 2006). Due to this resistance, it is not possible to inactivate spores by PEF applying the same conditions used for inactivation of vegetative microorganisms. Some researchers described the use of high electric field strength up to more than 30 kV/cm as the main requirement for spore inactivation by PEF (Hamilton et al. 1968). Others combined PEF with a higher inlet temperature of 60 to 70 °C to achieve a reduction in spore count (Qin 2001, Bermúdez-Aguirre et al. 2012; Reineke et al. 2013). However, no systematic study analyzing the influence of different process and product parameters was performed on inactivation of bacterial endospores by PEF.

The aim of the study presented is detection of inactivation kinetics of various spore types by PEF in combination with thermal energy. Three spore types were chosen based on their resistance and occurrence in food products; *B. subtilis*, *A. acidoterrestris* and *G. stearothermophilus*. Various experiments at different conditions were performed from which a predictive model was developed. Those mathematical expressions were useful to compare the resistance among different strains and evaluate the influence of different process parameters in the inactivation of bacterial endospores. The use of isolog graphs according to the inactivation predicted by the models in a range of conditions is a very useful tool to study the resistance of spores and to set the correct process parameters for a PEF process. Due to the combination of PEF with thermal energy, the impact of temperature on inactivation has to be considered. The development of a mathematical model describing the thermal inactivation kinetics of the spores used in this study permitted to differentiate between the thermal inactivation due to the thermal load during the PEF treatment and the PEF induced inactivation. The principle of thermal inactivation of spores is mainly based on protein denaturation and enzyme inactivation (Coleman et al. 2007). Whereas the mechanism of inactivation of vegetative microorganisms by PEF is termed as electroporation and is widely accepted (Saulis et al. 2010), the principle of spore inactivation by PEF is unclear. Only a few studies analyzed PEF treated spores by microscopic techniques, with result of a different appearance of PEF treated spores compared to thermal treatment (Jin 2001). Within this study, microscopic analysis and inactivation of decoated *B. subtilis* mutant were used to confirm the already published data and to have an approach which components are involved in inactivation mechanism.

The industrial applicability of the developed process was tested by treatment of yeast extract and carrot juice. In each study a safety validation, shelf life and analysis of important quality parame-

ters were performed with a thermal treatment as a reference. Based on these results, the implementation of the developed process in industry is discussed with regards to scale – up capability, implementation and existing HACCP concept and costs analysis.

2. Literature review

2.1 Bacterial endospores and their importance in food products

Bacterial endospores are of high importance because of the potential pathogenicity and therefore risk for the human health. The main difference between vegetative microorganisms and spores is their resistance to environmental parameters, such as heat or extreme pH values, which is based on the structural composition. Due to this high resistance of spores, they can survive over a long period of time. A recovery of spores from environmental samples, from hundreds to thousands of years ago, was observed (Nicholson et al. 2000). Spores can also be found in all parts of the earth's surface and subsurface as well as in soil and regolith surfaces of rocks (Nicholson et al. 2000). Possible contamination of food products comes from ingredients. These ingredients have been in direct or indirect contact with soil. Due to the high resistance of spores, they can survive for a long time. They show a high resistance to inactivation procedures. Normal pasteurization conditions are never sufficient to inactivate spores. Mainly strains from the genus *Bacillus* are able to form endospores. The genus *Bacillus* is separated in different strains in accordance to similarities in 16S rRNA sequencing. In general, *Bacillus* are rod-shaped, aerobic and facultative anaerobic, as well as Gram-positive bacteria with the ability to form spores. They have a higher heat resistance compared to the vegetative form (Nazina et al. 2001). Owing to their intrinsic resistance, spores are the perfect vehicles for infections. In the past, spores have been shown to be the cause of diseases, sometimes leading to fatalities.

One example for such a disease is botulism. The disease is caused by *Clostridium botulinum* and is of high interest. The so called botulinum toxin produced by *Clostridium botulinum* produces a distinct clinical syndrome of symmetrical cranial nerve paralysis of the voluntary muscles (Sobel 2005). First cases were reported in Germany in the 18th century related to the consumption of raw meat or blood sausage (Erbguth 2004). According to the records, in 1793 the government sent a warning not to consume blood sausages. The medical officer Justinus Kerner collected all cases and published them including a complete clinical description. Kerner found that the disease was related to a toxin, which he called "sausage poison" and termed the disease as Botulism. In 1895, 4000 inhabitants in a Belgian village were infected by botulism. Emile Pierre Marie Van Ermengen

studied the cases and was able to isolate *Bacillus botulinus*. This was later named *Clostridium botulinum*. Since then, it is known, that Botulism is related to the presence of *Clostridium botulinum*, which demonstrates how important preservation of food is in terms of the prevention of food poisoning.

Another disease caused by infection with spores is anthrax. In the 7th and 19th centuries, anthrax epizootics were responsible for devastating domestic livestock losses in Europe. In 1769 the researcher Nicolas Fournier separated the disease into two types—"spontaneous" and "contagious." "Spontaneous" is sporadic cutaneous anthrax. "Contagious" includes the internal variety beside a single cutaneous legion and is often fatal. The infection occurs after touching or eating animal products. In 1881 Louis Pasteur published 5 live attenuated anthrax vaccines (Torred 2012). Three clinical presentations of anthrax caused by *Bacillus anthracis* in humans are known so far. They are cutaneous, orogastric and inhalational anthrax (Spencer 2003). Typical symptoms of cutaneous anthrax are edema with black eschar. The orogastric anthrax causes severe throat sores or ulcers in the oropharyngeal cavity associated with neck swelling, fever, toxicity and dysphagia. At the beginning of inhalational anthrax, symptoms of malaise, fever and non-productive cough are seen. These progresses to diaphoresis, cyanosis and chest pain are observed after 2 to 3 days (Friedlander 1999). Due to various routes of exposure, the minimum infection dose cannot be precisely stated. But it is know that only a small number of spores are required to initiate a cutaneous anthrax infection (WHO 2008).

These different diseases indicate the importance of inactivation of spores in food products. The target strains of this study come from three different Bacillus genus:

Bacillus subtilis
Geobacillus stearothermophilus
Alicyclobacillus acidoterrestris

The first Bacillus discovered was *Vibrio subtilis* in 1835 by Ehrenberg. In 1872 the strain was renamed *Bacillus subtilis* by Cohn (Gibson 1944).

In 1920 a thermoresistant strain was discovered in samples of "Standard Maine Style" corn, which was thermally treated at 118 °C for 75 min. The same microorganisms were found in spoiled cans of string beans and corn on the cob. The microorganisms were named *Bacillus stearothermophilus* (Donk 1920). 16S rRNA analysis performed by Nazina et al. (2001) indicated similarities between some bacilli species. These species were then named Geobacillus.

Cerny et al. (1984) published the occurrence of a thermo acidophilic spore forming bacteria in apple juice. The apple juice presented an off flavor and turbidity. After isolation of the microorganisms, a strain was obtained. The strain could grow in a pH range from 2.5 to 5.5 and at temperatures from 40 to 50 °C. The specific microorganism was identified as *Bacillus acidocaldarius* DSM 2498, because of its ability to form spores and the presence of ω-cyclohexane and hopanoids in the cell membrane. In 1971 and 1973 de Rosa et al. analyzed the thermoresistance of *Bacillus acdiocaldarius* with regard to the substance in the cell membrane. ω-cyclohexane and hopanoids were found in this bacilli, which was unique for this strain. At the same time, the researcher Hippchen et al. (1981) analyzed different soil samples. He found different bacilli strains, such as *Bacillus coagulans* and *Bacillus acidocaldarius*. One strain could not be identified. The presence of ω-cyclohexane, a strong relation to *Bacillus acidocaldarius* was assumed. Later in 1987, Deinhard et al. (1987) analyzed the strains discovered by Hippchen et al. (1981) and Cerny et al. (1984) and determined through various physiological and biochemical tests that the strains were not *Bacillus acidocaldarius.* The guanine and cytosine content of *Bacillus acidocaldarius* is higher than the discovered strain. Moreover, different growth temperatures for both strains were observed. The discovered strain was termed *Bacillus acidoterrestris*. Wisotzkey et al. (1992) performed a comparative 16S rRNA sequence analysis, which was the most appropriate method to determine taxonomic relations. As a result, sufficient differences were found between *Bacillus acidocaldarius* and *Bacillus acidoterrestris* compared to *Bacillus stearothermophilus* and *Bacillus coagulans*. The strains delivering equal results in 16S rRNA analysis were termed *Alicyclobacillus*. The classification is listed in Table 2-1.

Table 2-1: Classification of *Alicyclobacillus*, *Bacillus* and *Geobacillus.*

	Alicyclobacillus	Bacillus	Geobacillus
Kingdom	*Bacteria*	*Bacteria*	*Bacteria*
Class	*Bacilli*	*Bacilli*	*Bacilli*
Order	*Bacillales*	*Bacillales*	*Bacillales*
Family	*Alicyclobacillacea*	*Bacillacea*	*Geobacillacea*
Genus	*Alicyclobacillus*	*Bacillus*	*Geobacillus*

A. acidoterrestis is still of interest in juice producing industry (Spinelli et al. 2009), because of its high heat resistance and ability to grow in an acidic environment. The spore can be found in passion fruit (McKnight et al. 2010), orange (Pettipher et al. 1997) or apple juice (Pettipher et al. 1997). The main explanation for contamination is that the bacteria are coming from the surface of the fruit which has had contact with the soil during production or harvesting (Groenewald et al. 2009; McKnight et al. 2010). The problem of *A. acidoterrestris* contamination is the high heat resistance and the ability to grow in an acidic media. This can be explained by the presence of ω-

alicyclic fatty acid in the membrane (Yamazaki et al. 1996). A contamination can be detected by an off odor, which occurs at high concentrations of > 10^6 cfu/ml (Pettipher et al. 1997).

2.1.1 Sporulation process

The bacterial replicative growth depends strongly on the presence of nutritional substances. In the case of less nutrition available, the cell´s first priority is to express functions to scavenge alternative nutrients in order to compete with other species for scarce nutrients. In the case of starvation for carbon and/or nitrogen, the cells initiate the sporulation process as a survival pathway of last resort. This allows a survival over a long time without any nutrition. Due to high energy consumption, sporulation is avoided under mild conditions and if it is initiated, it is irreversible. Intensive research has been done and many reviews written on the sporulation procedure of *B. subtilis* (Errington et al. 1993; Phillips et al. 2002; Duerre et al. 2004; Piggot et al. 2004; Robleto 2012) (Figure 2-1).

Generally the sporulation is separated in 7 parts controlled by 5 distinct sigma factors (σ). They bind directly to RNA polymerase and direct it to transcribe only from specific promoters (Driks 1999).

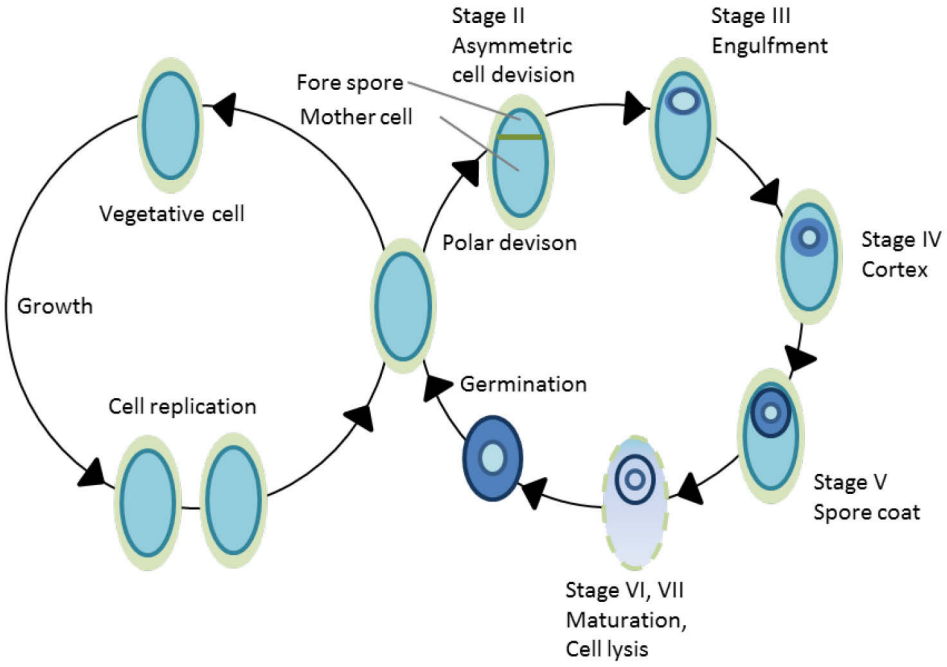

Figure 2-1: Schematic overview sporulation process (adapted from Errington (1993)).

The sporulation is initiated by different inputs driven by nutritional factors, population density and natural cell cycle (Errington 1993, 2003). The initiation (stage I) includes DNA coiling along the central axis of the cell and is controlled by a complex assembling of protein kinases, phosphorylated proteins and phosphatases. The complex Spo0A is phosphorylated to Spo0A-P (Philipps et al. 2002). In this state, the complex is able to bind to the DNA affecting transcription of 121 genes (Robleto et al. 2012). The σ^H is expressed as a response to the binding and promotes together with Spo0A the development of the asymmetric septum. This is the second sporulation stage (stage II). Within this stage, the cell is separated into 2 parts - a small part termed fore spore and a larger part called mother cell. The polar septation starts before the chromosome segregation is completed. Temporarily, a small portion of chromosome is trapped in the fore spore compartment. After a short period, the remaining DNA is pumped across the septum into the fore spore by DNA translocase activity (Phillips et al. 2002). After septation, a temporal cascade of sigma factors starts. The σ^F activates the transcription of 48 genes to facilitate different functions in the fore spore compartment. The expressed genes are involved in regulation of DNA binding, synthesis of germination receptors, detoxification, DNA repair and communication between the mother cell and the fore spore. Moreover, the σ^F mediates the engulfment, which means degradation of wall material at the center of the septum (stage III) (Robleto et al. 2012). At the same time, σ^F activates σ^E which is only active in the mother cell. Around 260 genes are expressed influencing the engulfment, coat and cortex formation as well as the mother cell metabolism. The genes mediate the degradation of the peptidoglycan layer between the mother cell and the fore spore starting at the midpoint of the septum and subsequently move towards the edges of the septum. At the same time, the mother cell is growing around the fore spore and synthesising the cortex (stage IV), the fore spore loses water and ions prompting a volume and simultaneous pH decrease. The water loss is a response to cation uptake including calcium-, magnesium- and manganese ions as well as pyridine-2,6-dicarboxylic acid (DPA). These were all synthesized in the mother cell. The formation of DPA in the mother cell is driven by the activation of σ^K by σ^E (Piggot et al. 2004). Beside the DPA synthesis, σ^K expresses 144 genes mediating the final steps (stage VII) in spore formation. These are formation of spore coat proteins, mother cell lysis and spore release. Furthermore, σ^K activates *spoVA* transferring the in the mother cell synthetized DPA to the fore spore (stage V) (Robleto et al. 2012). When the engulfment is finalized, the σ^F active in the fore spore triggers the activation of σ^G expressing 90 genes and mediating continuous morphogenesis of spore cortex, resistance properties and germination functions. The resistance of the spore is related to DNA protection, which is accomplished by the synthesis of small acid-soluble proteins (SASPs). The fore spore nucleoid is remodeled into a ring due to the SASPs.

The active σ^K induces the last steps of an 8 to 10 hours sporulation process, mainly mother cell lysis and spore release. The spores have no metabolism and are able to survive over a long period of time including extreme environmental conditions (Robleto et al. 2012).

2.1.2 Germination of spores

Spores are able to survive over a long period of time without any metabolism, but the environment is continually monitored for available nutrients to germinate. This process starts the exit of the dormancy stage and transforms the spores into vegetative cells. The process from dormant spore to replicable vegetative cells is termed germination and includes a series of biophysical and degrading reactions leading to the loss of high resistance to extreme environmental conditions (Figure 2-2). As well as the sporulation, the germination process for *Bacillus* is well described in literature and is summarized below (Moir et al. 2002, Setlow et al. 2003, Moir 2006, Paredes-Sabja et al. 2011, Christie 2012).

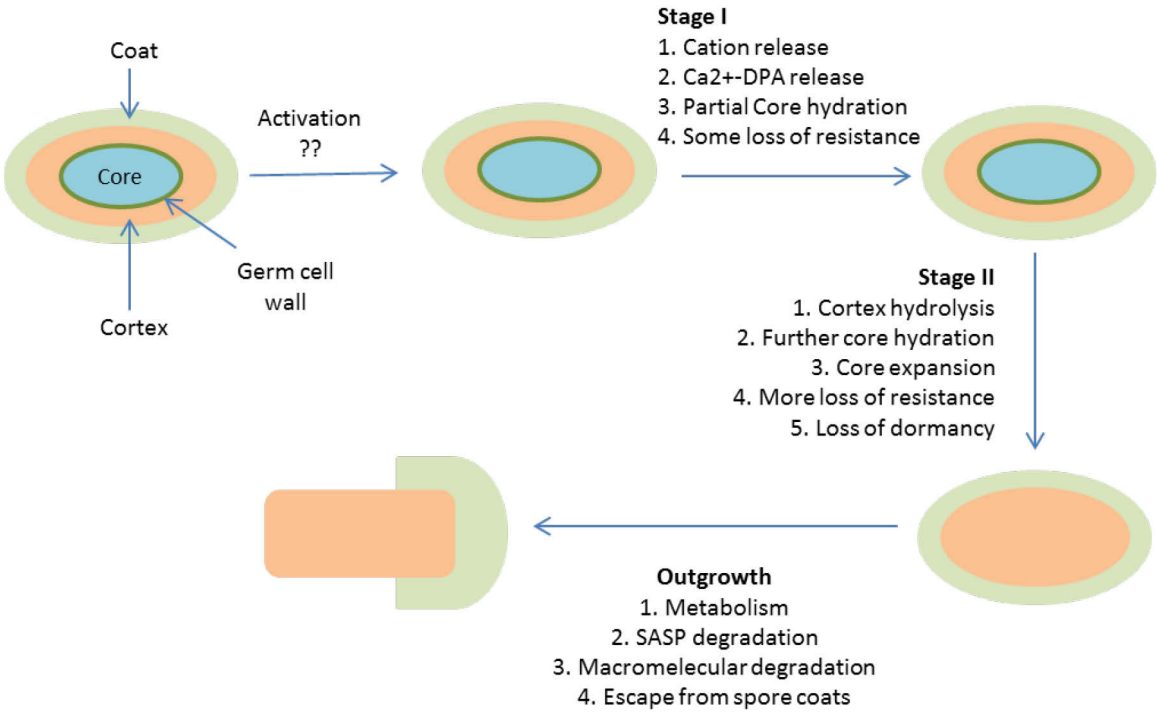

Figure 2-2: Steps in spore germination (adapted from Setlow et al. (2003)).

Generally, two different germination procedures were described in literature; nutrient and non-nutrient germination.

The nutrient induced pathway is triggered by so called germinants, which are substances of lower molecular weight, such as amino acids, ribosoids, sugar or ion binding to receptors (Christie 2012). The contact between receptor and germinant takes place for a few seconds, but is a sufficient indicator for resumption of vegetative growth. The interaction is poorly understood on a molecular level. The receptors are localized in the inner spore membrane. Therefore, the germinants have to traverse the protective layers (coat and cortex) to bind at the cognative receptor site (Christie 2012). The receptor proteins are encoded by tricistronic *gerA* operon and expressed in the fore spore during sporulation (Setlow et al. 2003). Each receptor is composed of three proteins with a potential to associate with the membrane. The A type consists of a large hydrophilic N-terminal domain. The B type is membrane associated and the C type is a lipoprotein; probably located on the outward facing aspect of the spore inner membrane. It is not clear how many receptor-ligand contacts have to occur to trigger the germination (Christie 2012). Thus, individual germination receptors can respond to a single germinant or an individual germination receptor or multiple germinants can respond to several germination receptors.

After germinant binding, which belongs to the first germination step, changes of permeability of the inner spore membrane are induced. As a result, selected ions (H^+, Na^+ and K^+) and other small molecules move out of the spore core, whereas water moves into the core (Christie 2012). Due to the ion movement, the pH of the core increases from 6.5 to 7.7. It is assumed that channels in the inner spore membrane allow the ion transport, but the exact mechanism and what kinds of proteins are involved is not clear. At that stage, the rehydration is not enough for any enzyme activity and the general protein mobility is restricted. Afterwards, a release of DPA is induced, predominately chelated with calcium ions, and other divalent metal ions. SpoVA proteins involved in the DPA transport from mother cell in fore spore during sporulation, are also active during germination and therefore they may be responsible for the DPA release. At the same time, the core is partly rehydrated with water resulting in a reduced heat resistance of the partly germinated spores. Due to the water uptake, the spore core volume increases in the range of 2 to 2.5 fold. Enzyme activity, initiation of metabolism, macromolecular synthesis as well as spore outgrowth is allowed.

Within the second germination stage, mainly initiated by Ca-DPA release, the spore specific structure (coat and cortex) are degraded by specific enzymes. The main enzymes involved in degradation of peptidoglycan cortex are CwlJ and SleB. For their action, muramic-S-lactam is required. This ensures that the spore´s germ cell wall, which is a thin layer of vegetative cell type peptidopgylcan, is not degraded and becomes the cell wall of the outgrowing spore. The enzyme CwlJ is responsible for cortex lysis as well as colony formation. It is synthesized in the mother cell during sporulation,

whereas the SleB as a transglycosylase is formed in the fore spore. CwlJ is located in the spore coat fraction and is removed by the decoating procedure. SleB can be found in the spore integuments, such as coat or cotex. Both enzymes were synthesized in mature during sporulation (Setlow et al. 2003). The mechanism to keep them inactivate in the dormant spore is unclear.
During the outgrowth phase, the SASPs, which protect the spore DNA, were degraded by specific proteases and allows a source of amino acids for outgrowth.

The non-nutrient germination can be categorized in a second physiological pathway and a non-physiological route.
A second physiological pathway is mediated by an eukaryotic-like serine/threonine kinase (PrkC). A peptidoglycan fragment binds to peptidoglycan domain located at the inner spore membrane and hence initiates the germination (Christie 2012).
The non-nutrient induced germination is mediated by lysozyme, Ca-DPA, cationic surfactants, such as dodecylamine, High Pressure Processing (HPP) or salts. If the coat is removed, the cortex is hydrolyzed by the lysozyme causing a germination-like rehydration of the spore core. The treatment with dodecylamine interferes with the inner spore membrane and causes a rapid Ca-DPA release leading to germination. The application of HPP stimulates the germination of spores (Reineke et al. 2013b). At pressure levels from 100 to 400 MPa and temperatures from 20 to 50 °C, the germination receptors are triggered and the nutrient induced germination pathway is started. The different germination receptors indicate different pressure resistance. At pressures higher than 500 MPa and temperatures of 25 °C, Ca-DPA channels are opened. These are most likely located in the inner spore membrane. This causes the release of DPA and degradation of cortex. No effect on the SASPs is detected, which is probably due to the inactivation of germination protease due to the HPP treatment (Wuytack et al. 1998).

2.1.3 Spore structure and important spore specific components

Some bacteria form a dormant cell, if the availability of nutrients becomes scarce. After sporulation, the dormant spore can survive for long time and can withstand chemical and physical agents as well as extreme environmental conditions. The reason for the high resistance is based on the spore architecture and on spore specific components.

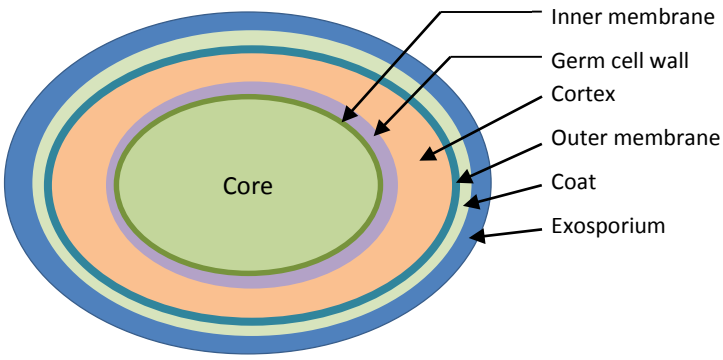

Figure 2-3: Schematic structure of a bacterial spore (adapted from Setlow (2003)).

The structure of a dormant cell (Figure 2-3) starting from the outside is exosporium, coat, outer membrane, cortex, germ cell wall, inner spore membrane and spore core.

The exosporium is a large loose-fitting structure mainly composed of proteins (Seltlow 2006) and cannot be found in each spore species. It is not attached to the spore and acts like a multilayered shell around the spore. In *B. cereus* spores, an exosporium composed of specific glycoproteins, was identified, whereas *B. subtilis* spores do not possess an exosporium (Driks 1999).

The spore coat can be regarded as multilayer with a proteinaceous structure, which varies for different species. It is mostly composed of tyrosine and cysteine rich proteins and small amounts of carbohydrates and lipids. In *B. subtilis* spores, two major coat layers were discovered and termed as inner and outer coat (Driks 1999). The coat is made of more than 50 different proteins of which most are spore specific gene products. Moreover, the coat represents the first barrier and is therefore very important for the first defense. It is found that the coat plays a key role in the resistance to chemicals, such as peptidoglycan lytic enzymes, such as lysozyme or predation by protozoa (Driks 1999). In contrast the coat is no barrier for small molecules. This is important for the germination, where small substances of small molecular weight traverse through the coat to bind to the germination receptors. Also, the outer membrane does not constitute a significant permeability barrier for small molecules.

The outer membrane surrounds the next layer, which is the spore cortex. The cortex has a similar structure to the peptidoglycan layer of vegetative cells, but with spore specific modifications (Popham 2002). The main difference is the replacement of around 50 % of peptide side chain muramic acid residues by muramic acid lactam in order to gain a higher flexibility (Popham et al. 1996). It serves as a retaining structure and can therefore withstand the turgor pressure generated by the high concentration of solutes in the core (Atrih et al. 1999, Meador-Patron et al. 2000). The germ

cell wall has a similar structure to that of vegetative cell wall peptidoglycan. Therefore, it constitutes the initial cell wall during spore outgrowth.

The inner spore membrane can be regarded as an analogue of a growing cell cytoplasmic membrane and protoplast. It is a strong permeability barrier and plays a key role in the high resistance of the spores. In the dormant status, the inner spore membrane is very compressed and expands during the germination procedure in the absence of ATP. Moreover, the inner spore membrane is important for nutrient induced germination, because the receptors are located in it (Nicholson et al. 2000).

The inner spore membrane protects the core, where the DNA, ribosomes, tRNA and spores enzymes are stored. Spore specific properties of the core are: low water content about 27 -55 % of wet weight, which causes the enzymatic dormancy and the high heat resistance, high mineral level, decreased spore core permeability and saturation of spore chromosome with SASPs. Due to the saturation of the DNA and therefore protection of the DNA backbone, the structure of the DNA changes. This is the main factor for UV-radiation resistance (Setlow et al. 1993). SASPs of the α/β type are extremely abundant and constitute 3 to 6 % of the total spore protein. They are highly preserved within and across the species, but were not found in non spore-forming species (Setlow et al. 2006). The SASPs are formed in the fore spore and encoded by the sspC gene during late sporulation (Carillo-Martinez et al. 1994) and degraded within minutes of spore germination (Fairhead et al. 1994).

Another important substance in the core increasing the resistance of bacterial spores is pyridine-2,6-dicarboxylic acid (DPA). Around 5 to 15 % of the dry weight of spores consists of DPA. Proteins located in the inner spore membrane are encoded by the *spoVA* operon, which is proposed to be involved in the DPA uptake in the fore spore. DPA is 1:1 chelated with calcium and the levels of Ca-DPA varies among the individual spore in a population. The accumulation of DPA causes a reduction of core water content, which is important for the wet heat resistance (Pedraza-Reyes et al. 2012).

2.1.4 Resistance of bacterial endospores

The inactivation of bacterial endospores is required for preservation of many food products. Different defense mechanisms are active in spores to survive at different extreme conditions, such as high temperatures or chemicals. The most important factor for this resistance is based on the spore structure and spore specific components. This chapter describes the defense mechanism against radiation, chemicals and heat.

Compared to vegetative bacteria, spores are 10 to 50 times more resistant to UV radiation at 254 nm (Nicholson et al. 2000). The high resistance can be explained by the novel photochemistry of the spore and the spore DNA repair system. The UV photoproducts of vegetative cells are mainly cyclobutane dimers and (6-4) photoproducts, which have a massive lethal effect on the cells. Due to the saturation of spore DNA with α/β type SASPs, the DNA structure is changed and, therefore the UV photochemistry is changed as well. In spores, the unique photoproduct 5-thyminyl-5,6-dihydrothymine is formed with less lethal properties. During outgrowth, the thymidyl-thimidine adduct is repaired by a photoproduct, namely lyase, which monomerizes the photoproduct (Setlow et al. 2006). Moreover, some spore types have UV-absorbing pigments on the outer layer to shield the UV-sensitive spore components (Nicholson et al. 2005).
Also, a high resistance of spores to γ-radiation is observed. The reason for the high resistance is not clear yet, but there is some evidence that the low core water content is important. Because of the low water content, the amount of hydroxyl radical formation by γ-radiation is reduced. So far, α/β type SASPs and DPA are not involved in γ-radiation resistance of spores (Nicholson et al. 2000; Setlow et al. 2006).

In the area of disinfection, the resistance of bacterial spores to chemicals plays an important role. Spores show an increased resistance to various chemicals, like acids, bases, oxidizing agents, aldehydes, phenols or organic solvents. The activity and therefore the sporicidal effect strongly depend on the environmental conditions such as temperature or concentration (Russel 1990). Many studies have been done studying the influence of different chemicals on spore inactivation and investigating these mechanisms (Russel 1990; Nicholson et al. 2000; Setlow 2006). The main components responsible for chemical resistance are (Nicholson et al. 2000):

- Spore coat
- Spore inner membrane
- DNA saturation by SASPs
- Low spore core water content

The spore coat protects the spore against large molecules, such as chlorine dioxide or hypochlorite (Setlow et al. 2000), but for some chemicals the coat plays only a minor role. So far, no specific coat proteins have been identified as being responsible for the chemical resistance. It is assumed that it detoxifies the chemicals before penetrating through the coat into the layers (Setlow et al. 2006). The main characteristic of spore inner membrane is the impermeability for molecules larger than 200 Da and the slow uptake of molecules passing the membrane. Several chemicals cannot pass this barrier because of their size. If small chemicals pass the membrane, they lose toxicity

due to the slow pass through. The DNA located in the spore core is protected against chemicals, such as formaldehyde or peroxidase, by SASPs by binding directly to the DNA (Nicholson et al. 2000; Setlow et al. 2000). For sporicidal or sporostatic activity of chemicals, water is required, because of the solubility in water and most reactions were carried out in water (Russel 1990). Due to the low spore core water content, a deceleration of the toxic reaction occurres resulting in an increased chemical resistance. However, the chemical resistance is complex, because of the complexity of different chemical reagents and the reaction of the spore. No general explanation exists describing the chemical resistance.

A markedly high heat resistance compared to vegetative cells is observed for spores. *B. subtilis* spores can be exposed for a long time without inactivation, resulting in D_T values around 20 to 30 min. The thermal resistance is different depending on the different structures and species. Basically, thermophiles show a higher wet heat resistance compared to mesophiles, but this is not valid for dry heat resistance. Moreover, the spores can survive for a longer time at dry heat compared to wet heat. The inactivation mechanisms for dry and wet heat are based on different mechanisms (Nicholson et al. 2000; Setlow et al. 2006).

The spore inactivation by dry heat is accompanied by accumulation of DNA damage due to base loss and mutation. As the DNA is protected by SASPs, the α/β type SASPs are also involved in dry heat resistance. Studies show an increase in dry heat sensitivity of α^-/β^- mutants (Setlow et al. 1995). During outgrowth of spores, the DNA repair system such as RecA is working and influenced by dry heat. Mutants with a lack in their DNA repair system indicate a higher sensitivity to heat. Moreover, the spore mineralization is involved in dry heat resistance. The exact mechanisms in which these factors, mainly role of α/β type SASPs or mineralization, are acting during dry heat treatment is not known.

When the spores are suspended in aqueous suspension, the thermal resistance is termed wet heat resistance. Literature shows that spores can be exposed to a temperature of 40 °C or higher temperature than vegetative cells (Nicholson et al. 2000). The main structural element of a spore responsible for high wet heat resistance is the low core water content. An inverse correlation between the spore core water content and the wet heat resistance exists (Nicholson et al. 2000; Setlow et al. 2006). The heat resistance can be affected by different parameters. By increasing the temperature during sporulation, a decrease of spore core water content occurs followed by an increase in wet heat resistance (Setlow et al. 2006). Besides sporulation, temperature and spore core water content, the core mineralization and α/β type SASPs contributes to the resistance. The core contains mostly divalent cations such as Ca^{2+}. An increase in mineral content causes displacement of water and therefore a decrease of water content (Bender et al. 1985; Marquis et al. 1985;

Paidhungat et al. 2000; Cazemeier et al. 2001). Also, the DPA present in the core is affecting the heat resistance. In mutants without DPA, a higher heat sensitivity is observed due to the lower water content compared to spores containing DPA. In contrast to dry heat treatment, the DNA is not affected, which is mainly based on the occurrence of α/β type SASPs protecting the DNA. Studies using mutants with a lack of α/β type SASPs show a higher heat sensitivity (Setlow et al. 1995).

The inactivation of spores in moist environments is based on protein denaturation and enzyme inactivation, which is contrary to the inactivation of spores by dry heat, where the principle of action is the DNA. During exposure of the spore to wet heat, the heat penetrates into the spore and causes a rehydration of the spore core. As a result, the proteins denature and DPA is released when the heat treatment is high enough (Coleman et al. 2007). Due to the heat inactivation of these crucial proteins, the spore is inactivated. The DPA can be retained in the spore and therefore germination is possible, the germination process is very slow compared to unheated spores, and the outgrowth is not possible because of lack of key proteins and enzymes (Coleman et al. 2009). As outgrowth is not possible, it can be assumed that key proteins or enzymes responsible for intermediary metabolisms are affected by the heat treatment (Coleman et al. 2010). It is not clear which proteins are affected and cause spore death. The DPA release after longer heat treatment is clear evidence of the breakdown of the major permeability barrier for small molecules, which is the inner spore membrane (Tabit et al. 2010). During germination of unheated spores, the DPA is released through channels in the membrane. It might be that due to the heat treatment, the channels were triggered to be opened or the heat causes a drastic damage of the inner spore membrane allowing release of small core components (Zhang et al. 2009). No clear evidence is available to prove the assumption. This inactivation mechanism was found for *B. subtilis* (Coleman et al. 2007; Zhang et al. 2009), *B. sporothermodurans* (Tabit et al. 2010), *B. megaterium* and *B. cereus* (Coleman et al. 2010; Zhang et al. 2009).

2.1.5 Parameters affecting the thermal resistance of spores

The thermal resistance is influenced by many environmental parameters, such as pH, fat content, sugar or ion concentration, and structural parameters, such as sporulation temperature. The effect on thermal resistance is mainly described by analyzing the decimal reduction time (D_T value) indicating a higher heat resistance with high D_T values. In many studies, the influence of a single parameter is analyzed in model solution. In food products, the results may vary (López et al. 1996).

Free fatty acids/ Lipids

The presence of lipids has an additional protective effect in order to increase the heat resistance. The D_{112} of *B. subtilis* spores suspended in phosphate buffer is 1 min in contrast to 275 min when suspended in soybean oil (Molin et al. 1967). The inactivation data of spores suspended in less fatty media shows a so called shoulder, which is the initiation of the inactivation. By increasing the fat content, the shoulder is getting shorter and disappears at a specific level (Senhaji et al. 1977). The inactivation data shows only a linear part with a much smaller slope compared to buffer solution. The reason for the increase in heat resistance is based on the lower heat conductivity of lipids compared to buffer. The free fatty acids present in the lipids have an additional stabilizing effect (Molin et al. 1967). Moreover, the water activity is reduced at higher fat levels causing a heat protection (Jagannath et al. 2003). Different D_T values were obtained for *B. subtilis* spores in whole and skim milk. A higher D_T value was obtained in whole milk, which is based on the higher fat concentration (Jagannath et al. 2003). Contrarily, Senhaji et al. (1977) reported no effect of fat in food matrices, because of the small oil droplet size. This assumption was confirmed by Rodrigo et al. (1999) studying the influence of vinegar and vegetable oil in tomato sauce on the heat inactivation. Lower D_T values were obtained after adding vinegar resulting in a pH drop from 5.3 to 4.8, but the addition of 16 % oil showed no effect on the inactivation of G*. stearothermophilus* spores by heat. Other studies analyzing the effect of single free fatty acids showed a decrease in heat resistance with increased free fatty acid content. A 30 % reduction of D_T value was obtained for *B. cereus* and *C. sporogenes* suspended in 2.0 mM palmitic, palmotoleic, stearic or oleic acid (Lekogo et al. 2010). An even higher reduction of 75 % was investigated for *G. stearothermophilus* suspended in 0.6 mM oleic acid (Tremoulet et al. 2002). The free fatty acids vary in occurrence of double bonds and the number of double bonds as well as the length of the carbon chain. A higher sensitivity of the spores to free fatty acids was observed with increasing unsaturated bonds in the carbon chain (Lekogo et al. 2013). It is reported that the free fatty acids cause an additional stress on the spores in order to decrease their heat resistance (Lekogo et al. 2013).

pH value

For thermal inactivation of vegetative microorganisms, the pH value can be used as an additional hurdle to reduce the thermal load. This is because most microorganisms are sensitive to acid pH. In the area of spore inactivation by heat, a dependency of the heat resistance on the pH is observed. Lowering the pH causes a decrease in heat resistance of

spores, such as *B. subtilis*, *C. botulinum* or *A. acidoterrestris*, which can be estimated by lower D_T values (Hutton et al. 1991; Ababouch et al. 1995; Palop et al. 1996; Pontius et al. 1998; Mazas et al. 1998; Silva et al. 1999; Fernandez et al. 2002; Setlow et al. 2002; Bevilacqua et al. 2008). It is assumed that the acid has an effect on spore layers in order to reduce the permeability (Setlow et al. 2002). Moreover, the conformation and the stability of nucleic acids may be affected by acid (Hutton et al. 1991). The basic studies on effect of acids on heat resistance were performed in buffer solutions. Analysis in food matrices indicates even lower D_T values compared to buffer solution. The D_{120} for *G. stearothermophilus* 7953 in McIlvaine buffer at pH 5.0 is 0.59 min. This is in contrast to 0.27 min in tomato puree at pH 5.0. The reason for this is based on the higher complexity of food matrices (López et al. 1996). Because of differences in sporulation and recovery conditions as well as spore strains, the effect of pH may vary, as i.e. Murakami et al. (1998) detected no effect of the pH ranging from 3 to 8 on the heat resistance of *A. acidoterrestris* spores suspended in McIlvaine buffer. Furthermore, the effect of pH depends on the applied temperature. At lower temperatures (< 97 °C for *A. acidoterrestris* spores) a higher effect of the pH on the lethality of the spores was observed (López et al. 1996; Pontius et al. 1998; Bahçeci et al. 2007). The D_{91} of *A. acidoterrestris* in fruit juice decreases from 54.3 to 31.1 min at pH 3.7 and 3.1, respectively. An increase in temperature up to 97 °C causes less difference in D_{97} value (8.8 min at pH 3.7 and 7.9 min at pH 3.1) (Pontius et al. 1998).

Soluble solids

The addition of sugar to the medium results in a lower water activity and therefore a higher heat resistance of spores (Silva et al. 1999; Bevilacqua et al. 2008). Hence, juice concentrates, rich in soluble solid content, show an inhibitory effect against spores (Murakami et al. 1998) or are even free from contamination (Chmal Fudali et al. 2011). The effect is mainly dependent on the sugar concentration. At soluble solids contents of 10 to 20 °Brix, no effect of sugar on the D_T value of *A. acidoterrestris* spores was obtained (Ceviz et al. 2009), whereas at 50 to 68 °Brix an increase of D_T value was observed (Maldonado et al. 2008). However, the sucrose exerts a similar osmotic pressure that exists in the spore cortex and induces a protoplast dehydration. A lower water content results in a higher heat resistance (Komitopoulou et al. 1999).

Furthermore, the same effect of temperature dependent effect of the soluble sugar as already observed for the effect of pH on the heat resistance was obtained. At low temperature (< 97 °C for *A. acidoterrestris* spores), the protecting effect of sugar is higher compared to high temperatures (Silva et al. 1999).

Ion concentration

The heat resistance of spores is affected by the salt content of the surrounding medium. It is noted that with an increasing salt content, the heat sensitivity increases resulting in lower D_T values (Roberts et al. 1966; Hutton et al. 1991). Moreover, a higher salt concentration represents a more complex medium, which causes an increase in heat resistance (López et al. 1996; Jagannath et al. 2003; Chmal-Fudali et al. 2011).

2.1.6 Thermal inactivation of bacterial endospores

Thermal processing aims at inactivating food spoilage microorganisms and/or spores. The food is exposed to a specific temperature-time combination causing reduction in microbial load. Two main thermal processes are applied: pasteurization or sterilization. Traditional thermal pasteurization process applies temperatures between 75 and 85 °C (Kessler 1996) and inactivates vegetative microorganisms. A higher temperature of typically 121 °C is used for sterilization (Kessler 1996), which inactivates bacterial endospores and results in a longer shelf life compared to pasteurization.

Generally a thermal procedure depends on (Singh 2007):

- Nature of food (pH value, water activity)
- Heat resistance of microorganisms
- Heat transfer to the food
- Initial microorganism/spore concentration

According to these factors, the thermal process can be designed. Thermal calculations rely in general on the microbial inactivation at specific temperature-time combinations. At higher temperatures, less time is required for sufficient inactivation and vice versa. Microbial inactivation is represented by the so called survival graphics. They show the logarithm of the survival fraction during the treatment time for a given temperature. The thermal resistance of microorganisms is characterized by D_T and z values.

The decimal reduction time (D_T value) describes the time required at a constant temperature (T) to inactivate 90 % of the population (Fellow 2009). The determination of the value is based on the first order kinetic calculation (Equation 1).

$$log\left(\frac{N}{N_0}\right) = -\frac{t}{D_T} \qquad \text{Equation 1}$$

By plotting the inactivation against the time, the D_T value is given by the negative reciprocal slope. The D_T value is temperature dependent. At higher temperature, less time is required for an inactivation resulting in a lower D_T value. A high D_T value is obtained at lower temperatures, which means more time for microbial reduction is required.

The thermal death time (z value) describes the temperature dependence of the D_T value. It indicates the temperature required to cause a tenfold change in D_T value and can be calculated by the following equation with D_1 observed at temperature T_1 and D_2 at temperature T_2.

$$z = \frac{T_2 - T_1}{logD_1 - logD_2} \qquad \text{Equation 2}$$

The thermal resistance of spores can be affected by various environmental factors, such as water activity or pH value (section 2.1.5). Moreover, the strain and the determination method lead to different thermal resistances. An overview of D_T and z values for *B. subtilis*, *A. acdioterrestris* and *G. stearothermophilus* spores in different products is shown in Table 2-2.

Table 2-2: Overview thermal resistance *B. subtilis*, *A. acidoterrestris* and *G. stearothermophilus* spores in various products obtained by different methods.

Spore type	Strain	Product	pH	Temperature [°C]	D_T [min]	z [°C]	Method	Reference
B. subtilis	5230	Olive oil	nr	130	39.32	22.02	Tube(1)	Ababouch et al. 1995
	5230	Butterfield buffer	nr	105	16.91	9.05	Tube(1)	Ababouch et al. 1995
	4673	Distilled water	nr	100	45.1	7.8	nr	Beaman et al. 1986
	ATCC 6633	Distilled water	nr	90	45	nr	Tube(1)	Blank et al. 1987
	nr	McIlvaine	nr	104	3.08	9	nr	Condon et al. 1992
	NCIB 8054	Soya oil	nr	112	275	nr	Tube(1)	Molin et al. 1967
	NCIB 8054	Phosphate buffer	nr	95	125	nr	Tube(1)	Molin et al. 1967
	5230	Phosphate buffer	nr	121	0.46	6.4	Injection(2)	Odlaug et al. 1981
	nr	Citrate buffer	7	110	0.25	8.5	Injection(2)	Sala et al. 1995

	nr	Citrate buffer	4	110	0.06	8.5	Injection(2)	Sala et al. 1995
	nr	Distilled water	nr	100	1.6	6.6	Capillary(3)	Serp et al. 2002
	nr	Milk	nr	100	1.6	7.3	Capillary(3)	Serp et al. 2002
A. acidoterrestris	DSM 2498	Citrate buffer	4	100	0.5	8.5	Tube(1)	Bahçeci et al. 2007
	DSM 2498	Apple juice	3.6	100	0.7	8.5	Tube(1)	Bahçeci et al. 2007
	AB-1	Citrate buffer	nr	90	14.4		Capillary(3)	Baumgart et al. 2003
	DSM 2498	Apple juice	4	100	11.1	16.4	Tube(1)	Ceviz et al. 2009
	DSM 2498	Orange juice	nr	100	2.3	9.4	Tube(1)	Ceviz et al. 2009
	DSM 2498	Malt ex-tract broth	nr	100	2.7	9.8	Tube(1)	Ceviz et al. 2009
	Z CRA 7182	Apple juice	nr	80	41.15	12.2	Injection(2)	Komitopoulou et al. 1999
	Z CRA 7182	Grapefruit juice	nr	80	52.35	10.48	Injection(2)	Komitopoulou et al. 1999
	Z CRA 7182	Orange juice	nr	80	54.3	12.9	Injection(2)	Komitopoulou et al. 1999
	DSM 3922	Ringer´s solution	7	95	10.7	24.1	Injection(2)	Oliver-Daumen et al. 2009
	NCIB 13137	Orange juice	nr	85	65.6	7.8	nr	Silva et al. 1999
	NCIB 13137	Cupuaçu	nr	85	17.5	9	nr	Silva et al. 1999
G. stearothermophilus	7953	Distilled water	nr	100	311	nr	nr	Beaman et al. 1986
	0121	Distilled water	nr	100	128	nr	nr	Beaman et al. 1986
	NCIB 8919	Water	nr	115	8.5	nr	Tube(1)	Cook et al. 1968
	ATCC 12980	Distilled water	6.2	115	23.28	7	Capillary(3)	Fernández et al. 1994
	ATCC 12980	Mushroom extract	6.2	115	13.05	7.32	Capillary(3)	Fernández et al. 1994
	ATCC 12980	McIlvaine buffer	7	115	12.36	nr	Injection(2)	López et al. 1996
	ATCC 15951	McIlvaine buffer	7	115	8.89	nr	Injection(2)	López et al. 1996
	ATCC 15952	McIlvaine buffer	7	115	9.1	nr	Injection(2)	López et al. 1996
	ATCC 7953	McIlvaine buffer	7	115	10.2	nr	Injection(2)	López et al. 1996
	ATCC 7953	ACES buffer	7	121	2.1	7.66	Capillary(3)	Mathys 2008
	ATCC 7953	ACES buffer	7	130	0.21	7.66	Capillary(3)	Mathys 2008
	NCIB 8924	Soya oil	nr	121	8	nr	Tube(1)	Molin et al. 1967
	NCIB 8924	Phosphate buffer	nr	90	28	nr	Tube(1)	Molin et al. 1967
	nr	Citrate buffer	nr	121.1	3.7	7.6	Capillary(3)	Serp et al. 2002

	ATCC 7953	Sorensen buffer	7	110	60	8.2	Tube[(1)]	Tremoulet et al. 2002

nr - not reported; [(1)]Tube method: Small/Medium sized medium test tube was heated up in a water or oil bath and cooled down on ice after holding time, lag time until the final temperature is reached; [(2)]Injection method: Suspension with target spores is injected into the preheated medium, almost no delay in heating; [(3)]Capillary method: The medium was filled in a small glass capillary, hold in hot water or oil and cooled on ice.

Different thermal resistances in terms of D_T and z values were obtained for the different spore types. The reason for differences in D_T value is based on the strain and the sporulation method. Moreover, the product has a major impact on the heat resistance. The D_{100} of *A. acidoterrestris* spores is 11.1 min in apple juice compared to 2.3 min in orange juice (Ceviz et al. 2009). The pH and soluble solid content in the juices have in this case had an impact on the D_T value. Furthermore, the fat content in the media (Molin et al. 1967) and the method of determination can result in different heat resistances. The main methods are tube, capillary and injection method. Differences in heat transfer might occur. Basically for investigations dealing with thermal resistances, the D_T and z value should be determined in each new study.

Another fundamental approach to determine the heat resistance of spores is based on the Arrhenius equation (Equation 3), which describes the dependence of the inactivation rate *k* on the temperature *T* [°C]. The pre factor A_0 can be regarded as the inactivation rate at infinite temperature. The factor *R* is the universal gas constant (8.314 J K^{-1} mol^{-1}) and E_a the activation energy [kJ/mol]. Plotting ln k against 1/T, the slope is $-E_a/R$ and allows calculation of activation energy.

$$k = A_0 \cdot e^{-\frac{E_a}{RT}} \qquad \text{Equation 3}$$

To design a sterilization process, the thermal kinetics of the target spores have to be determined. The process is based on application of heat in a specific temperature and temperature holding time combination. Therefore, a high temperature for short time can result in the same inactivation level as low temperature for a longer time. The selected combination is dependent on the product, such as heat sensitive components, and on the available equipment. Moreover, the target set up could also be based on enzyme kinetics. The combinations can be described by the so called sterilization value (F value). The equation for calculating the F value includes the heating up phase (A), temperature holding time (B) and cooling phase (C) (Equation 4) (Figure 2-4) (Singh 2007).

$$F = \int_0^t 10^{\left(\frac{T(t)-T_R}{z}\right)} dt \qquad \text{Equation 4}$$

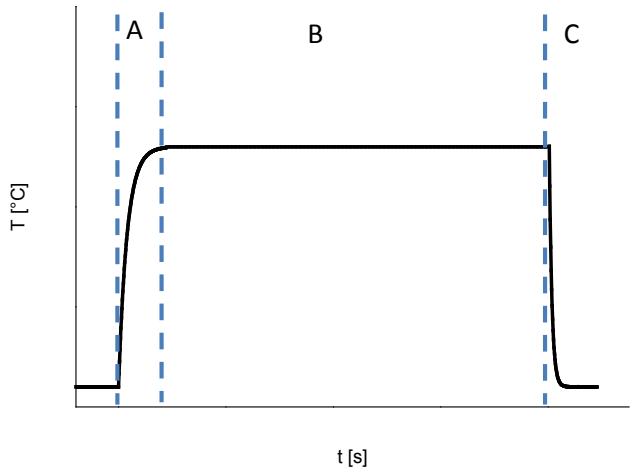

Figure 2-4: Thermal process including (A) heating up, (B) temperature holding time and (C) cooling phase.

The F value is calculated by integration of the temperature time profile of the process (*T(t)*) and the reference temperature (T_R). An overview of a sterilization process for a commercial application is shown in Table 2-3. Besides the inactivation of spores, the sterilization process causes the destruction of valuable components related to color or flavor change. A way to avoid high quality loss due to the thermal treatment is the addition of valuable components after the treatment under aseptic conditions (Singh 2007).

Table 2-3: Possible sterilization conditions for commercial sterilization process (adapted from Singh (2007)).

Process	Storage	Conditions	F value [min]*
Ultra pasteurization	Refrigerated	138 °C, 2 s	1.50
Minimum destroy *C. botulinum* spores	Non refrigerated	138 °C, 4 s	3.00
Commercial sterility	Non refrigerated	138 °C, 8 s	6.00
European UHT range	Non refrigerated	135 °C, 3 s	1.22
		140 °C, 4 s	3.87

*A z value of 10 °C was assumed for calculation.

2.2 Application of Pulsed Electric Fields (PEF)

2.2.1 Historical background

The use of electricity to achieve a bactericidal effect has been known since the 19th century (Krüger 1893, Thiele et al. 1899), almost at the time when electricity was commercially available. The application of direct or low frequency alternating current caused an inactivation of spoilage microorganisms based on thermal effects. In 1920 the so called process “Electropure” was introduced to Europe and United States (Beattie and Lewis 1925). The aim of the process was to pasteurize

milk by using electricity. Therefore, a 220 – 420 V not pulsed current was applied to a carbon electrode treatment chamber. The process can be described as a thermal treatment as the milk was first preheated up to 52 °C and due to the Joule effect heated up to 71 °C during 15 s holding time. Up to the 1950s about 50 plants were using this technology, but due to the rising energy costs and the competition with newer milder thermal technologies, plants were being replaced (Reitler 1990). At the same time, investigations into pulsed high voltage discharges across a treatment chamber consisting of two electrodes were done. The aim was to inactivate microorganisms and was termed as electrohydraulic treatment (Edebo et al. 1968). This process is based on submerging electrodes within a pressure level followed by application of electric arcs generated by high voltage pulses. The applied arcs lead to formation of transient pressure shock waves up to 250 MPa and ultraviolet light pulses. This process causes a microbial inactivation of 95 % of *E. coli*, *B. subtilis* or *Streptococccus cremoris* suspended in sterile water (Gilliland et al. 1967). It was observed based on the lethal effect of electrochemical reactions, shock wave and highly reactive radicals. Therefore, the process was regarded as fast and effective way to decontaminate water. Despite the high efficiency of the process, it never reached the point of industrial application, because of contamination with disintegrated food particles and electrodes (Jeyamkondan et al. 1999).

The German engineer Doevenspeck (1961) studied the disruption of cells by pulsed electric fields in food matrices and expanded the knowledge to inactivation of microbial cells. He found that the electrochemical reactions and the temperature increase, which is based on the Joule heating, is less relevant with short and homogenous electric pulses without causing arcing. During his studies from 1961 and 1971, he analyzed the change of pH after application of pulsed electric fields. After the treatment, a pH of 6.8 was measured at the anode and 8.0 at the cathode. After mixing, the previous pH of 7.2 was observed. Further analysis of PEF treatment of *Lactobacillus delbrückii* in beer stained with methylene blue indicated a color uptake by the bacteria after the treatment based on permeabilization (Doevenspeck 1975). Other studies showed an inactivation of microbial cells, such as *E. coli*, applying pulses with a high electric field strength and an accelerated growth of bacteria if pulses with low electric field strengths were applied. The system, with which the results were obtained, was described and recorded in a patent by Doevenspeck (1960). The setup included a pulse modulator and a continuously operating treatment chamber. The pulses were generated by a repetitive energy discharge stored in capacitors and use of switches to control the discharges. In his function as a consulting engineer, he was looking for potential new applications of PEF technologies. During this time, he connected with Münch, who was the technical director

for an animal material processing department at Krupp Maschinentechnik GmbH. Münch recognized immediately the high potential of Doevenspecks work and together they developed the process “ELCRACK” for the disintegration of cellular tissue and “ELSTERIL” for the decontamination of liquid materials (Sitzmann et al. 1987).

In 1968, the researcher Flaumenbaum, studied the effects of pulsed electric fields on apple mash and detected an increase of 10 to 12 % in juice yield. Furthermore, the product was light in color and less oxidized than after heat or enzyme treatment (McLellan 1991).

The first systematic studies of non-thermal lethal effects on microorganisms were conducted in the UK at the Unilever Research Centre by Sale and Hamilton (1967). A new system was built for their research consisting of a power generator connected to a batch treatment chamber. They applied a maximum voltage of 10 kV with an adjustable pulse width ranging from 2 to 20 µs during their research. The results of their experiments showed that the electric field strength, total treatment time, pulse number and pulse width are the main influencing parameters for inactivation of microbial cells. Their investigations also showed an irreversible damage of the cell membrane and therefore a loss of the permeability barrier followed by cell death. Permeability of the cell membrane is increased by the electric field, causing a leakage of ions and cytoplasma content (Sale et al. 1968; Kinosita 1997). Since these investigations, a high quantity of research activities have been carried out focused on analyzing the non-thermal effect of pulsed electric field processing. The studies focused on decontamination of liquid food products (Zhang et al. 1995; Grahl et al. 1996; Wouters et al. 1997; Barbosa-Cánovas et al. 1999) and the related effect on enzymes (Ho et al. 1997; Bendicho et al. 2003) as well as on disintegration of cellular tissue to increase the mass transfer processes (Eshtiaghi et al. 2002; Fincan et al. 2004; Lebovka et al. 2004) or to induce stress responses and secondary metabolite production (Guderjan et al. 2005). Patents for the application of PEF on food products were applied for by Krupp Maschinentechnik GmbH to develop the PEF process to allow industrial application. In the 1980s, Krupp developed a system based on Doevenspecks investigations; a system with a capacity of 200 kg/h for the treatment of meat or fish slurry, oil seeds or fruit mashes. According to the promising results, the system was successfully installed by Krupp in a fish company in Norway and a brochure was published (Figure 2-5).

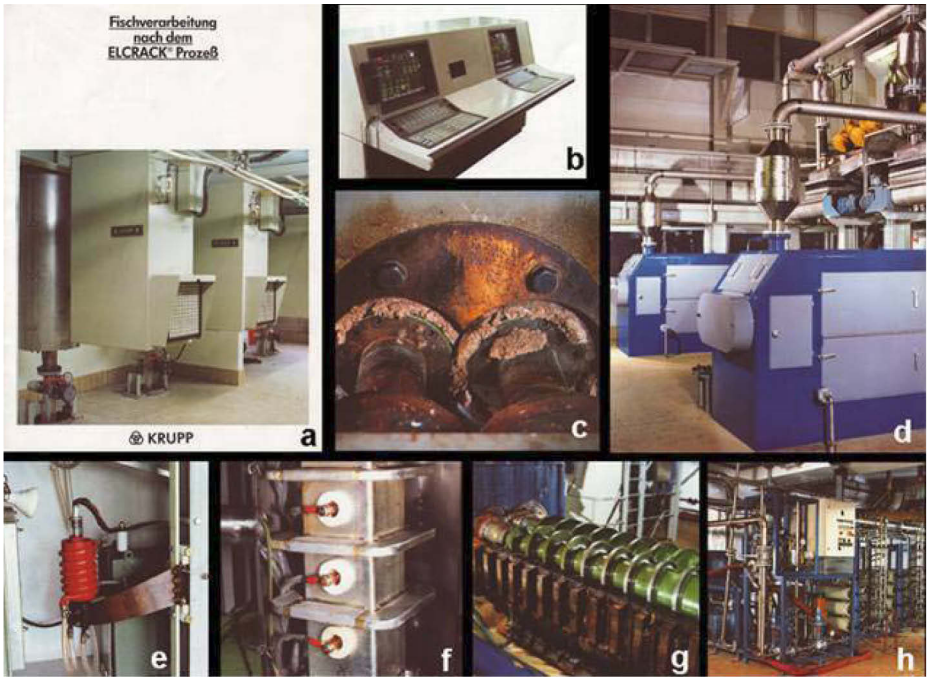

Figure 2-5: Fish processing by ELCRACK® – pictures of industrial equipment installed in Norway from a Krupp Maschinentechnik brochure (Krupp 1988) a) switch boxes; b) control unit; c) press outlet; d)screw presses; e) HV-switch; f) capacitor bank; g) screw press, dismantled; h) ultra filtration unit (Toepfl 2006).

The success of the developed system fails not only because of the system. Missing experience in the area of implementation into process line is the main reason for failure (Toepfl 2006). Due to this failure, the financial support was stopped and the Krupp group stopped working on PEF processing. The developed ELSTERIL® system was transferred to Berlin University of Technology for experiments studying the effect of PEF on carrot tissue (Geulen et al. 1992) and on potato (Angersbach et al. 1997).

In 1982, the research group led by Dunn developed a PEF treatment system and filed for a patent in 1987 under Maxwell Laboratories, San Diego, USA (Dunn et al. 1987). In 1995, a subsidiary of Maxwell Laboratories, called PurePulse developed a system termed CoolPure® with a capacity of 2.000 l/h. A lab scale system called CoolPure® Jr. with a lower capacity of 6 to 10 l/h was also developed. Using this specific system, a shelf life of fresh juice could be increased to about 1 week without significant quality changes (Dunn et al. 1987).

Nowadays, approximately 40 groups are working on PEF and more than 500 papers have been published on the topic. In 2006, the first commercial PEF unit was installed in the US (Clark 2006), but stopped in 2008 due to technical and commercial limitations. In Europe, the first commercial application was implemented in 2009 for preservation of juice by PEF with a capacity of 1500 l/h. One year later in 2010, the first application of PEF treatment on solid material was installed with a maximum capacity of 50 t/h. For scale up, the electric field strength, treatment temperature and delivered energy were identified as the most important parameters (Zhang et al. 1995; Heinz et

al. 2003; Amiali et al. 2007). The current maximum capacity of decontamination of liquid material is 5000 l/h and systems have been installed in Europe (Toepfl 2012; Irving 2012).

2.2.2 Application of PEF in permeabilisation of vegetative cells and cellular tissue

The application of PEF has been known since 1920, when electricity was first used to preserve food products. Since then, the potential application of PEF was studied in many research and commercial projects. It was found that PEF could be used for many fields (Figure 2-6).

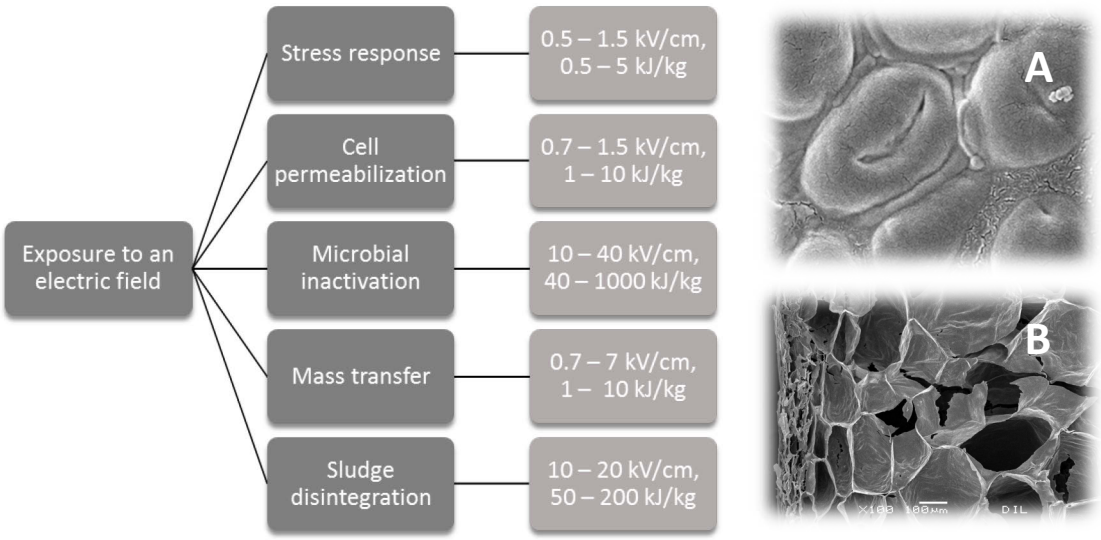

Figure 2-6: Applications of PEF treatment (adapted from Toepfl et al. 2006); (A) *S. cerevisiae* after PEF treatment (adapted from Aganovic et al. (2012)) and (B) tomato tissue after PEF treatment (unpublished data).

Food preservation

By applying PEF, spoilage microorganisms in liquid food products can be inactivated by affecting the permeability barrier of a cell, which is the membrane, leading to cell death. Due to the inactivation of spoilage microorganisms, the shelf life of the product can be extended and microbial safety of the product is ensured. Due to the operation at ambient temperature and even lower temperatures followed by temperature increase, which is based on the Joule heating, the heat load of the product is lower than thermal treatment. The process focussed on high quality products as the nutrients are not destroyed by heat. Food products rich in vitamins and other healthy components are mainly juices. Therefore, the first application tests and the main industrial applications were performed on juices.

Apple juice, as an example, is a qualified juice product for PEF treatment. Many studies indicate potential inactivation of various target microorganisms, such as *E. coli*, *S. cerevisiae* or *L. brevis* (Evrendilek et al. 2000; Aguilar-Rosas et al. 2007; Charles-Rodríguez et al. 2007; García et al. 2005a,

2007; Moody et al. 2014). After successful inactivation of spoilage microorganisms, the potential to achieve a higher juice quality was analyzed (Bi et al. 2013). For example, the polyphenol content is responsible for the color and the flavor development. After applying bipolar pulses with an electric field strength of 35 kV/cm, a considerable loss of phenols of 14.49 % was observed in contrast to 32.2 % after a thermal treatment at 90 °C for 30 s (Aguilar-Rosas et al. 2007). Moreover, a higher loss of volatile components after thermal treatment leading to destruction of flavor was observed. Other studies focused on the effect of PEF on pesticide reduction such as methamodophos or chlorpyrifos in apples. Research showed a successful reduction of pesticides was obtained (Chen et al. 2009; Zhang et al. 2012).

One of the most widely consumed juices is orange juice. It is temperature sensitive and hence the nutritional content is easily destroyed with normal pasteurization. PEF is a perfect opportunity to produce a shelf stable high quality orange juice. Many researchers studied the destructive microbiological effect of PEF on various indicator organisms, such as *E. coli*, *L. innocua* or *S. cerevisiae* (McDonald et al. 2000; Min et al 2003; Gurtler et al. 2010). An inactivation on a higher scale of 500 l/h inactivating natural flora in order to extend the shelf life could be shown by Min et al. (2003). Most important quality and health promoting substance in orange juice is vitamin C, which is known to be heat labile. After PEF application, the vitamin C retention is much higher based on the lower temperature used for preservation (Hodgins et al. 2002; Torregrosa et al. 2005). As an example, a vitamin C content of 55 mg/100 ml corresponding to the content of freshly squeezed orange juice was achieved after PEF treatment compared to a 19 % (44.5 mg/100ml) reduction of vitamin C after thermal treatment (Min et al. 2003). The flavor of orange juice changed during thermal pasteurization to a so called cooked flavor. A better flavor profile can be achieved by PEF treatment, due to the lower temperature. Myrene, for example, is a typical volatile component for orange juice has a retention of 88 % after PEF treatment in contrast to 63 % after thermal processing at 90 °C (Min et al. 2003). Other components like ethyl butyrate or decanal showed a 97 and 100 % retention after PEF treatment with an electric field strength of 30 kV/cm and 480 µs treatment time (Jia et al. 1999).

Nowadays, fruit purees, called smoothies are very common drinks, because of their health benefits. Smoothies are freshly squeezed and blended fruits, which are directly packed and retailed. In their fresh state, the shelf life is only a few days. A heat treatment to extend the shelf life would result in flavor destruction as well as quality loss. With the application of PEF process, an extended shelf life can be achieved without or only minimal effects on fresh characteristics, such as color, pH value or flavor compounds (Jin et al. 1999; Cserhalmi et al. 2006; Marsellés-Fontanet et al. 2009; Walkling-Ribeiro et al. 2009a; Morales-de la Peña et al. 2011). The presence of enzymes in

juice is important, due to their activity, the structure, color or physical properties of the juice changes. The study of Aguiló-Aguayo et al. (2008) focused on effect of PEF on lipoxygenase (LOX) responsible for lipid oxidation and β-glucosidase (GLU) responsible for color and flavor release in strawberry juice. The residual LOX activity after PEF treatment at 35 kV/cm decreases with increasing frequency and pulse width, whereas GLU showed an increased activity after the PEF treatment. Pectinmethylesterase and polygalacturonase activity in strawberry juice were lower after PEF treatment compared to thermal treatment (Aguiló-Aguayo et al. 2009). High temperatures strongly affect enzyme activity; it is worthy to analyze the effect of PEF on enzymes. Meneses et al. (2013) performed a mathematical model to separate the effect of PEF and temperature on the activity of polyphenoloxidase (PPO). The results show that PPO sensitivity to PEF strongly depends on the temperature. In temperature ranges between 55 and 59 °C PPO is affected by PEF. Another study analyzed the thermal inactivation of alkaline phosphatase (ALP) and lactoperoxidase (LPO) during PEF processing of milk. The model showed no thermal effect on ALP after PEF treatment at 97 kJ/kg with an outlet temperature of 42 °C. An increase of energy causes an enzyme activity reduction of 60 %, from which a large part is related to thermal inactivation based on the high outlet temperature. LPO activity was reduced by 50 % applying an energy of 251 kJ/kg. The 50 % enzyme reduction can be separated in 5-7 % PEF related inactivation and the rest thermal inactivation, which can be explained by the high outlet temperature of 85 °C (Jaeger et al. 2010).
An option to reduce the overall energy used for a specific microbial inactivation could be a combination of PEF with other technologies or ingredients. The application of PEF can be combined with thermosonication (Walkling-Ribeiro et al. 2009b) or high pressure (400 MPa, 1 min). Plaza et al. showed this in orange juice in a study in 2011. Furthermore, the combination of treatments or adding antimicrobials, such sodium benzoate (Hodgins et al. 2002; Wu et al. 2005; Nguyen et al. 2007; Gurtler et al. 2011) as chemical substances or natural antimicrobial substances, such as cinnamon bark oil, lysozyme or citric acid (Mosqueda-Melgar et al. 2008a, 2008b; Pina-Pérez et al. 2009; Ait-Ouazzou et al. 2011; Mosqueda Melgar et al. 2012).

Intracellular compounds extraction

Another effect which can be achieved by the application of PEF is the disintegration of cellular tissue. The treatment of products, such as potato or red beet root, causes the cells to disintegrate and the cell content containing valuable ingredients, such as colorants or health promoting ingredients, were easily extracted. Based on the fact that the cell content is easier to extract after PEF treatment, the mass transfer process is improved and therefore all processes where mass transfer is important can be improved.

Important colorants for the food industry are betalains, which are present in red beet root. A thermal treatment at 80 °C for 1 hour to open cell structure results in complete degradation of the colorants. After a PEF treatment at 30 °C with an electric field strength of 1.5 kV/cm and 20 pulses, a higher extraction yield can be achieved with less color degradation (Fincan et al. 2004, Loginova et al. 2011a). The effect of PEF on red beetroot is shown in Figure 2-7. After PEF treatment and cutting, a wet surface is visible and after centrifugation liquid release containing the colorants is obtained (Figure 2-7).

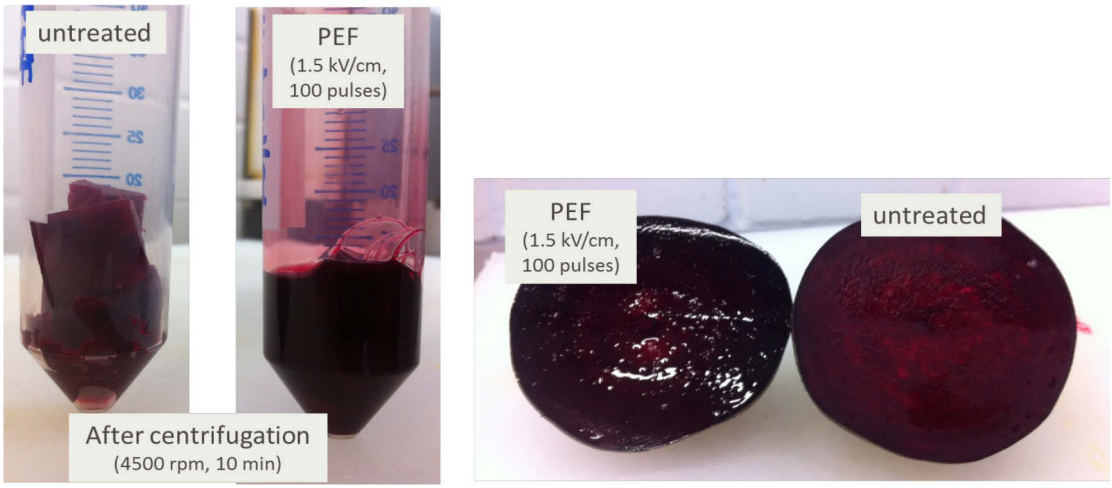

Figure 2-7: Effect of PEF on red beetroot.

The drying process can also be improved as the cells are opened and accelerate a liquid transfer. Different studies have been performed with various fruits and vegetables.

An overview on cell disintegration and PEF is listed according to the process and the treated material in Table 2-4.

Table 2-4: Overview application of PEF on various products divided in process groups.

Process	Product	Reference
Extraction	Algae	Toepfl 2006; Goettel et al. 2013; Grimi et al. 2014
	Apple	Bazhal et al. 2000, 2001; Schilling et al. 2007; Grimi et al. 2011; Toepfl et al. 2010; Turk et al. 2010; Jaeger et al. 2012; Turk et al. 2012a, 2012b
	Bones (calcium extraction)	Yin et al. 2008
	Chicory	Zhu et al. 2013
	Grape	Praporscic et al. 2007; Corrales et al. 2008; López et al. 2008; Bousetta et al. 2009a, 2009b; Donsi et al. 2010; Puértolas et al. 2010a, 2010b; Boussetta et al. 2011; Puértolas et al. 2011; Boussetta et al. 2012a, 2012b; Garde-Cerdán et al. 2013; López-Alfaro et al. 2013;
	Purple potato	Puértolas et al. 2013
	Red beetroot	Chalermchat et al. 2004; Fincan et al. 2004; López et al. 2009a; Loginova et al. 2011a

	Sugar beet	Eshtiaghi et al. 2002; Bouzrara et al. 2003; Jemai et al. 2003; El-Belghiti et al. 2004; Jemai et al. 2006; Lebovka et al. 2007; López et al, 2009b; Loginova et al. 2011b; Ma et al. 2013
	Oil extraction	Guderjan et al. 2005; Guderjan et al. 2007; Abenoza et al. 2013
	Orange peel	Luengo et al. 2013
Drying	Apple	Arevalo et al. 2004; Amami et al. 2006
	Carrot	Rastogi et al. 1999; Amami et al. 2007a; Amami et al. 2007b; Grimi et al. 2007; Gachovska et al. 2009
	Potato	Knorr 1999; Fincan et al. 2003; Lebovka et al. 2004; Lebovka et al. 2007b; Galindo et al. 2008, 2009; Jalté et al. 2009; Janositz et al. 2010, 2011; Boussetta et al. 2013a; Mhemdi et al. 2013
	Red pepper	Ade-Omowaye et al. 2003
	Strawberry	Taiwo et al. 2003
Distillation	Roses	Dobreva et al. 2010

2.2.2.1 Mechanism of electropermeabilization

The previous described effects of PEF on microbial cells or biological tissue can be explained by the structural membrane changes due to the electric field. The membrane is regarded as a semi permeable barrier involved in many metabolic activities. The electrical breakdown of the membrane has been explored in different model systems, such as phospholipid vehicle, and in microorganisms (Zimmermann et al. 1974; Ho et al. 1996; Barsotti et al. 1999). So far, no clear evidence is exists explaining the mechanisms of action of PEF on the membrane.

The application of PEF results in membrane changes and formation of pores. Therefore, the process is called electroporation, which can be divided in 4 parts (Saulis et al. 2007).

(1) Increase in trans membrane potential

The cell membrane can be considered as a capacitor filled with dielectric materials and a dielectric constant in the range of 2 (Zimmermann et al. 1974). Due to the difference in dielectric constant, free charges of different polarity accumulate and generate a membrane potential up to 10 mV. The exposure of the cell to an electric field causes an increase in membrane potential due to the higher accumulation of free charges at both membranes sides. The potential is increased up to a maximum, which depends on various factors such as cell size and shape as well as media factors.

(2) Pore formation

An increase in membrane potential occurs due to the exposure of the cell to an electric field. If the increase in potential exceeds a critical value, the membrane gets permeabilized, which is based on pore formation. The number of pores formed, depends mainly on the electric field strength applied, but also other factors such as temperature or membrane fluidity.

Currently two main explanations for initiation of pore formation are available. The first suggestion is termed the electromechanical model (Zimmermann et al. 1974; Dimitrov 1984). The basic idea of this model is formation of compressive forces due to the polarization. If the electric field strength exceeds a critical value, which is called critical electric field strength, the electro compression forces are getting so strong that the membrane is permeated (Figure 2-8). This model is well accepted due to its simplicity. Some effects are not that well explained though, such as, not all cells exposed to an electric field are electroporated (Kinosita et al. 1977). The second explanation model is based on statistical background. Due to naturally occurring fluctuations, hydrophobic pores are formed in the cell membrane. If the radius of the electric field induced pore exceeds a critical value, the pore becomes hydrophilic. This is because hydrophilic pores are more stable, because they are associated with lower energy. In this theory, the main reason for electroporation is the accumulation of hydrophilic pores and the related expansion (Glaser et al. 1988; Weaver 2000; Teissie et al. 2005).

(3) Evolution of number and size of the pores

The number and also the size of the pore are affected by the external electric field. For example pulses of nanosecond duration form smaller pores in a larger number compared to millisecond pulses (Joshi et al. 2004). Also, a higher voltage amplitude increases the size of the pores as well as the number (Joshi et al. 2004).

(4) Pore resealing

By applying PEF, cells were formed in the membrane. This can be reversible or irreversible. If a pore can be resealed depends on several factors, such as membrane thickness, cell size and shape as well as electric field strength. For irreversible pore formation a critical electric field strength has to be exceeded, which is in the range of 1 to 2 kV/cm for biological tissue and 10 to 14 kV/cm for microorganisms (Toepfl et al. 2005). If the applied electric field strength is close to the critical electric field strength and the treatment time is low, the pore formation is reversible.

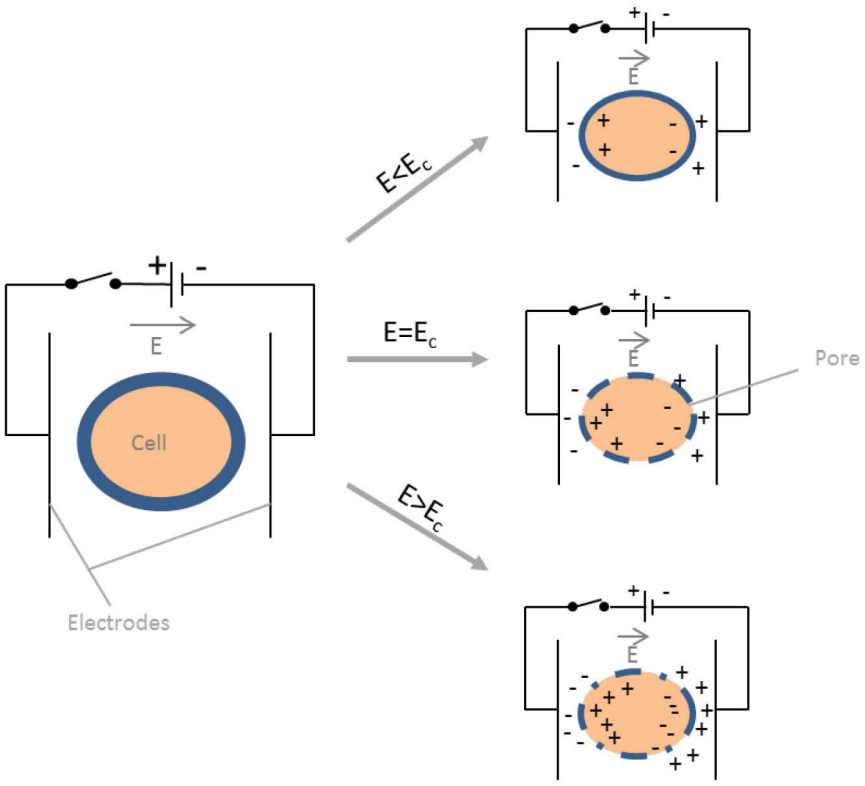

Figure 2-8: Electroporation of a cell exposed to an electric field; E = electric field strength, E_c = critical electric field strength (adapted from Toepfl 2006).

2.2.2.2 Influencing parameters for PEF treatment

The efficiency of the PEF treatment depends on different parameters, which can be divided into 3 parts. The process parameters, such as electric field strength or pulse width, are process dependent. In contrast, the product and microbiological influencing parameters are process independent. All influencing parameters have to be taken into consideration when applying a PEF treatment in order to achieve the most efficient treatment in terms of microbial inactivation, product quality and energy costs (Wouters et al. 2001).

Process parameters

Electric field strength (E)

One of the main process parameters is the electric field strength. *E* is defined as the voltage applied between two electrodes. When analyzing the electric field strength, the value itself and also the electric field distribution has to be considered.

To achieve an inactivation of relevant microorganisms, irreversible pores have to be formed causing leakage of cell material as well as cell lysis. Irreversible pore formation

occurs if a, so called critical electric field strength (E_c) is exceeded. Due to structural differences, different E_c values were obtained for various microorganisms and therefore the E_c can be used as parameter comparing resistances of microorganisms (Heinz et al. 2001; Bazhal et al. 2003). The cell size and the cell orientation in the electric field are very important for the critical electric field strength (Heinz et al. 2001). The smaller the cells, the higher the E_c and rod shaped cell orientation along the electric field leading to a higher E_c. Generally, an increase in electric field strength causes an increase in treatment efficiency (Hülsheger et al. 1983; Heinz et al. 2000; McDonald et al. 2000; Álvarez et al. 2003a; Gómez et al. 2005; Toepfl et al. 2007; Riener et al. 2008), but it is limited by the dielectric strength of the food material (Ho et al. 2000). Very high electric field strengths, up to more than 30 kV/cm were performed on a lab scale, which cannot be transformed on industrial level, because of system limitations as well as high risk of arcing inside the treatment chamber.

A homogenous electric field distribution allows a perfect control of the applied electric field in the treatment area. In the case of hetergenous electric field distribution, areas of very high electric field strengths might occur resulting in an over treatment. Mathematical simulations can be used to define these heterogenous situations to guarantee, sufficient treatment intensity (Qin et al. 1998; Fiala et al. 2001).

Treatment time and specific energy

The process intensity is described by the number of pulses applied and the related pulse width giving the treatment time. Generally, a higher treatment time results in a higher microbial efficiency (Sale et al. 1967). The pulse width is fixed by the impulse generation and describes the voltage applied over a specific time. For exponential decay pulses, it is the time until the decay of 37 %. For a batch treatment, the number of pulses can be very precisely adjusted, but for a continuous flow an average in pulse number has to be assumed. Furthermore, due to treatment chamber geometry the applied electric field strength might differ. This can be observed for a colinear configured treatment chamber, where the electric field distribution is not homogenous. A more precise parameter to describe the process intensity is the specific energy.

The energy input (W) can be calculated by the process induced temperature increase and the specific heat capacity of the medium. For this calculation, an adiabatic system is assumed, where the delivered energy is totally converted to heat. A more accurate calculation is represented by the following equation, which is based on the voltage and current measurement close to the electrodes.

$$W_{Pulse} = \int U(t)I(t)dt \qquad \text{Equation 5}$$

For exponential decay and rectangular pulses, the specific energy can be calculated by

$$W_{specific} = f \cdot \frac{1}{\dot{m}} \cdot \int_0^{\infty} \kappa(\mathrm{T}) \cdot \mathrm{E(t)^2 dt} \qquad \text{Equation 6}$$

The parameters *E*, *f*, $\dot{m}$ and $\kappa(T)$ denote the electric field strength, frequency, mass flow rate and media conductivity. The specific energy can be used as a control parameter and also for indicating the operation costs. However, studies showed an increase of inactivation with increasing specific energy (Heinz et al. 2001; Álvarez et al. 2003a). Nevertheless, indicating the specific energy is not enough to describe the PEF process. Applying the same specific energy and different electric field strengths can result in different effects, as a PEF treatment for inactivation of vegetative bacteria is more efficient at higher electric field strengths. Moreover, the specific energy gives no information about the energy delivered per pulse or the pulse number per volume element.

Pulse shape and pulse width

The basis of PEF treatment is the application of high voltage pulses. These pulses can be applied in different shapes. Mostly rectangular or exponential decaying pulses were used. Studies show that rectangular pulses are more efficient, which means less energy for microbial inactivation, compared to exponential decaying pulses, because of the high voltage is applied for the complete pulse width. The efficiency is strongly related to the electric field strength (Álvarez et al. 2006). Moreover, the possibility of applying mono polar or bipolar pulses, which means applying a positive pulse followed by a negative pulse, exists. It is generally accepted that bipolar pulses are more efficient, due to the change in polarity. The cells are exposed to an additional stress resulting in a higher sensitivity to the treatment (Leadley et al. 2006).

The length of the pulses or the time the voltage is applied to the product is given by the pulse width. In literature, controversial information is available regarding the influence of the pulse width. In some studies, the inactivation was claimed as independent from the pulse width (Hülsheger et al. 1981; Álvarez et al. 2003a; Sampedro et al. 2007) and other obtained a higher inactivation when long pulses were applied (Wouters et al. 1999; Aronsson et al. 2001). Again, other researchers showed a high efficiency of the treatment when applying short pulses and a high electric field strength at the same specific energy

(Schoenbach et al. 1997). The application of short pulses is more advantageous, because less heating based on Joule effect is observed (Sale et al. 1987; Evrendilek et al. 2004).

Temperature

An increase of inlet temperature of the product allows reduction of PEF treatment intensity to obtain similar effects. This effect has been observed both in non lethal (Aronsson et al. 2001; Heinz et al. 2003; Fleischman et al. 2004) or lethal (Sepulveda 2005, 2006) temperature range. The reason for the lowered energy requirement for microbial inactivation at higher temperatures is based on the effect of moderate temperatures on the membrane fluidity and stability. At lower temperatures, the phospholipid structure of the membrane is packed in a gel-like structure. With increasing temperature, this status is shifted to a liquid crystalline structure (Stanley et al. 1991). Therefore, an increase in temperature results in a decrease in energy applied by PEF for inactivation (Zhang et al. 1995; Wouters et al. 1999; Smith et al. 2002; Amiali et al 2005, 2007; Fernández-Molina et al. 2005; Sampedro et al. 2007; Toepfl et al. 2007; Fox et al. 2008). Beside the inlet temperature, the increase in temperature has to be considered. Recent mathematical simulations indicate that the temperature in the treatment zone is higher than the one measured outside the treatment chamber (Jaeger et al. 2009; Buckow et al. 2010). The final temperature should be considered when the lethal effect of a PEF treatment is studied. However, the use of the synergistic effect of mild and non lethal temperature allows a gentle and energy efficient PEF treatment.

Product parameters

Conductivity

The conductivity is one of the main influencing product parameters affecting the PEF treatment. The effect of conductivity has to be distinguished from the influence of process parameters and the influence of salt and/or ions on the cell membranes. It is measured in Siemens per length and is the inverse of the resistivity.

In media with high ion concentrations, such as vegetable juices or soups, problems with achieving the correct voltage arise, because smaller peak field strength is generated across the treatment chamber. According to Ohms law (R=U/I), an increase in conductivity results in a decrease of resistance and the current increases, as the voltage is constant. The electrical current is limited by the system (Toepfl 2006). As the conductivity is dependent on the temperature, a change of conductivity has to be assumed during the PEF treatment as

the temperature is increasing. At higher temperatures, the mobility of the ions is increasing resulting in a higher conductivity.

However, the ionic strength also has an effect on the cells, which should be destroyed by the treatment. Based on the difference in ionic strength between the media and the cytoplasma, the membrane will be weakened at higher conductivity rates (Jayaram et al. 1992). The weakening causes structural changes and a higher permeability, which results in a higher sensitivity of the membrane to the treatment. The energy for an inactivation of specific microorganisms can be reduced at higher ionic strength of the media (Hülsheger et al. 1981; Vega-Mercado et al. 1996). Contrary results were obtained by Álvarez et al. (2000) and Heinz et al. (2002), where a negligible effect of the conductivity of the media was observed.

pH value of the treatment media

Among the influence of pH value on the inactivation of bacteria by PEF, controversial results were available in literature. Some studies observed a higher sensitivity of the microorganisms when suspended in acid media (Wouters et al. 1999; Aronsson et al. 2005) and some observed a higher sensitivity at neutral pH value (Álvarez et al. 2000). Also, no effect of pH on the sensitivity was observed (Sale et al. 1967; Heinz et al. 2000; Smith et al. 2002; Álvarez et al. 2003a).

The correlation of the pH and the microorganism strain has to be taken into consideration. Studies from García et al. 2005b indicated a higher resistance of gram positive bacteria in neutral media compared to gram negative. However, in acid media, the inverse, a higher resistance of gram negative was observed. The difference in resistance might be related to the repair capability of sub lethally injured cells.

Water activity

The water activity a_w of the media influences the PEF efficiency regarding inactivation of microorganisms. The a_w does not directly influence the PEF treatment, it affects the resistance of microorganisms to the treatment. At low a_w value, a water flux of cell liquid is caused based on different osmotic pressures leading to a cell volume reduction. Due to the smaller cell, a higher PEF resistance results. Moreover, due to the cell shrinkage the cell membrane is compressed and is therefore thicker, which gives the cell a higher resistance (Neidhardt et al. 1990). Hence, at low a_w the sensitivity of microorganisms to the treatment is decreased, then a higher energy input is required for a specific inactivation (Álvarez et al. 2000, 2003; Aronsson et al. 2001). The influence is dependent on the solute

added to the media. Based on the lower molecular weight of glycerol compared to sucrose, an addition of 10 % (w/v) glycerol and 50 % (w/v) sucrose is required to reduce the water activity to 0.93. After PEF treatment, a higher sensitivity of *Salmonella* Senftenberg and *Listeria monocytogenes* was observed when glycerol was added. The addition of sugar instead of glycerol causes the opposite, which means the analyzed microorganisms showed a higher resistance. The reason for the different results can be explained by the different molecular weights. As glycerol is small, it can diffuse through the membrane in the cell. Due to the diffusion, the cell swells and enlarges, which results in a lower resistance. As the molecular size sucrose is larger than glycerol, it cannot diffuse through the membrane and cause a diffusion difference. Instead it causes a flux of cell constituents out of the cell resulting in a lower size and a higher resistance (Álvarez et al. 2006).

Besides sugar, other substances such as fat influence the a_w. The study of Grahl et al. (1996) observed a higher resistance of *E. coli* cells, when the media contains fat. Other authors indicate no effect of fat on the PEF sensitivity of microorganisms (Mañas et al. 2001; Pol et al. 2001).

Microbial parameters

The target of PEF treatment is the inactivation of spoilage microorganisms in different food matrices. As the microorganisms vary along their composition, size and shape, different resistances to the PEF treatment were observed.

The type of microorganism affects the energy of the PEF treatment for a specific inactivation. Generally, bacterial endospores were the most resistant (Knorr et al. 1994, Barbosa-Cánovas 1999) type of microbial cells. The high resistance is mainly based on the structural composition, which is explained in chapter 2.1.3. More specifically, gram positive bacteria were more resistant than gram negative bacteria (Sale et al. 1967; Hülsheger et al. 1983; Dutreux et al. 2000; Aronsson et al. 2001; Fernández-Molina et al. 2005; García 2005a; Sharma et al. 2014). Nevertheless, the pH value and the strains are important factors when comparing the resistance of gram positive and negative bacteria. The differences in inactivation can be explained by the different membrane compositions. Gram positive bacteria contain a thick cell wall made of several peptidoglycan layers and teichoic acids conferring rigidity and a physical resistance of the cell. The cell wall of gram negative bacteria is thinner and the cell has an outer membrane, which differs from the inner membrane as it is made of lipopolysaccharides instead of phospholipids.

The inactivation of yeast require less energy compared to bacteria or even spores. The reason is the size. Yeasts are spherical cells with an average diameter of 5 to 10 µm. *E. coli*

cells, for example, are rod-shaped with a typical length of 2 µm and a diameter of 0.25 to 1 µm. Yeast cells are much bigger, but also differ in shape. The influence of the size and the shape is mainly related to the trans membrane potential created by the external electric field. A lower potential is induced by the electric field, when small cells are exposed to this field and thus cause a higher resistance of the cells (Zimmermann et al. 1974; Hülsheger et al. 1983). Non spherical cells orient in the electric field along their axis, so that the potential is mainly induced at the poles of the cell. Based on the fact that the poles have the smallest area, a high resistance results (Heinz et al. 2001).

The status in growth phase of the microorganisms has an effect on the energy required for an inactivation. Cells in the exponential growth phase are more sensitive compared to cells in the stationary growth phase (Hülsheger et al. 1981; Pothakamury 1995; Gásková et al. 1996; Wouters et al. 1999; 2001, Álvarez et al. 2002; Rodrigo et al. 2003). Cells in this growth phase are more electromechanically unstable because of the continuous cell division and the larger size of the cells (Jacob et al. 1981).

The resistance to PEF treatment correlates with a resistance to other inactivation processes, such as thermal ones. Strains with a high heat sensitivity could show a high resistance to the PEF treatment (Unal et al. 2002; Lado et al. 2003; Aronsson et al. 2005). The identification of target microorganisms for PEF studies cannot be based on correlation to thermal processes.

2.2.3 Inactivation bacterial endospores by PEF

A successful application of PEF for inactivation of vegetative microorganisms is widely described in literature, whereas only limited information is available for inactivation of bacterial endospores by PEF. Moreover, controversial results were obtained as to whether a spore reduction is possible or not. The reason for opposing results could be mainly explained by the different treatment conditions and PEF systems.

The study of Marquez et al. (1997) demonstrates a possible inactivation of *B. subtilis* and *B. cereus* spores. Applying an electric field strength of 50 kV/cm and 30 pulses, a 3.4 log reduction for *B. subtilis* spores and after 50 pulses a 5 log reduction for *B. cereus* was obeserved. Furthermore, it was found that the inlet temperature of the product was important. The treatment performed at the same PEF conditions at inlet temperatures of 5 and 10 °C indicated a lower inactivation compared to 25 °C. As the treatment chamber was cooled, no temperature increase was observed. The same effect of higher inactivation at higher inlet temperatures was observed by Bermúdez-Aguirre et al. (2012). They studied the inactivation of *B. subtilis* spores suspended in whole and skim milk at

different PEF treatment conditions. A 3 log reduction in skim milk at 50 °C inlet temperature applying 20 pulses with an electric field strength of 35 kV/cm and 2.5 µs pulse width was achieved. Due to the Joule heating effect, the temperature after the treatment increased, but 80 °C was the maximum temperature. Under pressurized conditions, higher temperatures can be achieved. Uemura et al. (2003) studied the inactivation of *B. subtilis* spores at temperatures of more than 100 °C. A sufficient inactivation could be achieved by applying an electric field strength of 16.3 kV/cm and a temperature increase up to 121 °C in 1 s. A thermal treatment caused less inactivation under these temperature time conditions. Beside the inlet temperature, the electric field strength is also important for successful spore reduction by PEF. Jin et al. (2001) studied the inactivation of *B. subtilis* spores at electric field strengths of 30, 37 and 40 kV/cm. The highest inactivation of spore was observed at 40 kV/cm and 3500 µs treatment time, an inactivation of 98% was realized. Microscopic pictures after PEF treatment showed a different appearance of the spores, compared to thermal treated spores (Figure 2-9). Untreated spores had a smooth surface. After thermal as well as PEF treatment, the spore appeared shrunken und showed wrinkles, although the PEF treated spores still had a different shrunken and wrinkled appearance. Other authors also recorded differences in shape and surface of PEF treated spores (Yonemoto et al. 1993; Marquez et al. 1997). It can be assumed that the spore inactivation by PEF has a different mechanism than thermal treatment. A direct comparison of thermal and PEF treatment was performed by Dunn (2001). They observed no inactivation of *G. stearothermophilus* spores suspended in phosphate buffer, when the temperature was lower than 100 °C. The inlet temperature was set at 70 °C and the increase in temperature was measured. The overall temperature holding time of the PEF process was 8.7 s. Analysis of thermal treatment at maximum temperature and holding time of 9 s showed a lower inactivation than PEF treatment.

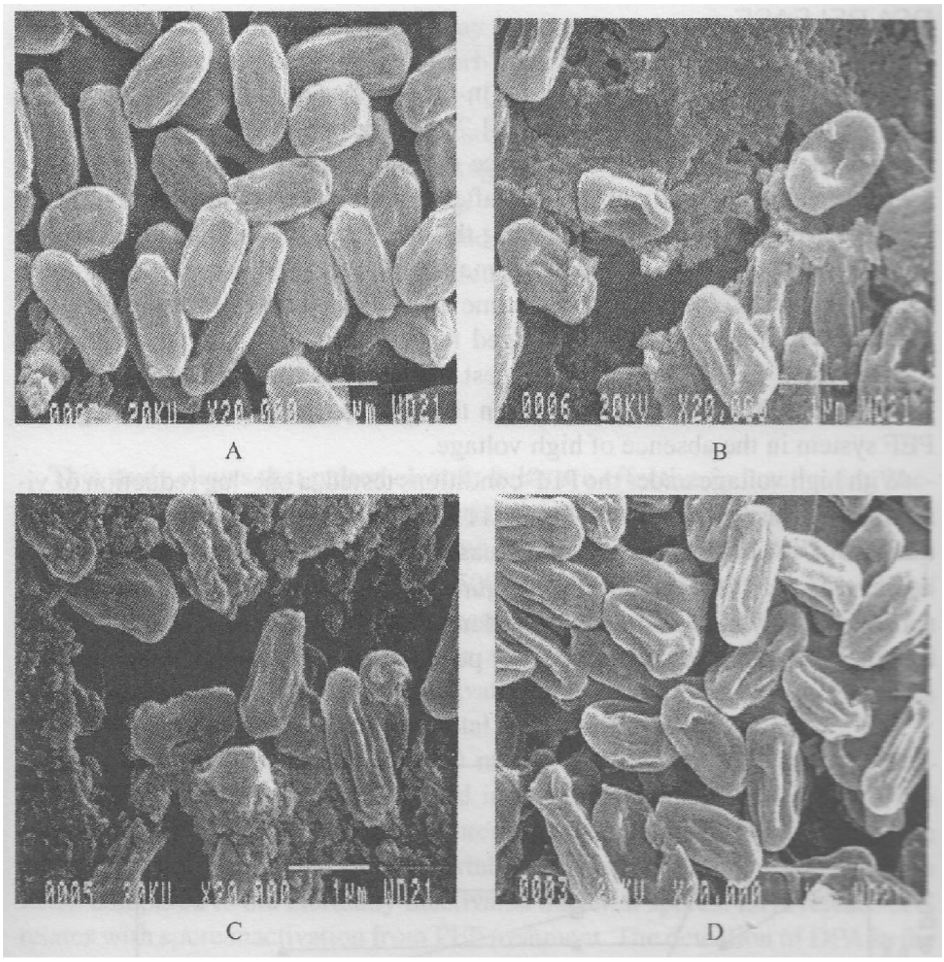

Figure 2-9: SEM micrographs of (A) untreated, (B) PEF treated at 30 kV/cm and 100 pulses, (C) PEF treatment at 30 kV/cm and 600 pulses and (D) thermal treated (121 °C, 20 min) *B. subtilis* spores (Jin et al. 2001).

Contrarily, other researchers detected no inactivation of bacterial spores by PEF. Pagán et al. (1998) observed no inactivation of *B. subtilis* spores after PEF treatment at 60 kV/cm and 75 pulses. Even when the treatment was performed with an inlet temperature of 60 °C, no reduction of spore concentration was observed. At lower electric field strengths and inlet temperatures, no inactivation was obtained (Knorr et al. 1994; Knorr et al. 1999; Cserhalmi et al. 2002; Shin et al. 2008). Hamilton et al. (1968) reported no spore inactivation by PEF treatments up to 30 kV/cm. When *B. cereus* spores were suspended in 0.1 % NaCl solution, no or only limited inactivation was observed. The addition of alanine-inosine mixture to the suspension induced a germination of spores, which made the cells more sensitive to the treatment. With increasing incubation up to maximum 90 min, an increase in inactivation after PEF treatment was observed. After germination, certain inactivation could be achieved (Pol et al. 2001), but PEF itself induced no germination (Knorr et al. 1994; Jin et al. 2001).

As a summary of available information in literature, the most important parameters for inactivation of bacterial endospores by PEF are electric field strength, specific energy and inlet temperature. The PEF treatment conditions used for inactivation of vegetative microorganisms are not sufficient for inactivation of spores. Therefore, the process has to be combined with other hurdles, such as temperature or additives. Spilimbergo et al. (2002) analyzed the combined process of PEF and supercritical CO_2. The combined process caused a higher inactivation compared to each process performed by its own.

2.2.4 Mathematical modeling of inactivation kinetics

For studying the microbial resistance against different inactivation methods, such as thermal or PEF processing, so called survival graphs or inactivation graphs were created. The graphs showed the survival fraction or inactivation at a given treatment intensity. These graphs were often described using different mathematical models from which different parameters were obtained to quantify and compare antimicrobial resistance. The shape of the survival or inactivation graph can be linear, concave upwards, concave downwards or sigmoid (Xiong et al. 1999; Periago et al. 2002). In case of a linear relationship between the logarithm of the number of survivors and the treatment time /treatment energy, a constant inactivation rate is obtained. If the graphs are non-linear, the inactivation rate depends on the process parameter (time or energy) used to describe the inactivation. To describe these cases, a complex mathematical equation is required to describe the survival graph.

When microbial inactivation kinetics of vegetative microorganisms by PEF are investigated, a concave survival curve has been observed in most cases (Jayaram et al. 1992; Álvarez et al. 2000, 2002, 2003a, 2003b; Ohshima et al. 2002; Rodrigo et al., 2003, Gómez et al. 2005; Cebrián et al.; 2007; Puértolas et al., 2009; Saldaña et al. 2010 a,b; Mosqueda-Melgar et al. 2012). Some other studies have indicated a traditional first order kinetic for inactivation of vegetative microorganisms by PEF and therefore a linear relationship between the logarithm of the number of survivors and the treatment time (Mizuno et al. 1988; Martín-Belloso et al. 1997; Sensory et al. 1997; Heinz et al. 1999; Huang et al. 2012). The microbial inactivation in these studies usually is not exceeding 3 to 4 log cycles and so these studies are probably referring to the first part of the curves without considering the tailing effect.

Primary model

To study the microbial inactivation kinetics and therefore the PEF survival curves, different primary mathematical models have been proposed.

The first specific model to describe the kinetics of microbial inactivation by PEF depending on electric field strength and treatment time was developed by Hülsheger et al. (1981). This model is based on the existence of two linear relationships, one between the logarithm of the number of survivors and the logarithm of the treatment time and the other one between the logarithm of the number of survivors and the electric field strength:

$$S(E,T) = (\frac{t}{t_c})^{(-\frac{E-E_c}{k})} \quad \text{Equation 7}$$

where *S* is the fraction of surviving microorganisms, *k* is a constant for each microorganism, E_c [kV/cm] the critical electric field strength for a given treatment time, t_c the critical treatment time [µs] for a given electric field intensity. This model establishes a critical treatment time (t_c) and a critical electric field strength (E_c) above which the treatment begins to be lethal to microorganisms. The parameter E_c is used to state the minimum limit for inactivation of various microorganisms at a given treatment time (Álvarez et al. 2006).

Subsequent, Peleg (1995) developed a model based on the Fermi equation to describe the relationship between the survival fraction (*S*) and the electric field strength (*E*) for a given number of pulses (*n*) describing the sigmoid curve shape. The critical electric field strength (E_c), which corresponds to the inflexion point of the curve and represents the electric field strength needed to inactivate 50 % of the population, and the slope of the linear part in the graphic (*c*) are the main parameters used in this model:

$$S(E,n) = \frac{100}{1+e^{\frac{E-E_C(n)}{a_C(n)}}} \quad \text{Equation 8}$$

Both models from Hülsheger et al. (1981) and Peleg (1995) are models specially developed for the PEF treatment. By using the models, microbial inactivation can be adequately described permitting the comparison of microbial resistance to a PEF treatment of different microorganisms or estimating the microbial inactivation achieved for specific treatment conditions (Grahl et al. 1996; Sensoy et al. 1997).

Over the past 15 years various mathematical equations were proposed to describe graphs with concave survival in both PEF treatment, such as treatment with other agents or technologies (Table 2-5). There have been purely empirical equations (Cole et al. 1993; Peleg et al. 2000) and equations with some biological basis. Among the latter, there exist equations based on the presence of two subpopulations of different resistance PEF (Pruitt et al. 1993), as well as models based on

the existence of a distribution of resistance within the microbial population (Augustin et al. 1998; Peleg et al. 1998; Mafart et al. 2002; van Boekel 2002).

Table 2-5: Mathematical equations used by different authors to describe the microbial inactivation kinetics by PEF (adapted from Álvarez et al. (2006)).

Model	Mathematical equation	Reference
Cole et al. 1993[a]	$Log_{10}S(t) = \frac{\alpha + (\omega - \alpha)}{1 + e^{4\sigma(\tau - \log_{10} t)/\omega - \alpha}}$	Raso et al. 2000
Peleg et al. 2000[b]	$Log_{10}S(t) = -a\ln(1 + ct)$	Álvarez et al. 2006
Pruitt et al. 1993[c]	$S(t) = pe^{-k_1 t} + (1-p)e^{-k_2 t}$	Álvarez et al. 2006
Augustin et al. 1998[d]	$S(t) = \left(1 + e^{(t-m)/s^2}\right)^{-1}$	Álvarez et al. 2003b
Peleg et al. 1998[e]	$Log_{10}S(t) = -bt^n$	Rodrigo et al. 2003
Mafart et al. 2002[f]	$Log_{10}S(t) = -\left(\frac{t}{\delta}\right)^{\rho}$	Gómez et al. 2005b
van Boekel 2002[g]	$Log_{10}S(t) = -\left(\frac{1}{2{,}303}\right)\left(\frac{t}{\alpha}\right)^{\beta}$	Álvarez et al. 2003a, b, c

a; α: upper asymtote ($\log_{10} S(t)$); ω: lower asymptote ($\log_{10} S(t)$); σ: maximum slope of the inactivation curve; τ: $\log_{10}$ of the time at which the maximum slope is reached; t: tme (µs); S: surviving fraction.
b; a y c: model constants; t: treatment time (µs); S: surviving fraction.
c; P: surviving fraction of the sub population 1; k_1: inactivation rate of sub population 1; k_2: inactivation rate of sub population 2; t: time (µs); S: surviving fraction.
d; m: $\log_{10}$ time to inactivate 50% of the population (µs); s Parameter proportional to the standard deviation of PEF resistance ($\mu s^{0,5}$); t: $\log_{10}$ of treatment time (µs); S: surviving fraction.
e; b: scale parameter; n: shape parameter; t: time (µs); S: surviving fraction.
f; δ: scale parameter; ρ: shape parameter; t: time (µs); S: surviving fraction.
g; α: scale parameter; β: shape parameter; t: time (µs); S: surviving fraction.

Because of the simplicity and flexibility, the models based on the Weibull distribution (Weibull 1951) have been used by many authors (Álvarez et al. 2006; Huang et al. 2014). Survival graphs with linear, concave upwards or concave downwards profiles can be described by using only two parameters, which are the scale parameter (b, δ or α in the equations of Table 2-5) and the shape parameter (n, ρ and β in equations in Table 2-5). Shape parameter refers to the profile presented by the survival graph. A shape parameter value lower than one corresponds to a concave upwards profile, higher than one with a concave upwards profile, and equal to one with a linear graph. In contrast, the scale parameter depends on the mathematical equation. Thus, in the equation of van Boekel (2002), it refers to the time required to inactivate the first napierian log cycle microbial population. While in the equation of Mafart et al. (2002), the scale parameter corresponds to the time required to inactivate the first decimal log cycle. In 2005 a tool called GInaFiT (Geeraerd and Van Impe Inactivation Model Fitting Tool) was developed by Geeraerd et al. (2005). Using the tool as an add-in in Microsoft Excel allows a simple application of 8 different equations according to

the experimentally obtained curve shape (Figure 2-10). Beside the classical Weibull distribution a model describing survival curve containing a shoulder and/or tailing part developed by Geeraerd et al. (2000) could be used.

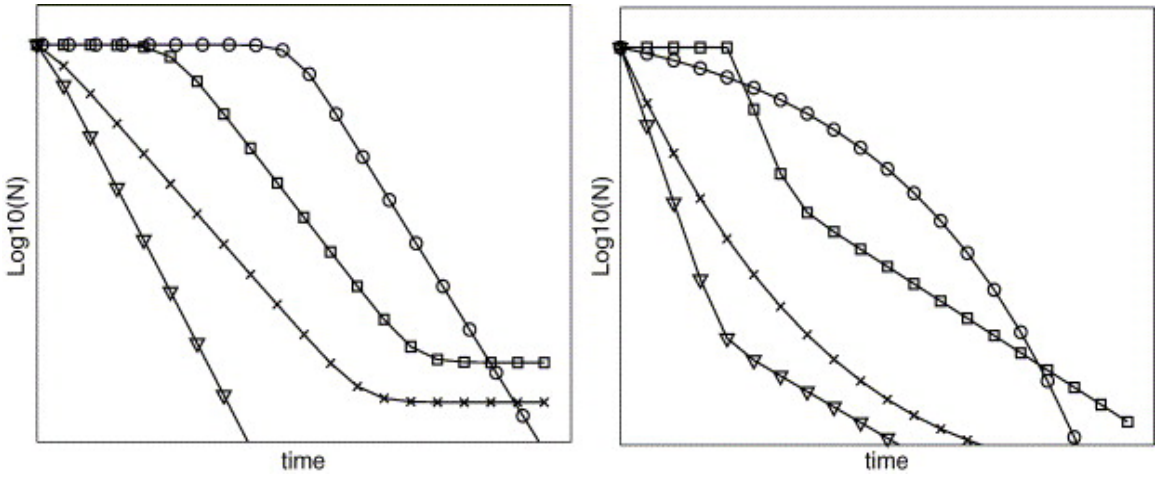

Figure 2-10: Commonly observed types of inactivation curves. Left plot: linear (▽), linear with tailing (×), sigmoidal-like (□), linear with a preceding shoulder (○). Right plot: biphasic (▽), concave (×), biphasic with a shoulder (□) and convex (○). Adapted from Geeraerd et al. (2005).

The model is based on three parts (shoulder, linear and tailing) and can be described as follows:

$$\frac{dN}{dt} = -k_{max} \cdot N \cdot \left(\frac{1}{1+C_c}\right) \cdot \left(1 - \frac{N_{res}}{N}\right) \qquad \text{Equation 9}$$

The first factor on the right side of the equation describes the linear part of the survival curve and follows the classical first order inactivation kinetic including the factor k_{max} as specific inactivation rate. The second factor implies the shoulder effect. The reason for occurrence of a shoulder can be described by three statements (Mossel et al. 1995).

1. The cells, mainly vegetative cells, suspended in an environment can be present as single or clump. The length of the shoulder coincides with the time before all, but one microorganism in such a clump has been killed.
2. The shoulder represents a time period, where the cells are able to resynthesize vital components and therefore not directly inactivated by the process, such as temperature.
3. Components in the media, such as fats or proteins, may have a protective effect on the inactivation. The cell is protected by the components and the resistance is increasing.

In summary, the cells are protected by components C_C outside the cells (statement 1 and 3) and/or components in the cell (statement 2). It is based on the hypothesis of a pool of protective or critical components C_C describing the physiological state of the cell, around or in each cell.

The last factor in the equation describes the tailing, showing the existence of a more resistant population N_{res}. The occurrence of tailing can be explained as a consequence of inactivation or resistance mechanism. Furthermore, the tailing can be an artifact due to genetic heterogeneity, heterogeneity of the treatment, clumping or enumeration of survivors (Cerf 1977).
To allow application of the model, the factor C_0 is replaced by the following term including the factor *Sl* (shoulder length).

$$C_0 = e^{k_{max} \cdot Sl} - 1 \qquad \text{Equation 10}$$

$$N(t) = (N(0) - N_{res}) \cdot e^{-k_{max} \cdot t} \cdot \left(\frac{e^{k_{max} \cdot Sl}}{1 + (e^{k_{max} \cdot Sl} - 1) \cdot e^{-k_{max} \cdot t}} \right) + N_{res} \qquad \text{Equation 11}$$

The model is applied in several studies analyzing the survival data of different microorganisms such as *L. monocytogenes* and *Lactobacillus* during a mild thermal treatment (Geeraerd et al., 2000) or *Monilinia fructigena* and *Botrytis cinerea* during pulsed white light treatment (Marquenie et al., 2003).
In those examples modeling inactivation with modified Weibull distribution or other models, treatment time as a comparing factor was used. The treatment time of a PEF treatment or the specific energy as well as other process parameters can be used to describe the graphics. In this case, the units of the scale parameter change, referring to the specific energy required to reduce the first napierian or decimal log cycle depending on the model.

Secondary model

To compare microbial resistance in a wide range of experimental conditions, the mathematical relationship between the parameters from the primary model with different variables investigated, such as electric field strength, pH of the medium or the initial temperature of the product, is studied (Álvarez et al. 2003a; Gómez et al. 2005a, b; García et al. 2009; Saldaña et al. 2010c). The equations used for this purpose are termed as secondary models.

For models based on the Weibull distribution, the shape parameter is independent of the electric field strength for the same microorganism. In this case, the model is readjusted to the initial survival curves using one single value as a parameter, the average value obtained for the different treatments. In such cases, the final model is simplified, since the equation is defined by the scale parameter, because the shape parameter is constant for all treatments (Mafart et al. 2002; Álvarez et al. 2003a, Monfort et al., 2010a, b; Saldaña et al. 2010a). Nevertheless, this is not always the case, because of dependence of strain and media. To characterize microbial resistance to PEF, Álvarez et al. (2003a) proposed the use of, together with the half-scale value, the value Z_{PEF}. This

is defined as the increase in the electric field strength necessary to reduce the scale parameter one log cycle. However, the relationship between the scale value and the electric field is not always linear, so the use of parameter Z_{PEF} is not generalized.

The ideal situation would be that these secondary models had biological bases, were simple and permitted the study of the influence of the process parameters on the primary model. However, it is possible to find relationships between the parameters and the studied variables of different types (linear, sigmoidal, polynomial, etc.). For example, Gómez et al. (2005a, b) and Saldaña et al. (2010b) used secondary models to describe the relationship between the pH of the product and the treatment and the shape parameter (ρ), an empirical model based on the Gompertz equation (Gompertz 1825):

$$\rho = A + C \cdot e^{-e\cdot(-B\cdot(pH-M))} \qquad \text{Equation 12}$$

where *a*, *b*, *c* and *M* are model parameters. It is also possible to approach the study of several variables of influence in the primary model, with the use of multiple regression analysis. Multiple regression analysis allows not only the influence of individual variables, but also their interactions (Gómez et al. 2005a, b).

Tertiary model

The inclusion of secondary models in the general expression model, allows the creation of the corresponding tertiary or final models. Once the final expression is obtained, the model should be validated with new experimental data, using different conditions to show the accuracy of the fit (Whiting 1995).

To validate the final model, different statistical parameters can be used. Some of them are the coefficient of determination (r^2), the mean square error (RMSE), accuracy factor (A_f) and the bias factor (B_f). The meaning and mathematical expressions of these statistical parameters are shown below in section 3.4.3. Other graphics, such as the comparison of real data versus predicted data, are useful to check that there is proper alignment between the estimated values and the real answer.

The final model can be used to predict the microbial inactivation by PEF considering the treatment parameters (electric field strength, treatment time or specific energy applied) and environmental variables studied (pH, sugar, the presence of antimicrobial agents, fat, treatment temperature, etc.). On the contrary, it is also possible to determine the processing conditions for a given level of inactivation in the microbial population (Gómez et al. 2005a, b; Saldaña et al., 2011). Therefore,

the use of mathematical models is a basic tool, not only to study the kinetics of inactivation of microorganisms, but also from a practical side. Several industrial applications can be applied here to ensure the safety of food, or to ensure efficient treatments in terms of energy applied (Álvarez et al. 2003a).

3. Materials and Methods

3.1 Spore preparation

3.1.1 *Bacillus subtilis* spores

The strain *Bacillus subtilis* subsp. *subtilis* PS832 from BGSC (Bacillus Genetic Stock Centre, Ohio State University, Columbus, USA.) was used. The first step of spore production was the cultivation of vegetative cells by inoculation of Standard I nutrient broth with *B. subtilis* cells. The culture was incubated for 12 to 18 h at 37 °C until it reached its stationary growth phase. After incubation, 200 µl was spread on Difco-sporulation agar plates. For preparation of Difco-sporulation agar plates, a solution containing 5 g/l peptone, 3 g/l meat extract, 1 g/l potassium chloride (Carl Roth GmbH + Co. KG, Karlsruhe, Germany.), 0.12 g/l magnesium sulphate heptahydrate and 20 g/l agar agar was prepared. The solution had a pH of 7.0 to 7.2 and was sterilized in an autoclave (Systec VX-95 Autoklav, Systec GmbH, Wettenberg, Germany.) at 121 °C for 15 min. The agar was cooled, then 1 ml/l 1 M calcium nitrate, 1 ml/l 1 M manganese-II-chloride and 1ml/l 1 mM ferrous-II-sulphate was added and the agar was poured. The inoculated plates were stored at 37 °C for 5 to 7 days. The sporulation was observed by analyzing the culture under phase microscope (Axiostar Plus, Carl Zeiss Jena GmbH, Goettingen, Germany.). When more than 75 % of the cells had sporulated, they were harvested. To remove the spores from the plate, 30 ml distilled water was added and the spore suspension was filled in sterile falcon tubes. The vegetative cells of the suspension were inactivated by a heating step (80 °C for 10 min) and then removed by centrifugation at 4816xg for 20 min (Thermo Scientific Multifuge XR3, Thermo Fisher Scientific Inc., Waltman, USA.). The supernatant was discarded and the pellet was suspended in 30 ml cold (4 °C), sterile and distilled water. The washing procedure was repeated in triplicate to achieve a clean spore suspension. The spores were stored at 4 °C until they were ready to be used.

To evaluate the effect of PEF on *B. subtilis* spores, the concentration (spo/ml) before and after treatment was analyzed. Therefore, the cold samples were heated to 80 °C for 10 min to inactive vegetative *B. subtilis* cells. The spore suspension was diluted in saline solution and spread out on Standard I nutrient agar plates. After incubation for 24 h at 37 °C the numbers of colonies were counted and the spore concentration (spo/ml) estimated. Each sample was analyzed in triplicate.

3.1.1.1 Lysozyme assay

A decoated spore was used in the study to analyze the influence of the coat on the resistance to PEF treatment. The mutant strain ΔcotE::tet from *Bacillus subtilis* subsp. *subtilis* PS832 was used.

To check, if the mutantdoes not contain a coat, a lysozyme test was performed, because the coat is responsible for the lysozyme resistance of spores (section 2.1.3). The trial was performed according the method established by Riesenman et al. (2000). The lysozyme resistance of the wild type and mutants were tested.

A spore suspension with a concentration of approx. 10^8 spo/ml in Ringer´s solution with a conductivity of 4 mS/cm was prepared and 0.5 mg/ml lysozyme was added. All samples including a control sample without lysozyme were incubated at 37 °C for 10 min. The spore concentration was also determined as described above. The spore concentration after treatment, without the lysozyme was set to 100 % and the spore survival fraction was calculated.

3.1.2 *Alicyclobacillus acidoterrestris* spores

As representative spore in low or high acid juices *Alicyclobacillus acidoterrestris* was analyzed. In this study, *Alicyclobacillus acidoterrestris* DIL 5132 (DSM 3922T) was used.

For sporulation, the vegetative cells were first spread on agar. The agar comprised of 0.25 g/l calcium chloride, 0.5 g/l magnesium sulfate, 0.2 g/l ammonium sulfate, 3.0 g/l mono potassium phosphate, 2.0 g/l yeast extract and 5.0 g/l glucose (DSMZ- Medium No. 402). For the second solution, agar in a concentration of 15 g/l was diluted with water.

After sterilization in autoclave (Systec VX-95 Autoklav, Systec GmbH, Wettenberg, Germany.) at 121 °C for 15 min and cooling down to 50 °C, both solutions were mixed. Additionally 100 µl/l trace element solution (DSMZ- Medium No. 27) and 1 ml of 5g/50ml manganese sulfate solution were added.

The vegetative cells were spread on this agar and cultivated for 1 to 3 days at 45 °C. After incubation one single colony was taken from the plate and put into 25 to 30 ml of the medium (without agar). The flasks containing the *A. acidoterrestris* cells were shaken for 16 to 30 h at 45 °C and 250 rpm. 10 ml of this pre cultivation was taken and put in 500 ml fresh medium (without agar) and shaken for 5 days at 45 °C for 200 rpm.

The sporulation of cells was obeserved by using phase contrast microscopy (Axiostar Plus, Carl Zeiss Jena GmbH, Goettingen, Germany.). When the spore concentration was about 80 to 90 %, the spores were harvested. Therefore the solution was centrifuged (Thermo Scientific Multifuge XR3, Thermo Fisher Scientific Inc., Waltman, USA.) at 4816 g for 30 min at 4 °C. The supernatant was removed and the pellet was suspended in 50 ml cold sterile demineralized water. After second centrifugation step at 4 °C and 4816 g for 30 min, the pellet was resuspended in 50 ml 70 % ethanol to inactivate the vegetative cells. The suspension was centrifuged (4816 g, 4 °C, 30 min) to have a clean spore suspension.

The spores were stored in demineralized water at 4 °C. A spore count was done to calculate the amount of spores required for the trials. Therefore, the suspension was plated on the previously described medium 402 (DSMZ- Medium No. 402). After incubation at 45 °C for 3 days, the spores were count and the spores per ml (spo/ml) were estimated.

3.1.3 *Geobacillus stearothermophilus* spores

For the preparation of *Geobacillus stearothermophilus* (DIL 5133, DSM 5934) spores, a vegetative culture, previously stored at -20 °C was spread out on Standard I nutrient agar and incubated at 55 °C for 24 h was used. The plates were stored in a bag to avoid drying out of the agar. After incubation one colony was picked from the plate and put in a test tube containing 10 ml TSB medium. The tube was incubated at 55 °C and shaken at 250 rpm. During incubation, the optical density (OD_{600}) was controlled by UV/visible spectrometer (UltroSpec10, Amersham Biosciences Europe GmbH, Freiburg, Germany.) at a wavelength of 600 nm. When the OD_{600} reached a value between 1.6 and 1.8 200 µl of the cell suspension was spread on Difco- sporulation agar (section 3.1.1) to start sporulation of the vegetative cells. The plates were stored in a bag to avoid drying and incubated at 55 °C. The sporulation was observed under phase contrast microscope (Axiostar Plus, Carl Zeiss Jena GmbH, Goettingen, Germany.). If the spore concentration was higher than 95 %, the spores were harvested. This was typically achieved 5 – 7 days after incubation. Therefore, 5 ml cold (4 °C), sterile and distilled water was poured on each plate. Afterwards, the suspension was collected in 50 ml Falcon tubes. For cleaning the spore suspension, which means separation of vegetative *G. stearothermophilus* cells and *G. stearothermophilus* spores, the suspension was centrifuged at 3074 g for 20 min at 4 °C (Thermo Scientific Multifuge XR3, Thermo Fisher Scientific Inc., Waltman, USA.). The supernatant was discarded and the pellet was suspended in 5 ml cold, sterile and distilled water. The purity of the suspension was observed under phase contrast microscope. When approximately, 95 % free spores were detected, the suspension was heated up to 80 °C for 15 min to inactivate vegetative cells. The final spore suspension was stored at -20 °C.

To estimate the spore concentration of the spore suspension, the sample was first diluted and spread on Standard I nutrient agar plates. After incubation at 55 °C for 24 h, the colonies were counted and the spore concentration was estimated.

3.1.4 Germination control

At specific conditions, e.g. heat or availability of nutrients, bacterial endospores start to germinate. This means, the spore is converted to a vegetative form.

To determine the amount of germinated spores, the samples were analyzed with and without an additional heat treatment of 80 °C for 10 min. The results without heat treatment indicate the number of spores and germinated/vegetative cells in the sample. After the heat treatment at 80 °C for 10 min, the germinated/vegetative microorganisms were inactivated, and the results of the count represent the spore count. The difference of count with and without heat treatment indicates the amount of geminated spores.

3.2 PEF equipment

3.2.1 Continuous operating PEF system

For all trials performed the Elea® 5 kW system (Elea®, Quakenbrueck, Germany.) developed by DIL was used. The technical data can be found in Table 3-1.

Table 3-1: Technical data PEF system Elea® 5 kW.

Size(Height-Width-Depth)	1600 mm – 1475 mm – 1100 mm
Rated Power	5 kW
Output Voltage	0 … 25 kV
Output Current	0 … 200 A
Pulse Repetition Rate	0 …. 1000 Hz
Pulse Duration	4 … 32 µs
Pulse Power	3 MW
Pulse Shape	Rectangular Pulse, Alternating polarity

The principle for pulse formation is the generation of low voltage pulses by a high voltage generator, which transforms into high voltage pulses.

The start of the intermediate circuit diagram is the power supply (Figure 3-1). Capacitors, acting like accumulators slowly charge by the power supply. The power rate is at a very high level (6 MW). In total, 4 switches are installed to control the maximum output current of the generator. They consist of IGBT (Insulated Gat Bipolar Transistor) and are able to switch many thousands of amperes. When switched off, or during a pulse break, all transistors are locked. For emitting a pulse, two transistors that are diagonally arranged are switched on. By these diagonally arranged switches, it is possible to apply bipolar pulses. By using bipolar pulses, the treatment efficiency is increased and also helps avoid the electrolysis process, which damages the treatment chambers. The circuit is completed by the connection of the capacitors to the transformer. The transformer transmits the voltage in the ratio of 1:24, which means 1000 V are transformed into 24 kV in the treatment chamber. At the same time, the current is reduced, in the ratio of 1/24.

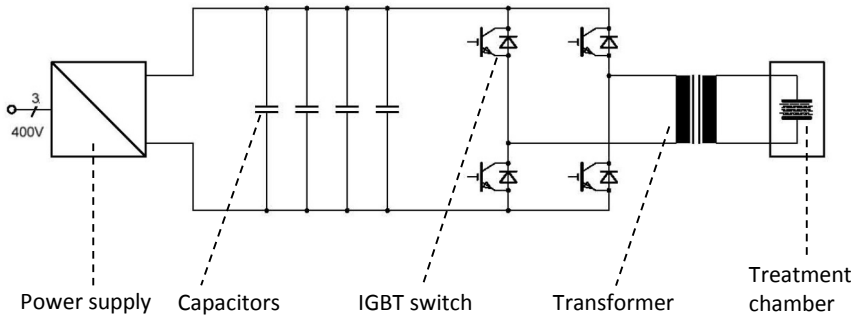

Figure 3-1: Circuit diagram of PEF system.

The transformer delivers the high voltage directly to the treatment chambers. Two treatment chambers with a colinear configuration are installed in this unit. Therefore, a stack of 5 rings is installed consisting of the following arrangement: metal, isolator, metal, isolator and metal ring. The metal rings, representing the electrodes, are made of titanium and the isolator made of ceramic. The metal ring in the middle is connected to the pulse transformer and the other connector of the transformer is connected to both other outer metal rings (Figure 3-2). The product is pumped through the stack and exposed to the electric field in the area of both isolators. The diameter of each treatment area is 10 mm and the inter electrode gap is 10 mm. The total volume of the treatment zones is 1.58 cm³.

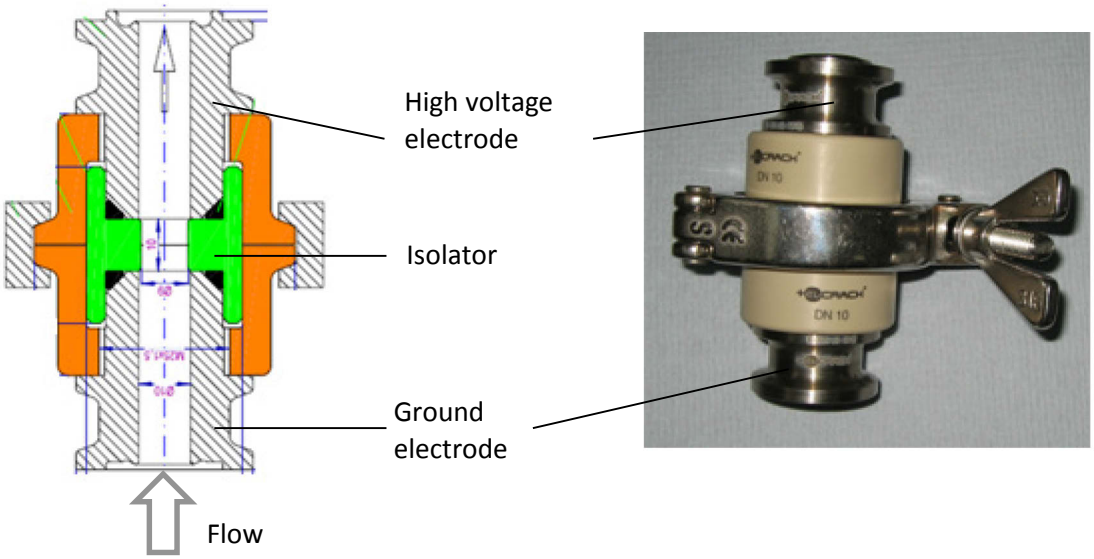

Figure 3-2: Colinear treatment chamber consisting of two electrodes made of titanium and an isolator made of ceramic with a dielectrode gap of 10 mm.

Thus, using a colinear treatment chamber configuration inhomogeneous electric field distribution occurs (Figure 3-3). The highest applied electric field strength appears at the shortest possible

distance between high voltage electrodes. Due to the colinear design, the highest field strength is applied at the inner surface and the lowest in the center of the treatment chamber.

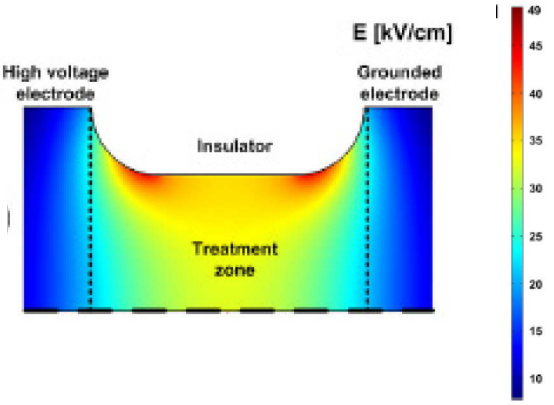

Figure 3-3: Electric field distribution in a co linear treatment chamber (adapted from Meneses et al. (2011)).

As the energy applied is directly transformed to thermal energy a temperature increase can be measured. When estimating the temperature time profile of the PEF process, only the average temperatures are measured. The temperature increase is a function of applied energy, inlet temperature and specific heat capacity of the product (Equation 13).

$$T_{out} = \frac{W_{specific}}{c_p} + T_{in} \qquad \text{Equation 13}$$

3.2.2 Experimental design – Basic studies

Each trial was performed using an Elea® 5 kW PEF system with a flow rate of 30 l/h. Ringer´s solution was prepared prior to the trials consisting of 8.6 g/l sodium chloride, 0.3 g/l potassium chloride and 0.33 g/l calcium chloride. The concentrations varied depending on the required conductivity. Afterwards, the product was inoculated with spores to a concentration of 10^5 spo/ml and pumped by a positive displacement pump (Sondex, Winsen (Luhe), Germany.) through plate heat exchanger to pre heat the product to 80 °C. Part of the study comprised of different pre heating temperatures that were studied. After pre heating the product, it was pumped through 2 colinear treatment chambers into the PEF system. Based on the total treatment area of 1.58 cm³, the treatment time is 0.19 s. After PEF treatment, the product is pumped through a plate heat exchanger and cooled to 12 °C. The samples taken were stored on ice prior to spore analysis. The line setup of the trials is shown in Figure 3-4. To avoid air bubbles in the system and to allow temperatures

higher than 100 °C, back pressure was applied. In case of *B. subtilis* and *A. acidoterrestris* spores a pressure of 3 bar was used and for *G. stearothermophilus* 4 bar.

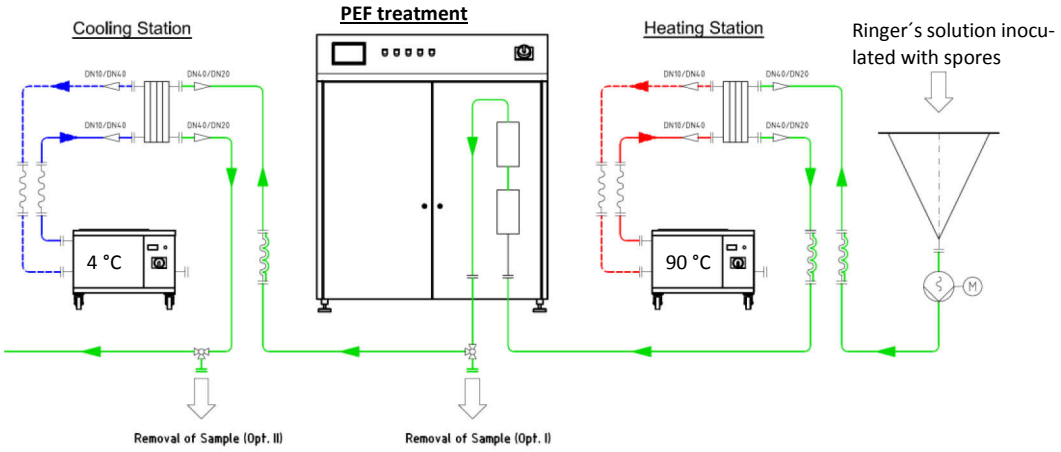

Figure 3-4: Line setup PEF trials consisting of pre heating and cooling by plate heat exchanger and PEF system (Elea® 5 kW).

This experimental design was used to analyze the influence of process- and product parameters on the inactivation of bacterial endospores by PEF in combination with thermal energy. As process parameters, the electric field strength, specific energy and inlet temperature were considered. Depending on the conductivity of the product the electric field strengths varied from 4 to 15 kV/cm. The maximum applied electric field strength is directly linked to the conductivity, which is the reciprocal of the resistance of the product, and therefore to the maximum current output. At high conductivity, the resistance is low and therefore a high current is required to apply high electric field strength. However, besides electric field strength, the specific energy varied from 100 kJ/kg to 300 kJ/kg. The specific energy was changed by increasing the frequency, which means, increasing the number of bipolar rectangular pulses with a pulse width of 20 µs. Different inlet temperatures from 56 to 80 °C were used to study synergistic effects between temperature and PEF treatment.

Beside process parameters, the influence of different ingredients was analyzed. Most importantly, product characteristics, such as salt-, sugar- and fat content were varied as well as pH environment was studied. To analyze the influence of salt content, Ringer´s solution in different concentrations was prepared. The conductivity varied from 1 to maximum 15 mS/cm. With respect to the pH value, the Ringer´s solution was used without adding acid to analyze inactivation at neutral pH. To analyze inactivation at lower pH, the Ringer´s solution was acidified to pH 4 by adding 1 M hydrochloric acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany.). Sugar levels from °Brix of 5 and 10

were analyzed in this study by adding the amount of sugar to the Ringer´s solution. To analyze the influence of fat on the inactivation, 5 and 10 % sunflower oil was added to the suspension. Before mixing lecithin as emulsifier in a ratio of 1 % from fat phase, was added to the oil. As an example, for the preparation of 10 l Ringer´s solution with 5 % fat, 5 g lecithin was mixed with 495 g oil. Afterwards, Ringer´s solution was homogenized by using a Kotthoff homogenizer (Fluid- Kotthoff Dispergierer Typ mS2, Essen, Germany.) and the fat-lecithin suspension was added very slowly. After 5 min of homogenization, the emulsion was inoculated with the spores and treated by PEF.

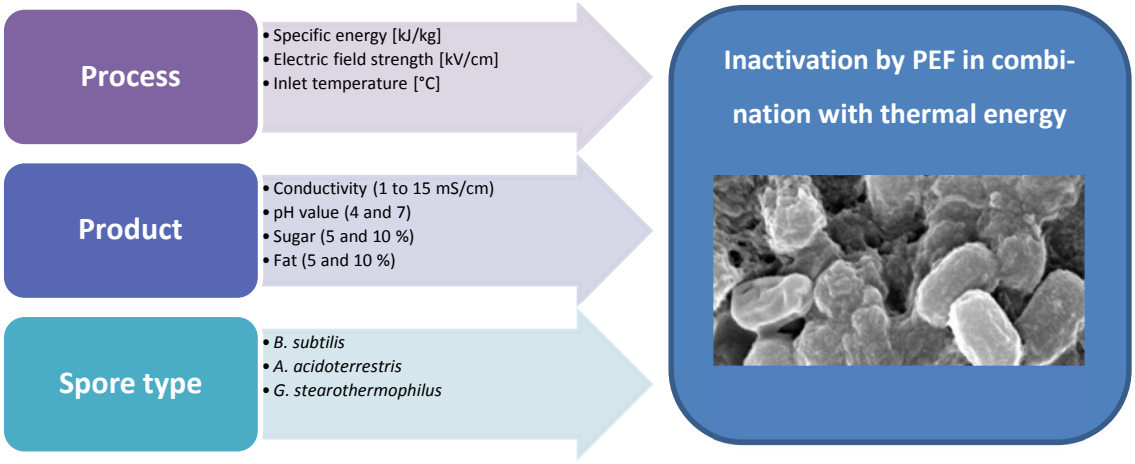

Figure 3-5: Overview of basic trials.

3.2.3 Batch operating PEF system

To treat the spores under isothermal conditions a PEF batch system developed by DIL (Quakenbrueck, Germany.) was used. A treatment chamber was designed consisting of a semi-spherical cup, where the samples are filled in and stamped into a cup (Figure 3-6). The stamp acts as the high voltage electrode and the cup as the ground electrode. This construction enables a quasi-parallel electrode configuration. The critical part within this treatment chamber is the filling area. Based on the capillary force, there might be some parts, which are not treated correctly.

The treatment chamber has a capacity of 4.2 ml and a gap of 0.2 cm, which allows the application of high electric field strengths. The ground electrode of the treatment chamber is connected to a water bath. In total, two water baths for cooling and heating were used, which can be switched by a three way valve. The temperature was controlled by a fiber optic temperature sensor in the high voltage electrode. The measurement part of the fiber optic sensor was placed 3 mm before the treatment area by drilling into the electrode.

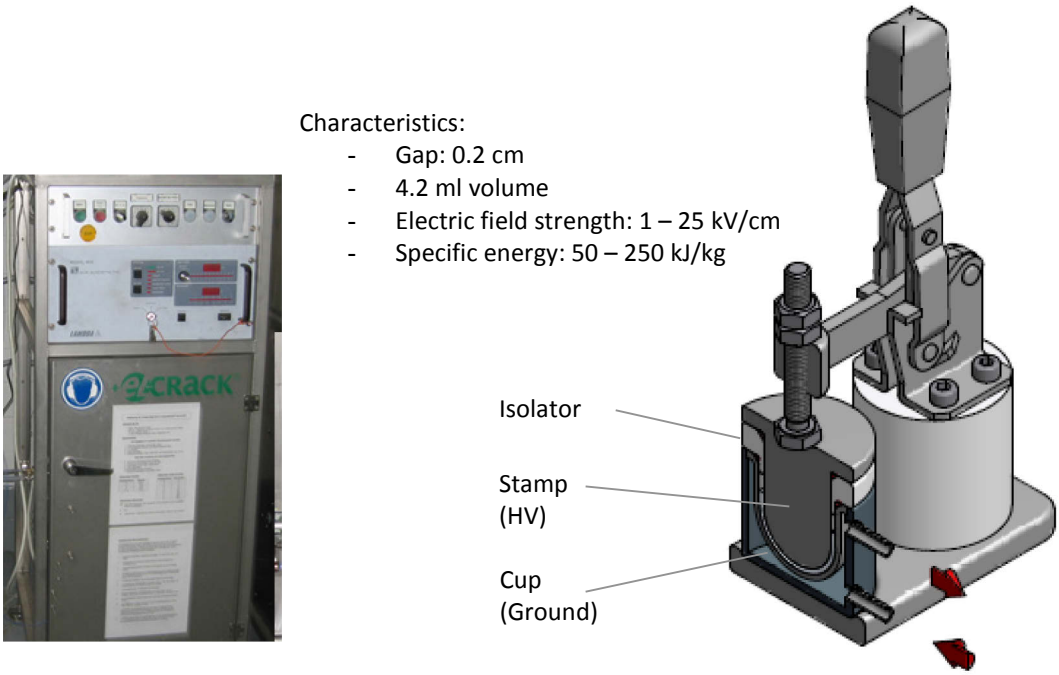

Figure 3-6: Treatment chamber for batch PEF system, which is shown on the left side (HV – High voltage).

For the trial (Figure 3-7), the treatment chamber was placed in the system and connected to the water bath. 4.2 ml of a spore suspension was filled in the cup of the treatment chamber. First the spore suspension was heated up to the desired temperatures. After PEF treatment, the second water batch was connected and the sample was cooled down.

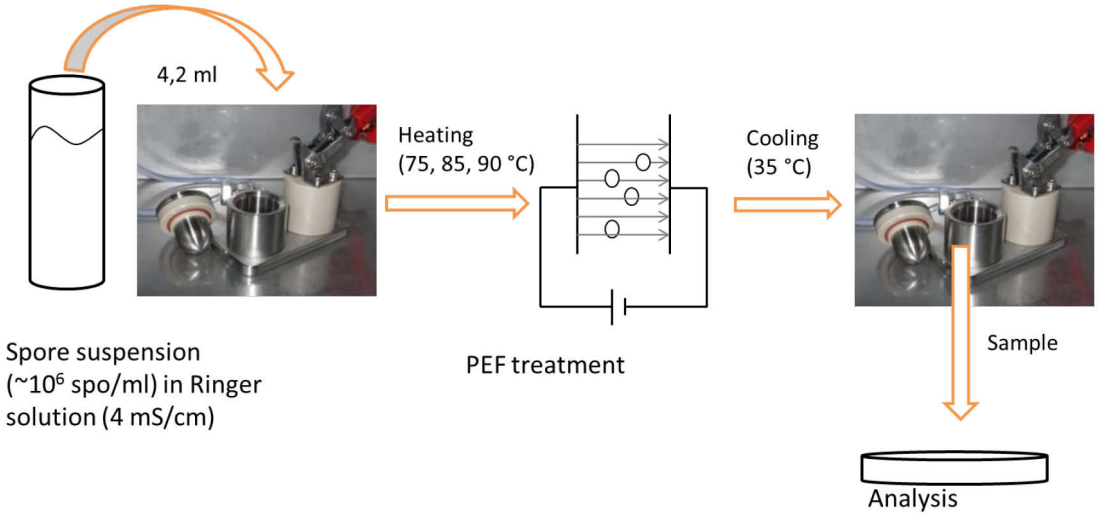

Figure 3-7: Trial performance with batch PEF system.

The temperature was controlled all times. As the heating bath is running, most of the generated heat is taken out by the water and almost isothermal conditions can be assumed. The temperature

is measured only at the surface of the electrode and on the surface of the product. Thus, it can be assumed that the actual temperature is higher than what is shown by the surface electrode reading. However, even if the temperature of the product increases up to for example 10 °C, this low temperature increase is not sufficient to inactivating the spores. Figure 3-8 presents a temperature profile pre heating up to 80 and 90 °C.

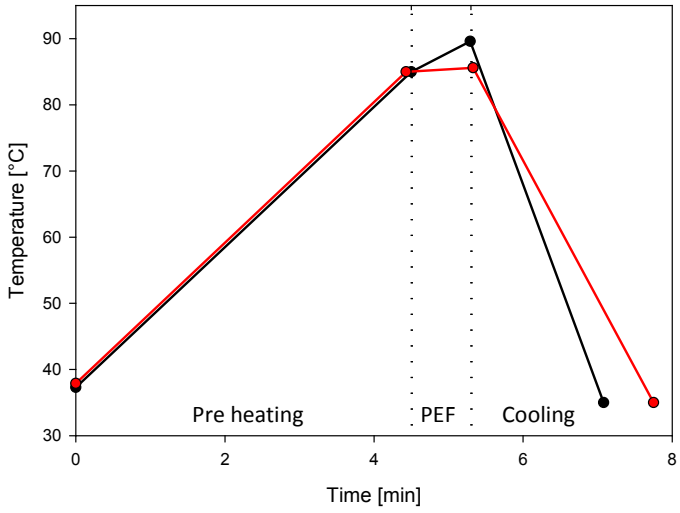

Figure 3-8: Temperature time profile with (●) and without (●) PEF treatment including pre heating, PEF treatment (250 pulses at 4 Hz) and cooling.

The electric field strength was adjusted by the output voltage. The specific energy was calculated based on the number of pulses applied. The batch system applies exponential decay pulses. Therefore, the equation used to calculate the specific energy per pulse (W_{pulse}) is:

$$W_{pulse} = \frac{1}{2} \cdot C \cdot U^2 \qquad \text{Equation 14}$$

where C is the capacity (0.5 µF for this system) and U the voltage in kV. Considering the volume and the pulse number, the specific energy can be calculated. As pulse shape, exponential decay pulses were applied.

3.3 Spore analysis

3.3.1 DPA assay

The DPA measurement was performed by measuring the Tb/DPA fluorescence based on the method developed by Rosen (1999). The fluorescence signal was detected by spectrophotometer (Jasco FP-8000, Groß-Umstadt, Germany.) and the data was recorded by Spectra measurement

software Spectra Manager Version 2.09.01 (Jasco Corporation, Rokyo, Japan.). The samples were measured at an excitation wavelength of 270 nm with a slit width of 5 nm. The emission was recorded from 400 to 600 nm with a scan speed of 50 nm/min (Figure 3-9). The peak area (PA) was determined by the software Spectra Manager Version 2.09.01 (Jasco Corporation, Rokyo, Japan.).

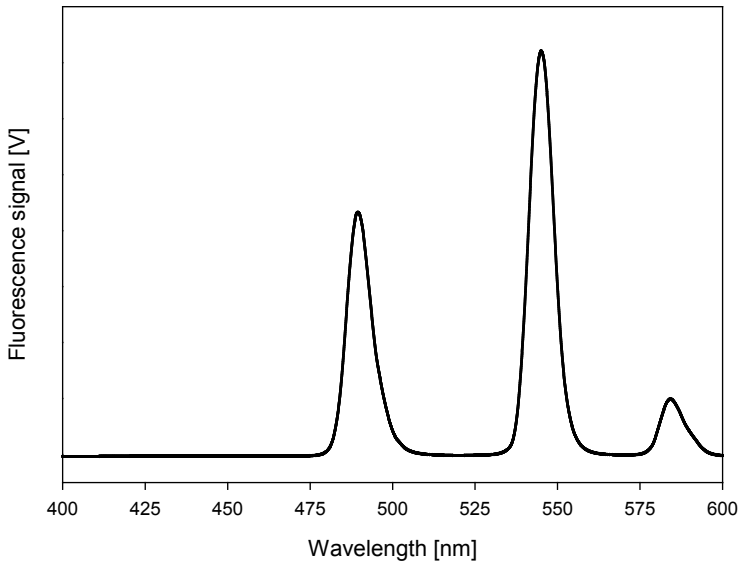

Figure 3-9: Scheme of DPA spectra of 12.5 µM DPA solution.

All samples including the samples for creating the calibration curve were treated the same way. Each sample was diluted 1:10 with Tris-HCl-$TbCl_3$ solution, which composed of 0.05 M Tris-HCl buffer (pH 7), 30 µM terbium(III)chloride and 4 % ethanol (v/v). After mixing the sample and the Tris-HCl-$TbCl_3$ solution, the mixture was sterilized filtered and measured. To determine the quantitative values for DPA concentration stock solution, it was measured. The linear correlation of the specific DPA concentration and the peak area, which is the area under the spectra, was estimated (Figure 3-10). The linear equation was used to calculate the DPA concentration of the samples.

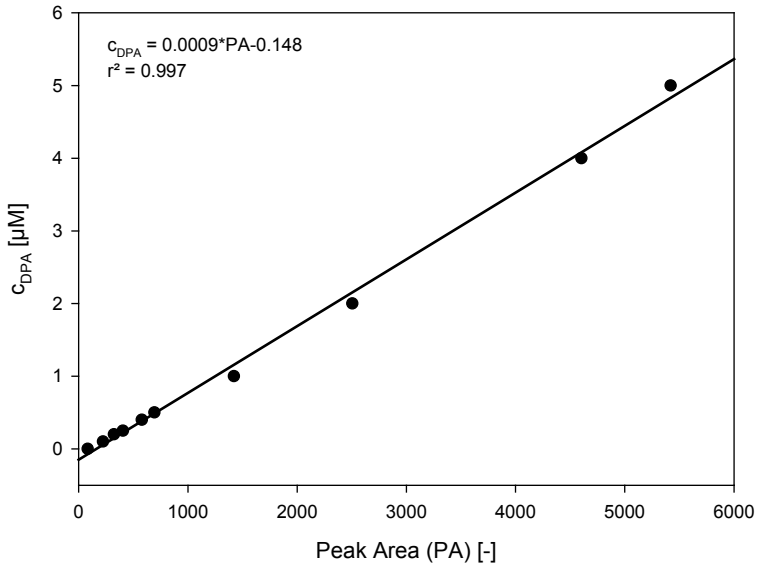

Figure 3-10: Linear correlation between the DPA concentration (c_{DPA}) and peak area (PA).

3.3.2 Microscopic spore analysis

Scanning electron microscopy

For SEM analyis, the samples were frozen with supercooled liquid nitrogen. Afterwards, the sample was sputtered on a thin gold film. During the investigation using high-resolution scanning electron microscope (JSM6460LV, Joel, Japan), the sample surface was scanned with a high energy electron beam in an isometric pattern.

Confocal laser scanning microscopy

To visualize the protein micrographs with confocal laser scanning microscopy (CLSM) the following procedure was performed. The spores were suspended in Ringer´s solution (4 mS/cm). After PEF and thermal treatment, the samples were centrifuged at 4816xg for 10 min at 4 °C (Thermo Scientific Multifuge XR3, Thermo Fisher Scientific Inc., Waltman, USA.) and the pellet was suspended in cold sterile, distilled water. One drop on each sample was put on a glass tray, where afterwards a few particles of rhodamine B isothiocyanate were added. The sample was covered by a cover slip and inspected under the microscope (Eclipse E600, Nikon) at a magnification of 100x. The pictures were edited by the program EZ-C1 (Nikon C1 Confocal Microscope Navigation). For each sample different sections were analyzed.

3.4 Mathematical modeling

3.4.1 Thermal inactivation kinetics

Thermal resistance of bacterial endospore is mostly described by D_T and z value. Within this study these values were estimated for all analyzed spore types (*B. subtilis*, *A. acidoterrestris* and *G. stearothermophilus*).

To estimate the D_T value, the inactivation of the spore at different temperatures and temperature holding times are required. The glass capillary method developed by Haas et al. (1996a, 1996b) was used to determine the thermal inactivation kinetics. Numerical simulations performed by Jaeger et al. (2010) analyzing the temperature time profile in the capillaries showed that with this method a fast heat transfer as well as accurate holding times in the range of seconds can be achieved. Ringer´s solution with a conductivity of 4 mS/cm and different pH (4 and 7) were prepared and inoculated with spores resulting in a concentration of approximately 10^5 spo/ml. The suspension (100 µl) was filled in glass capillaries with a length of 300 mm and a diameter of 0.9 mm (Kleinfeld Labortechnik GmbH, Stötefeld, Germany.), which were sealed afterwards and immersed in an oil bath (Type Haake B3 DC 50, PSL-Systemtechnik, Clausthal-Zellerfeld, Germany.). The temperatures ranged from 90 to 130 °C and the holding time from 2 to maximum 700 s. After the heating step, the samples were immediately cooled on ice. Every temperature time combination was repeated in triplicate.

The results of the glass capillary method were used to calculate the D_T value describing the time required to inactivate 90 % of the studied spore types. Therefore, the inactivation calculated by the logarithm of the spore count after treatment (N) divided the logarithm of the spore count before treatment (N_0) was plotted against holding times at a defined temperature. The D_T value for a specific temperature was graphically estimated by reciprocal slope of each plot. To estimate the z value for each spore type the logarithm of D_T value was plotted against temperature at which D_T value was obtained. The reciprocal slope of each plot gives the z-value.

3.4.2 Modeling thermal inactivation during PEF process

The separation of total experimental inactivation in thermal and PEF induced inactivation is calculated by developing a model which combines thermal inactivation data of spores and the temperature time profile. The method was firstly described by Jaeger et al. (2010).

As already discussed in section 3.4.1, the thermal inactivation of the analyzed spores is determined by using the glass capillary method. Different temperatures and holding times were tested

to calculate the thermal inactivation. To describe the thermal inactivation a first order kinetic was assumed by Equation 15.

$$\frac{dN}{dt} = -k \cdot t \qquad \text{Equation 15}$$

where *k* is the inactivation rate and *t* the treatment time (s). The inactivation rate for different temperatures can be estimated by plotting the natural logarithm of (N/N_0), where *N* represents the spore count after thermal treatment and N_0 the initial spore count, against temperature holding time *t*. The inactivation rates at different temperatures where inactivation rate is a function of activation energy E_a and factor k_0, the Arrhenius equation was used (Equation 16)

$$\ln k(T) = -\frac{E_a}{R} \cdot \frac{1}{T} + A_0 \qquad \text{Equation 16}$$

where *R* is the universal gas constant, *T* the temperature (°C) and A_0 the pre factor. By substituting Equation 16 into equation 15, the thermal inactivation of spores depending on temperature and temperature holding time can be calculated, resulting in Equation 17.

$$\frac{N}{N_0} = e^{-A_0 \cdot t \cdot e^{(-\frac{E_a}{R}\frac{1}{T})}} \qquad \text{Equation 17}$$

To estimate the contribution of thermal inactivation, the temperature time profile of PEF process was developed.

The PEF process can be divided in 4 parts: (A) pre heating, (B) PEF treatment, (C) temperature holding time in PEF unit and (D) cooling. For pre heating and cooling plate heat exchangers type S4A-IT10-16-TL-Liquid (Sondex Deutschland GmbH, Hamburg, Germany.) were used. Each exchanger with a heat transfer rate of up to of 12 kW consists of 16 plates with a filling volume of 0.188 l for each plate. The temperature time profile was determined by a numerical solution. Therefore Equation 18 was assumed.

$$\frac{dT_2}{dt} = (T_1 - T_2) \cdot n \qquad \text{Equation 18}$$

Temperature T_1 denotes the temperature of the heating (90 °C) and cooling medium (4 °C) and T_2 the temperature of the product. The proportionality factor, *n* is calculated by solving the equation knowing the start and end temperature of the product. For pre heating, *n* is assumed as 0.075 s^{-1} and for cooling 0.172 s^{-1}.The temperature evolution is calculated by solving the equation at different time intervals. Implementing temperature and related time values in TableCurve 2D version 4

(SPSS Inc., Chicago, USA.) an equation describing the pre heating (Equation 19) and cooling (Equation 20) phase can be estimated. The equation for pre heating is in all analyzed cases the same, because the pre heating temperature is the same. As the energy input varies, the temperature also varies. Different values for equations describing the temperature development in cooling process results are shown below for pre heating and cooling (Table 6-3).

$$T_{2,pre\ heating}(t) = 33 + 57 \cdot (1 - e^{-0.078 \cdot t}) \quad \text{Equation 19}$$

$$T_{2,cooling}(t) = a + b \cdot (e^{-\frac{t}{c}}) \quad \text{Equation 20}$$

After pre heating, the product is pumped through the PEF system. In contrast to the temperature time development in plate heat exchanger, temperatures are measured by fiber optic sensors (Polytec FOTEMP-4, Waldbronn, Germany.). The temperature profile in the PEF system consists of a temperature holding part at 80 °C (5.2 s), treatment chambers (1.3 s) and pipe until cooling (16.1 s). To measure the temperature in the system, a so called T-piece was installed before the entrance of the PEF unit. One opening was connected to the pump, so that the product is pumped through the PEF unit. The fiber optic sensor was put in the second opening using a silicon seal to prevent leakage. A second T-piece was installed at the exit of the system. The temperature was measured at defined points (Figure 3-11).

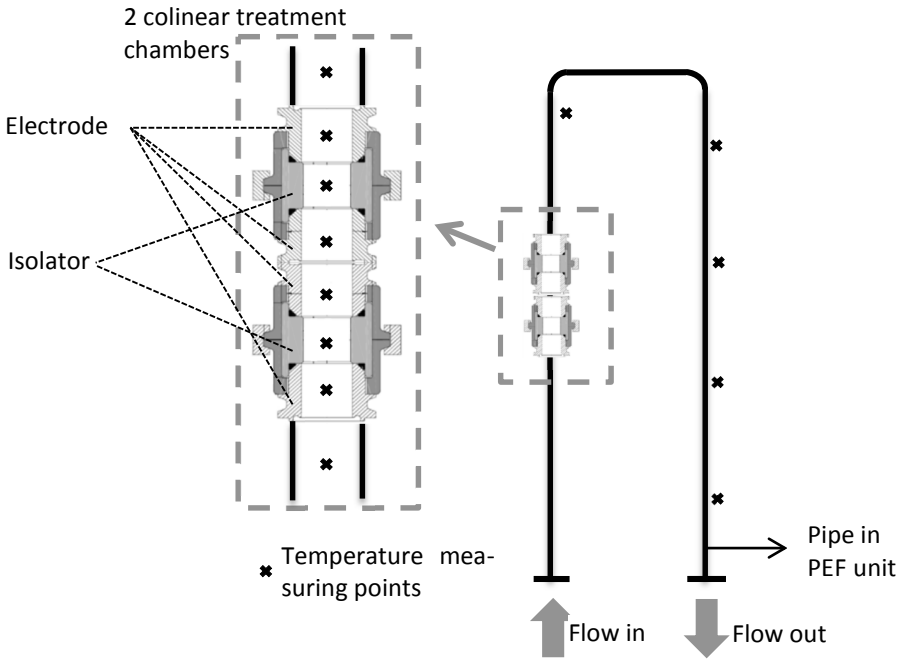

Figure 3-11: Temperature measuring points in the PEF unit with a pipe diameter of 10 mm and 2 colinear treatment chambers with an inter electrode gap of 10 mm.

The equations describing the time temperature profile in plate heat exchanger and experimentally measured temperatures in the PEF unit were merged using Mathcad Software version 13.1 (MathSoft Engineering and Education Inc., Cambridge, USA.) to form the complete temperature time profile of PEF process.

To model the contribution of thermal inactivation, the data was merged with temperature time profile of the PEF process (Equation 21). The result gives the thermal inactivation during the PEF process. The difference between experimentally determined inactivation and thermally modeled inactivation results in PEF induced inactivation.

$$\frac{N}{N_0} = e^{\left(\int_0^t -k(T(t))dt\right.} \qquad \text{Equation 21}$$

To describe thermal processes the F value, termed as thermal death time, is used. The F value is a function of temperature time profile (*T(t)*) of the analyzed process and a reference temperature ($T_{Reference}$), which is in this study was 121 °C. By integration of Equation 22 the F value is given, which means the time required to inactivate a known population in a defined medium at 121 °C as reference temperature. For calculation of thermal death time for thermal process ($F_{thermal}$), the Equation 23 is used, which represents the minimum F value for spore inactivation based on the D_T value.

$$F_{PEF} = \int_0^t \frac{T(t) - T_{Reference}}{z} dt$$ Equation 22

$$F_{thermal} = D_{121} \cdot (logN_0 - logN)$$ Equation 23

3.4.3 Formation of isolog graphs

To develop isographs in order to demonstrate the influence of the process and product parameters the GInaFiT (Geeraerd and Van Impe Inactivation Model Fitting Tool) developed by Geeraerd et al. (2005) was used. The inactivation at different conditions shows a smooth initiation of the inactivation (shoulder) followed by a faster linear inactivation. To connect these parts the dynamic GInaFiT tool applying the model developed by Geeraerd et al. (2000) was used.

The process to develop a mathematical model is separated into 3 steps.

1. Primary model

   The experimental data showed a nonlinear distribution. A linear and shoulder equation was appropriate to describe the exponential data according to Geeraerd et al. (2000).

   $$log\frac{N}{N_0} = -\frac{k_{max} \cdot W}{\ln 10} + log\left(\frac{e^{k_{max} \cdot Sl}}{1 + (e^{k_{max} \cdot Sl} - 1) \cdot e^{-k_{max} \cdot W}}\right)$$ Equation 24

   where k_{max} (kJ/kg)$^{-1}$) is the slope of the linear part and *Sl* (kJ/kg) is the specific energy required to reach the shoulder limit. K_{max} and *Sl* were calculated for each spore and process condition.

2. Secondary model

   To describe the relationship of k_{max} and *Sl* with process parameters (*E*, *W* and *T*), a multiple regression tool was used (TableCurve 3D, SPSS Inc., Chicago, USA.) resulting in a 2nd order mathematical expression.

3. Tertiary model

   The inclusion of the secondary models in the general expression or primary model (Equation 24) results in the final or tertiary model. The obtained equations described accurately the inactivation of spores by PEF in all the range of experimental conditions.

Each final model was validated by calculating the coefficient of determination r^2, the mean square error *RMSE*, accuracy factor A_f and the bias factor B_f.

The observed data was plotted against the predicted values and the fit of the line of equivalence was analyzed. A high coefficient of determination r^2 and a low mean square error *RMSE* indicate the precision of the model.

$$r^2 = \frac{\sum_{i=1}^{m}(x_{i,2}-\bar{x_i})^2}{\sum_{i=1}^{m}(x_{i,1}-\bar{x_i})^2} \quad \text{Equation 25}$$

$$RSME = \sqrt{\frac{\sum_{i=1}^{n}(x_{i,1}-x_{i,2})^2}{m}} \quad \text{Equation 26}$$

where $x_{i,1}$ is the observed inactivation, $x_{i,2}$ is the value estimated by the model and $\bar{x_i}$ with *m* number of analyzed cases.

The goodness of the fit can be described the accuracy factor A_f and the bias factor B_f modified by Baranyi et al. (1999):

$$A_f = exp\left(\sqrt{\frac{\sum_{i=1}^{m}(lnx_{i,1}-lnx_{i,2})^2}{m}}\right) \quad \text{Equation 27}$$

$$B_f = exp\left(\frac{\sum_{i=1}^{m}(lnx_{i,1}-lnx_{i,2})^2}{m}\right) \quad \text{Equation 28}$$

where $x_{i,1}$ indicates the observed inactivation and $x_{i,2}$ the predicted inactivation with m number of analyzed cases. The accuracy factor is based on “mean square differences” and the Bias factor on “arithmetic mean of the differences”. If the values are similar, the observations are over predicted or provide a higher prediction than another model.

3.5 Case studies

3.5.1 Microbiological analysis

Spore analysis

Yeast extract has a electrical high conductivity. Due to this, yeast extract delivered from Leiber GmbH (Bramsche, Germany.) was diluted with water in a 1:1 ratio. The trials were performed diluting the yeast extract in a ratio 1:10. The final conductivity was 12.5 mS/cm and the pH 5.8. As indicator spore, *B. subtilis* was used. 10 l yeast extract were prepared and inoculated with *B. subtilis* spores in a concentration of 10^5 spo/ml. For carrot juice *A. acidoterrestris* and *B. subtilis* were identified as target spores. A volume of 10 l was filled into a funnel and inoculated to a final concentration of 10^5 spo/ml. The same line setup as used for the basic studies analyzing inactivation

of spores was used (section 3.2.2). Different electric field strengths and specific energies were tested and analyze the effect of these process parameters. As a reference, approx. 100 ml of the inoculated suspension was filled in vessel and autoclaved (Systec VX-95 Autoklav, Systec GmbH, Wettenberg, Germany.) at 121 °C for 20 min. To analyze the efficiency of the thermal and PEF process, the yeast extract samples were stored after treatment at 4 °C as well as room temperature and were analyzed after day 0, 20, 50 and 68. The same was performed with the thermally treated samples. The carrot juice was analyzed every week over a time period of 10 weeks.

Shelf life

The shelf life of the PEF treated yeast extract and carrot juice was estimated. The yeast extract was diluted 1:10, and treated with PEF. The values for specific energy and electric field strength were taken from the trial analyzing the inactivation of the target spores. The yeast extract was treated at 244.9 and 257.0 kJ/kg at an electric field strength of 4.5 kV/cm. After PEF treatment, the samples were filled into sterile containers. The setting identified for PEF treatment of carrot juice was 198 kJ/kg at 9 kV/cm based on the results of spore inactivation by PEF. After treatment, the juice was filled into sterile containers and stored at 4 °C and at ambient temperature.

The shelf life was determined by analyzing the total plate count as well as yeast and moulds counts. The analysis was performed on day 0, 20, 50 and 68 for yeast extract and every week over a duration of 10 weeks for carrot juice. As a reference, the untreated yeast extract was autoclaved for 20 min at 121 °C (Systec VX-95 Autoklav, Systec GmbH, Wettenberg, Germany.) and analyzed in the same way as PEF treated and untreated samples.

To analyze the total plate count, the cold samples were first diluted in Maximum Recovery Diluent and second poured out onto Plate Count Agar. After the incubation time of 3 days at 30 °C the total viable count in colony forming units per gram (cfu/g) was determined.

For the yeast and molds analyses, the samples were diluted in Maximum Recovery Diluent. For efficient analysis, the samples were poured out onto YCG plates and incubated at 37 °C for 5 days. Due to the physical differences of the yeast and mold colonies on the plates, they can be easily identified on the plates and thus counted separately, as they appear different.

3.5.2 Quality analysis

The aim of the quality analysis was a comparison of the influence of thermal and PEF treatment on the quality of yeast extract and carrot juice. An overview of the performed analysis is shown in the following table.

Table 3-2: Overview of the quality analysis.

Yeast extract	Carrot Juice
Color	Color
pH	pH
	Brix
	Total phenol content
	Antioxidant capacity

Color

To ensure a good quality of liquid yeast extract, the color and pH value should not change over the shelf life. To compare the quality of PEF and thermal treated yeast extract, the color and the pH were taken as the main quality parameters and were analyzed in the same day interval as the microbial samples.

The color was measured using Minolta CM600D spectrometer (Konica Minolta, Hannover, Germany). The lightness to darkness (L*) (100 to 0), redness (+) to greenness (-) (a*) and the yellowness (+) to blueness (-) (b*) color parameters were determined. After calibration of the spectrometer, the sample was filled into a glass cell. Depending on the homogeneity of the product, 6 to 10 measurements were done. The net color difference (*ΔE* value) was estimated by the following equation using the parameters L*, a* and b* to allow evaluation of color difference.

$$\Delta E = \sqrt{(\Delta a^*)^2 + (\Delta b^*)^2 + (\Delta L^*)^2} \qquad \text{Equation 29}$$

The calculated net color difference can be assessed by DIN 53230, which is illustrated in the following table.

Table 3-3: Evaluation of the net color difference (ΔE-value) (DIN 53230).

ΔE	Evaluation of net color difference
0-1	No color difference
1-2	Slight color difference (trained eye)
2-4	Detectable color difference
4-5	Intensive color difference
>5	Very intensive color difference

pH value

An electrode based method (Mettler Toledo Seven Easy, Mettler Toledo GmbH, Giessen, Germany) was used to measure the pH of the yeast extract and the carrot juice. The pH was measured in triplicate for statistical significance.

Brix

The sugar content was measured by WINOPAL Multi Scale Automatic Refractometer RFM 81 (WINOPAL Forschungsbedarf GmbH, Elze, Germany.). The Brix value of each sample was measured 5 times and the average as well as standard deviation was calculated. To avoid measuring inconsistency air bubbles in the sample and during measuring were eliminated.

Antioxidative capacity

The method for measuring the resistance of lipids to oxidize in the presence of antioxidants is based on the reaction with a radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) in a methanol solution (Brand-Williams et al. 1995). The reduction of DPPH$^{\bullet}$ can be followed by decrease in its absorbance. In its radical form DPPH absorbs at 517 nm. During reaction (below) with an antioxidant (AH) or a radical species (R$^{\bullet}$) the absorption disappears.

$$DPPH^{\bullet} + AH \longrightarrow DPPH\text{-}H + A^{\bullet}$$

$$DPPH^{\bullet} + R^{\bullet} \longrightarrow DPPH\text{-}R$$

A mixture of 20 µl sample and 980 µl 0.1 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) was prepared and incubated for 10 min at ambient temperature. The absorbance was measured at a wavelength of 517 nm. For determining the antioxidative capacity in the sample, a calibration curve with different ascorbic acid concentrations (0, 10, 25, 50, 100, 150, 200 and 300 µg/ml) was prepared. The resultant regression analysis (r^2=0.993) was used to calculate the ascorbic acid equivalent (AAE [µg/ml]).

Total phenol content

The colorimetric method developed by Singleton et al. (1965) was used to determine total phenol content. The principle is based on the use of Folin-Ciocalteu reagent (FCR) consisting of complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids. The FCR oxidizes phenolates by reducing the heteropoly acids to a blue complex, which can be optically measured. The FCR is not specific and detects all phenolic compounds in the sample.

The samples were mixed with sodium carbonate (10-fold dilution). Afterwards, 125 µl of the diluted samples were combined with 625 µl 0.2 N FCR and incubated for 3 min at ambient temperature. After incubation, 500 µl sodium carbonate was added to the sample and incubated in a thermo mixer at 45 °C for 15 min. The absorbance of the sample was measured at a wavelength of 750 nm by a spectrophotometer (GeneQuant 1300, GE Healthcare Life Sciences, Cambridge, UK.).

To calculate the total phenol content, a calibration curve with different gallic acid concentrations (0, 10, 25, 50 and 100 mg/l) was prepared. From the regression analysis, (r^2=0.998) the gallic acid equivalent (GAE [mg/l]) was estimated.

3.6 Chemicals

For the different trials performed in this study, different chemicals were used. An overview about the chemicals is given in Table 3-4.

Table 3-4: Overview chemicals used for the trials.

Materials	Molecular formular	CAS	Supplier
DPPH (1,1-diphenyl-2-picrylhydrazyl)		1898-66-4	Sigma-Aldrich Co. LLC, St. Louis, MO, USA.
Agar agar			BD (Franklin Lakes, NJ, USA)
Ammonium sulphate	$(NH_4)_2SO_4$	7783-20-2	Carl Roth GmbH + Co. KG, Karlsruhe, Germany.
Ascorbic acid	$C_6H_8O_6$	50-81-7	
Calcium chloride	$CaCl_2\ 2H_2O$	22691-02-7	Carl Roth GmbH + Co. KG, Karlsruhe, Germany.
Calcium nitrate	$Ca(NO_3)_2$	10124-37-5	Sigma-Aldrich Co. LLC, St. Louis, MO, USA.
Ethanol	C_2H_6O	64-17-5	Carl Roth GmbH + Co. KG, Karlsruhe, Germany.
Ferrous-II-sulfate	$FeSO_4$		Carl Roth GmbH + Co. KG, Karlsruhe, Germany.
Folin-Ciocalteu			Sigma-Aldrich Co. LLC, St. Louis, MO, USA.
Gallic acid	$C_7H_6O_5$	149-91-7	Sigma-Aldrich Co. LLC, St. Louis, MO, USA.
Glucose	$C_6H_{12}O_6$	50-99-7	Fluka, Buchs, Switzerland.
Lysozyme		9001-63-2	OVOBEST Eiprodukte GmbH u. Co. KG, Neuenkirchen-Vörden, Germany.
Magnesium sulphate heptahydrate	$MgSO_4\ 7\ H_2O$	13778-97-7	Merk, Darmstadt, Germany.
Manganese II chloride	$MnCl_2$	7773-01-5	Merk, Darmstadt, Germany.
Maximum recovery Diluent			Oxoid Limited; Hampshire; UK.
Meat Extract			Carl Roth GmbH + Co. KG, Karlsruhe, Germany.
Mono potassium phosphate	KH_2PO_4	7778-77-0	Sigma-Aldrich Co. LLC, St. Louis, MO, USA.
Plate count agar			Oxoid Limited, Hampshire, UK.
Potassium chloride	KCl	7447-40-7	Carl Roth GmbH + Co. KG, Karlsruhe, Germany.
Rhodamine B isothiocyanate	$C_{28}H_{31}ClN_2O_3$	36877-69-7	Sigma-Aldrich Co. LLC, St. Louis, MO, USA.
Sodium carbonate	Na_2CO_3	497-19-8	Carl Roth GmbH + Co. KG, Karlsruhe, Germany.
Sodium chloride	NaCl	7647-14-5	Carl Roth GmbH + Co. KG, Karlsruhe, Germany.
Standard I nutrient			Oxoid Limited, Hampshire, UK.
Terbium(III)chloride	$TbCl_3$	10042-88-3	Sigma-Aldrich Co. LLC, St. Louis, MO, USA
Trace element			

TSB Medium	Sigma-Aldrich Co. LLC, St. Louis, MO, USA.
Yeast extract	
YGC Agar	Oxoid Limited, Hampshire, UK.

4. Results and Discussion

4.1 Influence of process parameters on spore inactivation by PEF

4.1.1 Inactivation of *Bacillus subtilis* spores

To describe the effect of inlet temperature, electric field strength and specific energy on the inactivation of *B. subtilis* spores by PEF the dynamic GInaFiT model developed by Geeraerd et al. (2005) was used. The inactivation graphs indicate an initial delay, which is called a shoulder (*Sl*), followed by a linear inactivation part, characterized by the slope (k_{max}). Also, the study performed by Bermúdez-Aguirre et al. (2012) detected occurrences of a shoulder in the inactivation of *B. cereus* by PEF. The used GInaFiT model describes correctly this kind of inactivation kinetics.

Based on Equation 24 the inactivation and the specific energy were fitted to obtain the k_{max} and *Sl* values for each inlet temperature and electric field strength.

Table 4-1: Specific inactivation rate k_{max} and shoulder length (*Sl*) at different electric field strengths and inlet temperatures, coefficient of determination (r^2) in brackets, nd - not detected.

		Electric field strength [kV/cm]		
	Temperature [°C]	6	9	12.5
k_{max} [(kJ/kg)$^{-1}$]	56	0.14 (0.968)	0.09 (0.980)	nd
	70	0.19 (0.967)	0.18 (0.993)	0.14 (0.994)
	80	0.49 (0.955)	0.27 (0.975)	0.27 (0.989)
Sl [kJ/kg]	56	317.63 (0.968)	286.08 (0.980)	nd
	70	236.79 (0.967)	223.95 (0.993)	224.86 (0.994)
	80	178.40 (0.955)	160.55 (0.975)	167.58 (0.989)

The slope of the linear part of the inactivation of *B. subtilis* spores is given by k_{max}. With decreasing temperature k_{max} was decreasing, indicating a higher resistance to PEF treatments at lower temperatures in the linear part of the graph. The effect was high at electric field strength of 6 kV/cm. At higher electric field strengths the difference was smaller, which indicates a lower temperature effect at higher electric field strengths. Comparing k_{max} at a constant inlet temperature and different electric field strengths, the k_{max} value tended to decrease. The highest decrease was obtained from 6 to 9 kV/cm at an inlet temperature of 80 °C. A further increase, from 9 to 12.5 kV/cm caused no change in the slope indicating limited effect of the electric field strength on the energy required for an inactivation. Also, the differences at inlet temperatures of 70 and 56 °C showed only small differences, which demonstrated a low effect of the electric field strength.

The same trend was observed for k_{max} was observed for *Sl*. The influence of the inlet temperature was more significant than the influence of the electric field strength. At inlet temperatures of

80 and 70 °C no change of *SI* was observed with increasing electric field strength. Only at 56 °C a higher *SI* value was obtained at 6 kV/cm compared to 9 kV/cm resulting in a longer shoulder length and therefore higher energy. As a result, the impact of the electric field strength on the inactivation is most obvious at low inlet temperatures. However, the effect of the electric field strength is smaller compared to the effect of the temperature. The difference of *SI* at different inlet temperatures and constant electric field strength is higher compared to the *SI* at constant inlet temperature and different electric field strengths. With increasing inlet temperature a lower *SI* was obtained. Therefore, at higher inlet temperatures less energy is required to start the inactivation.

The next step to develop the final model was to find a relationship between the process parameters and the k_{max} and *SI* values by using TableCurve 3D. The obtained mathematical expressions correspond to the secondary model.

$$k_{max} = (238.340 - 0.00002 \cdot T^3 - 65.931 \cdot \ln(E) - 518.764 \cdot \ln(E)/E)^{-1}$$

r²=0.986

$$SI = -596.076 + \frac{99812.285}{T} - \frac{1805.685}{E} - \frac{2702671.5}{T^2} + \frac{5146.399}{E^2} + \frac{50373.528}{T \cdot E}$$

r²=0.999

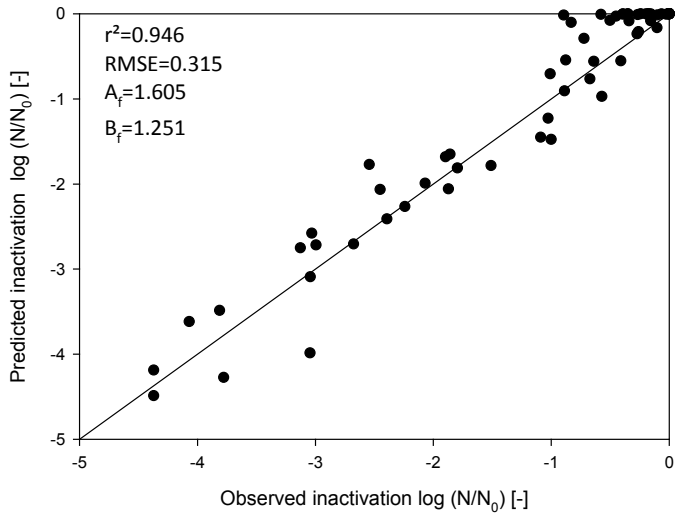

Figure 4-1: Regression analysis of calculated and experimental estimated inactivation of *B. subtilis* at different energy values, electric field strengths and inlet temperatures (r^2 = determination coefficient, *RMSE* = root mean square error, A_f = accuracy factor and B_f = bias factor).

By integrating the resultant equation for k_{max} and *SI* in the primary model, the inactivation at different specific energy inputs, electric field strengths and inlet temperatures can be calculated.

The comparison of the experiment observed and the data predicted by the tertiary or final model (Figure 4-1) indicates a good accuracy of the model with an r^2 of 94.9 %, *RMSE* of 0.315, A_f of 1.605 and B_f of 1.251. The highest deviations are detectable at small inactivation rates, which correspond to the shoulder. The calculation of the accuracy and bias factor provided good validation for the model, which was used to form the isolog graphs (Figure 4-2) presenting the effect of process parameters on the inactivation.

Figure 4-2a shows the combination of specific energy and inlet temperature at a constant electric field strength of 12.5 kV/cm for inactivation rates of 1, 2 and 3 log. With an increasing inlet temperature from 55 up to 80 °C, the energy for a specific inactivation rate is decreasing. The energy input required for a 3 log inactivation is 351 kJ/kg at an inlet temperature of 55 °C compared to 194 kJ/kg at 80 °C.

Furthermore, more energy is required for higher inactivation rates. To achieve 3 log inactivation a specific energy of 269 kJ/kg has to be applied in contrast to 238 kJ/kg for a 1 log reduction. At high inlet temperatures, the differences in required energy input to achieve a certain inactivation are smaller compared to the differences at lower inlet temperatures. This indicates the beneficial effect of the temperature on the inactivation.

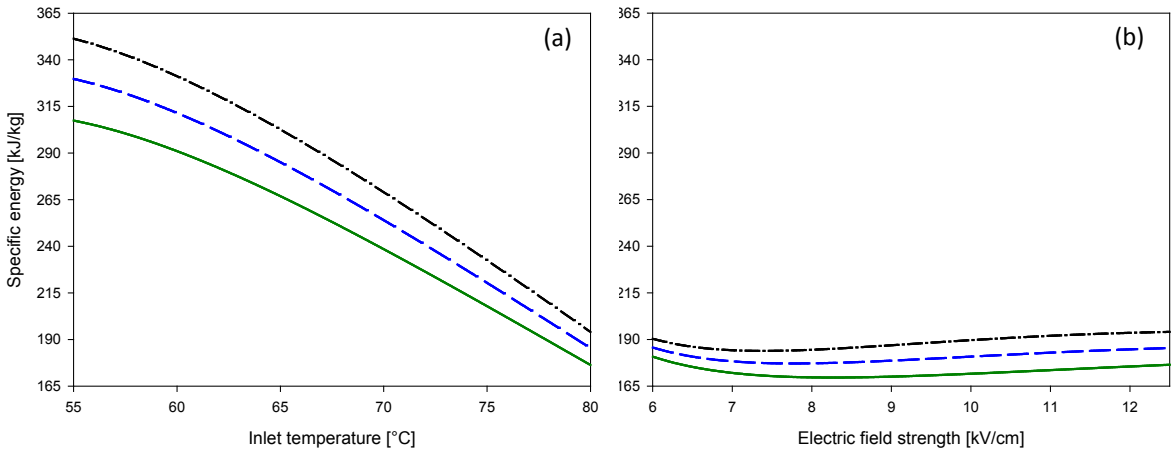

Figure 4-2: Isolog graphs for 1 log (—), 2 log (– – –) and 3 log (—·—) inactivation of *B. subtilis* spores depending on (a) specific energy and inlet temperature at an electric field strength of 12.5 kV/cm and (b) specific energy and electric field strength at an inlet temperature of 80 °C.

The analysis from Table 4-1 showing k_{max} and *SI* values for different inlet temperatures and electric field strengths, indicate only minor impact of the electric field strength on the inactivation on the range investigated. The graphic presenting the required energy at different electric field strength from 6 to 12.5 kV/cm for a certain inactivation (Figure 4-2b) attempt to clarify this fact. From 6 to

approximately 7.5 kV/cm, a slight decrease in energy with increasing electric field strength is observed. A further increase from 7.5 to 12.5 kV/cm leads to an slight increase in specific energy to achieve a specific inactivation. Comparing the different energy values at the lowest level and the highest level, the difference is small. For a 3 log reduction, the lowest energy level is 183 kJ/kg at an electric field strength of 7.3 kV/cm and the highest 194 kJ/kg at 12.5 kV/cm. Hence, the difference is 9 kJ/kg, which is small compared to the influence of the inlet temperature, where the difference in energy level to achieve a 3 log reduction is 163 kJ/kg at 55 and 80 °C.

4.1.2 Inactivation of *Alicyclobacillus acidoterrestris* spores

To analyze the effect of process parameters, mainly specific energy, electric field strength and inlet temperature on the inactivation of *A. acidoterrestris* spores, the experimental data were fitted with GInaFiT model (Geeread et al. 2005). Therefore, the specific inactivation rate k_{max} [kJ/kg^{-1}] and the shoulder length *Sl* [kJ/kg] were estimated for different inlet temperatures and electric field strengths using Equation 24.

Table 4-2: Specific inactivation rate k_{max} and shoulder length (*Sl*) at different electric field strengths and inlet temperatures, coefficient of determination (r^2) in brackets.

		Electric field strength [kV/cm]		
	Temperature [°C]	6	9	12.5
k_{max} [(kJ/kg)$^{-1}$]	56	0.08 (0.949)	0.06 (0.955)	0.27 (0.999)
	70	0.19 (0.980)	0.14 (0.992)	0.19 (0.981)
	80	0.37 (0.980)	0.20 (0.953)	0.19 (0.984)
Sl [kJ/kg]	56	236.36 (0.949)	235.57 (0.955)	271.89 (0.999)
	70	189.08 (0.980)	169.97 (0.992)	173.55 (0.981)
	80	107.80 (0.980)	90.24 (0.953)	88.65 (0.984)

The slope of the linear part of the total inactivation, termed as k_{max}, indicates the increase of energy required to increase the inactivation. The results show a dependency of k_{max} on inlet temperature. With decreasing temperature k_{max} decreased, except at an electric field strength of 12.5 kV/cm. In that case, an increase of k_{max} was obtained. A decrease of $k_{max,}$ indicated an energy increase to achieve higher inactivation. The differences between the k_{max} at different inlet temperatures decreased with increasing electric field strength. Therefore, the highest influence on the increase in energy for inactivation was observed at 6 kV/cm.

The initiation of inactivation is given by the *Sl* value. The results showed an increase of *Sl* with decreasing temperature. Thus, at lower inlet temperatures a higher energy input for an inactivation was required, which was represented as a longer shoulder. At an inlet temperature of 80 °C, a decrease of *Sl* was observed with increasing electric field strength. Therefore, at higher electric

field strengths, less energy to initiate the inactivation is required. At lower temperature of 70 and 56 °C, a decrease was observed for 6 to 9 kV/cm, followed by an increase at 12.5 kV/cm. The longest shoulder was obtained at 56 °C and 12.5 kV/cm. However, the differences between different electric field strengths at constant temperatures were smaller compared to the differences at constant electric field strengths and different inlet temperatures. Consequently, the impact of the temperature is higher than the effect of the electric field strength.

The mathematical expression describing the k_{max} and *Sl* values according to the process parameters were obtained by using TableCurve 3D. These secondary models were included in the primary model. To develop the tertiary or final model, the prediction of the inactivation during different processing conditions was calculated.

$$k_{max} = -0.009 - 0.004 \cdot T + 0.019 \cdot E + 0.0002 \cdot T^2 + 0.008 \cdot E^2 - 0.002 \cdot T \cdot E \qquad r^2=0.981$$

$$Sl = -1029.139 + \frac{161521.14}{T} - 47.077 \cdot E - \frac{4888762.7}{T^2} + 1.202 \cdot E^2 + 1672.479 \cdot \frac{E}{T} \qquad r^2=0.997$$

The accuracy of the model is shown in Figure 4-3. Here, the predicted inactivation is plotted against the experimental data and the validation factors are shown. Besides some outliers between 2 and 3 log cycles, the model fits with good precision. The coefficient of determination is 91.6 and the accuracy and bias factor are close to 1. This good fit allows the use of the developed model for further analysis.

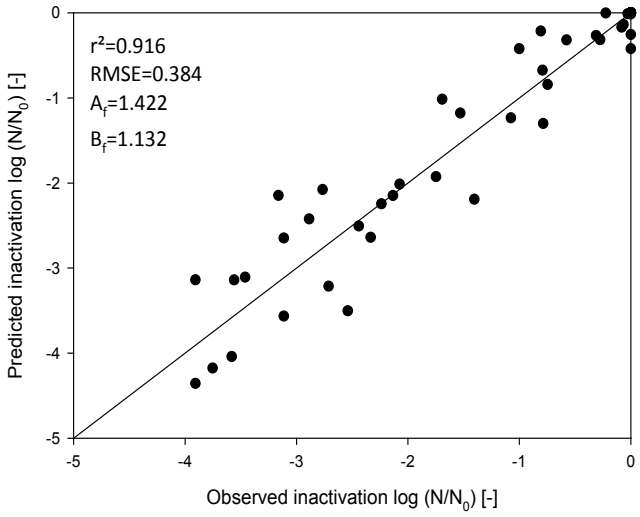

Figure 4-3: Regression of calculated and experimental estimated inactivation of *A. acidoterrestris* spores at different energy values, electric field strengths and inlet temperatures (r^2 = determination coefficient, *RMSE* = root mean square error, A_f = accuracy factor and B_f = bias factor).

To show the impact of electric field strength, inlet temperature and specific energy, isolog graphics were prepared (Figure 4-4). The graph on the left side (Figure 4-4a) showed the dependence of

the inactivation from the specific energy at different inlet temperatures and constant electric field strength of 12.5 kV/cm. With an increasing inlet temperature from 55 to 80 °C, the energy input for inactivation was decreasing. A 3 log reduction was achieved at an applied energy level of 281 kJ/kg at an inlet temperature of 60 °C, in contrast to 122 kJ/kg at 80 °C. Furthermore, the energy can be reduced, if less inactivation is required. At an inlet temperature of 70 °C, the energy to achieve a 3 log reduction is 214 kJ/kg compared to 189 kJ/kg for a 1 log reduction.

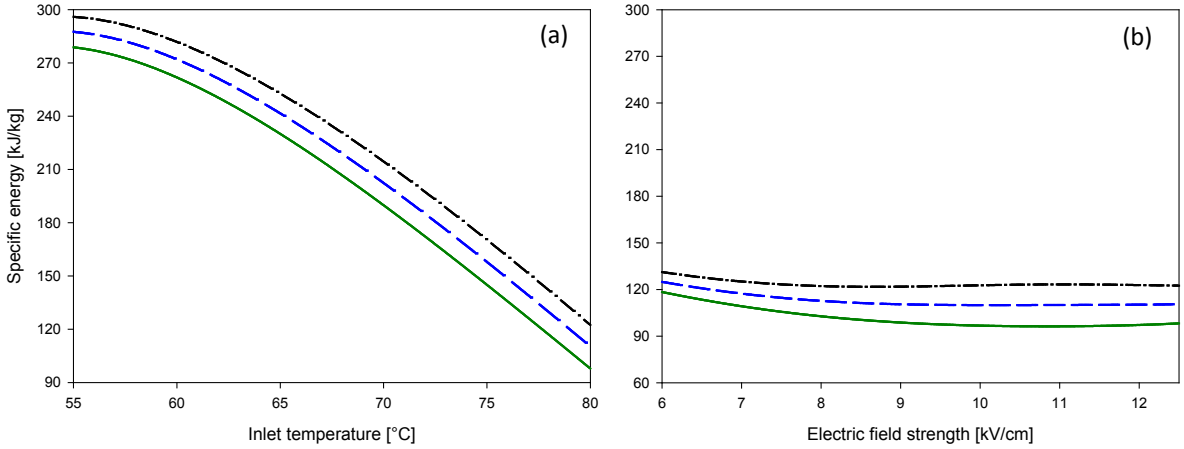

Figure 4-4: Isolog graphs for 1 log (—), 2 log (– –) and 3 log (—·—) inactivation of *A. acidoterrestris* spores depending on (a) specific energy and inlet temperature at an electric field strength of 12.5 kV/cm and (b) specific energy and electric field strength at an inlet temperature of 80 °C.

The effect of the electric field strength on the inactivation is shown in Figure 4-4b. The highest impact was observed for a 1 log reduction in the area of low electric field strengths from 6 to 9 kV/cm. Here, the energy required for inactivation decreased with increasing electric field strength. The same effect is observed for the inactivation of vegetative microorganisms by PEF (Grahl et al. 1996; Heinz et al. 2001; Álvarez et al. 2003a). With increasing inactivation rates of up to 3 log, the effect got smaller. This can be demonstrated by calculating the energy difference required for a specific inactivation rate at 6 and 9 kV/cm. For a 1 log inactivation the difference is about 20 kJ/kg, whereas the difference for a 3 log reduction is 9 kJ/kg. Generally, a higher energy is required for a higher inactivation rate, which results in a higher outlet temperature. Therefore, the low difference in energy can be explained by the higher temperature involved. Moreover, it also indicates the dimension of the energy difference. In total, the differences in energy between 6 and 12.5 kV/cm are small, especially when compared to the difference in energy at different inlet temperatures (Figure 4-4a). As an example, the energy difference for a 3 log reduction be-

tween 60 and 80 °C is 159 kJ/kg at electric field strength of 12.5 kV/cm. The higher energy difference when comparing different inlet temperatures and electric field strengths demonstrates the higher impact of the inlet temperature on the inactivation.

4.1.3 Inactivation of *Geobacillus stearothermophilus* spores

The main influencing process parameters (electric field strength, specific energy and inlet temperature) are included in the model to evaluate the influence of each parameter on the inactivation of *G. stearothermophilus* spores.

The first step was the application of the primary model developed by Geeraerd et al. (2005) (Equation 24).

Table 4-3: Specific inactivation rate k_{max} and shoulder length (*Sl*) at different electric field strengths and inlet temperatures, coefficient of determination (r^2) in brackets, nd – not detected.

		Electric field strength [kV/cm]		
	Temperature [°C]	6	9	12.5
k_{max} [(kJ/kg)$^{-1}$]	56	nd	0.10 (0.982)	0.06 (0.944)
	70	0.19 (0.905)	0.21 (0.998)	0.12 (0.988)
	80	0.19 (0.972)	0.26 (0.999)	0.28 (0.993)
Sl [kJ/kg]	56	nd	359.77 (0.982)	362.95 (0.944)
	70	313.69 (0.905)	308.06 (0.998)	310.85 (0.988)
	80	222.22 (0.972)	235.80 (0.999)	243.37 (0.994)

The factor k_{max} [(kJ/kg)$^{-1}$] is the specific inactivation rate and represents the slope of the linear part. With decreasing temperature, k_{max} was also decreasing. At high temperatures, the inactivation was faster compared to low temperatures. At higher electric field strengths, the difference of k_{max} at different temperatures was increasing. This means, the effect of the temperature is higher at higher electric field strengths. At 56 °C, 6 kV/cm, no k_{max} nor *Sl* value were obtained, as no shoulder nor linear part of the inactivation could be obtained from experimental data.

With increasing electric field strength, k_{max} was increasing at a temperature of 80 °C. Therefore, a faster inactivation was observed at high electric field strengths. By decreasing the inlet temperature to 70 °C, k_{max} at 9 kV/cm (0.21 (kJ/kg)$^{-1}$) was slightly higher than at 6 kV/cm (0.19 (kJ/kg)$^{-1}$), but a further increase in electric field strength resulted in a lower k_{max} of 0.12 (kJ/kg)$^{-1}$. It can be assumed, that there is not much difference in energy requirement at 6 and 9 kV/cm at an inlet temperature of 70 °C, but based on lower k_{max} at 12.5 kV/cm, more energy increase is required for a higher inactivation.

Sl indicates the shoulder length in kJ/kg and represents the initiation of spore inactivation. An increase of *Sl* with decreasing inlet temperature showed that more specific energy was required

to start the inactivation of *G. stearothermophilus* spores at lower temperatures. Comparing *Sl* at different electric field strengths, the effect of the electric field strength on the inactivation can be evaluated. Similar *Sl* values for each studied electric field strength at a constant inlet temperature were obtained, although a small trend could be observed. However, due to the fact of a higher k_{max} at 12.5 kV/cm compared to 6 kV/cm a faster inactivation can be observed. The *Sl* values at 56 and 70 °C indicated only small changes, which can be neglected. Thus, the shoulder length observed at 56 and 70 °C can be considered as independent from the electric field strength.
In summary, the inlet temperature has a higher impact on the required energy level for an inactivation than the electric field strength. The difference between k_{max} at different inlet temperatures are higher compared to the values obtained at different electric field strengths. The same relation is discovered for *Sl* values.

The second step in the model is the fitting of the previously estimated k_{max} and *Sl* values. The program TableCurve 3D was used to estimate equations for the values as a function of electric field strength (*E*) and inlet temperature (*T*).

$$k_{max} = -1.239 + 0.007 \cdot T + \frac{16.074}{E} + 0.0001 \cdot T^2 - \frac{11.62}{E^2} - 0.176 \cdot \frac{T}{E}$$

r^2=0.944

$$Sl = \frac{(506.212 - 6.733 \cdot T + 22.591 \cdot E - 1.042 \cdot E^2)}{(1 - 0.013 \cdot T + 0.086 \cdot E - 0.004 \cdot E^2)}$$

r^2=0.999

The final step is the inclusion of the secondary model in the general expression model (Equation 24). Figure 4-5 represents the correlation between the real data and the predicted values for the model in the same experimental conditions, showing the goodness of fit of the model.

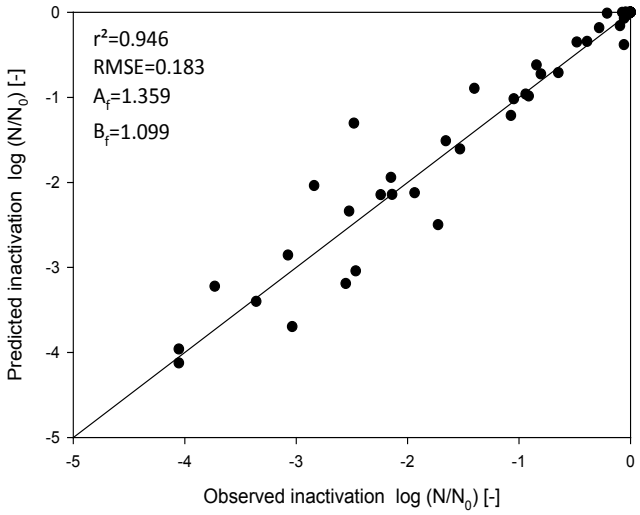

Figure 4-5: Regression of calculated and experimental estimated inactivation of *G.stearothermophilus* at different energy values, electric field strengths and inlet temperatures (r^2 = determination coefficient, *RMSE* = root mean square error, A_f = accuracy factor and B_f = bias factor).

The model is suitable to use it for further analysis, because of an r^2 of 94.6 % and accuracy and bias factor close to 1. In the region of low inactivation (1 log cycle) the model is more accurate as the points are more in the area of the line of equivalence. The highest deviations can be found between 1 and 2.5 log cycles, while the deviations get smaller above 2.5 log of inactivation. A comparison of the values (data not shown) explains the high deviations at 56 °C. At 56 °C and electric field strength of 6 kV/cm, no values for k_{max} and *SI* could be calculated, because no inactivation was achieved by the applied energy. However, the statistical correlation is considered high enough to allow the use of the model. To analyze the effect of specific energy, electric field strength and inlet temperature isolog graphs presented in Figure 4-6 were plotted. The influence of inlet temperature dependent on the specific energy needed to achieve a certain inactivation is shown in Figure 4-6a. With increasing inlet temperature from 56 to 80 °C, the energy needed to reach the end of the shoulder is decreased. For a 3 log reduction, an energy requirement of 590 kJ/kg at an inlet temperature of 60 °C was needed compared to 270 kJ/kg at 80 °C. The comparison of the energy for specific inactivation levels at a constant temperature showed certain differences. The model indicated a high influence of the initial temperature on the resistance of *G. stearothermophilus* spores. The specific energy required to increase the inactivation from 1 to 3 log was 160 kJ/kg, while at 80°C, the required energy input to increase the inactivation within the same range was only 17 kJ/kg.

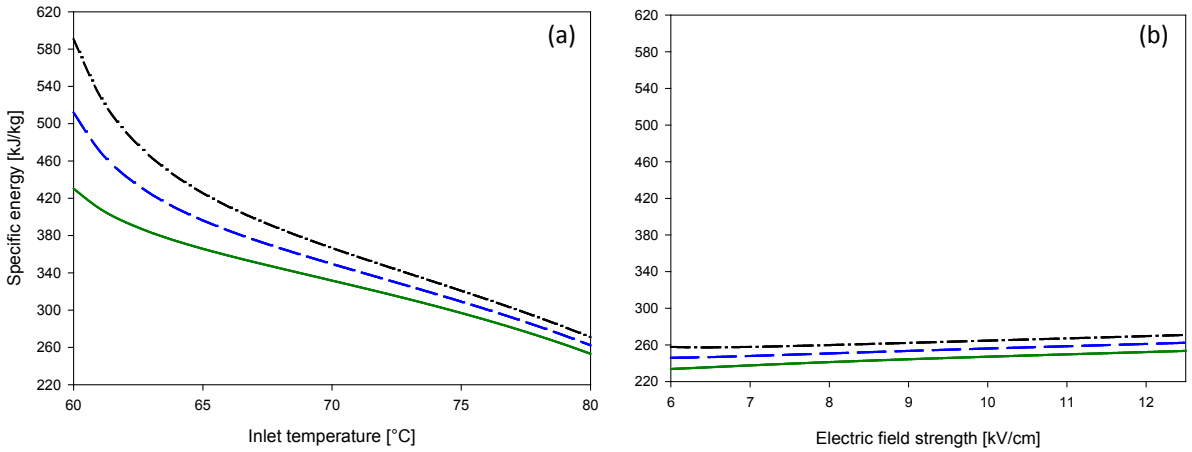

Figure 4-6: Isolog graph for 1 log (—), 2 log (– –) and 3 log (—·—) inactivation of G. *stearothermophilus* spores depending on (a) specific energy and inlet temperature at an electric field strength of 12.5 kV/cm and (b) specific energy and electric field strength at an inlet temperature of 80 °C.

The effect of the electric field strength on the inactivation is shown in Figure 4-6b. With increasing electric field strength from 6 to 12.5 kV/cm, a slight increase in energy is required for a specific inactivation. At low electric field strengths of 6 kV/cm, a slight difference in energy for a 1 log inactivation was observed. However, for a high inactivation rate higher energy was required. Comparing the energy at low electric field strengths (6 kV/cm) with the energy at high electric field strength (12 kV/cm), only low differences were observed. For a 3 log inactivation, 257 kJ/kg was required at an electric field strength of 7 kV/cm and 270 kJ/kg at 12.5 kV/cm. These results correspond to the result obtained in the analysis of the k_{max} values. The differences in k_{max} between the analyzed electric field strengths at constant inlet temperature were small compared to the differences at different inlet temperatures. Consequently, the previously obtained result of a higher effect of the temperature than of electric field strength can be confirmed.

4.1.4 Discussion and comparison of inactivation of different spores types by PEF and influence of process parameters on the inactivation

The previous sections analyzed the effect of specific energy, inlet temperature and electric field strength on the inactivation of three different spore types. The comparison of the impact of inlet temperature is shown in Figure 4-7. In all cases, the energy required for an inactivation, decreased with increasing inlet temperature. The curve shape of *B. subtilis* and *A. acidoterrestris* is similar, but the energy required for *B. subtilis* spore inactivation is higher. Applying an energy of 331 kJ/kg at an inlet temperature of 60 °C and 12.5 kV/cm a 3 log reduction of *B. subtilis* was achieved,

whereas an energy of 281 kJ/kg was required for the same conditions for inactivation of *A. acidoterrestris* spores. In contrast, the specific energy for inactivation of *G. stearothermophilus* spores was higher and indicated a different curve shape in the temperature range of 60 to 65 °C. At temperatures <65 °C, the energy demand for an inactivation was high resulting in a temperature increase according to Equation 13. Therefore, an effect of temperature on the inactivation can be assumed. The same effect of energy increase with decreasing temperature was detected for *B. subtilis* and *A. acidoterrestris* spores, but the increase in energy was lower compared to the inactivation of *G. stearothermophilus* spores.

Back to the developed model for *G. stearothermophilus* spores, no inactivation rates could be estimated at inlet temperatures below 60°C, because no inactivation was obtained by the experiment in this temperature range. Therefore, according to the model a very high specific energy for a 3 log reduction of this spore would be required. Within the temperature range from 65 to 80 °C, the curve shape is similar to the ones obtained for *B. subtilis* and *A. acidoterrestris* spores. As a result, the inlet temperature has an impact on the energy required for specific inactivation. The differences in energy when comparing the spore types indicate the general resistance of each spore to the applied treatment.

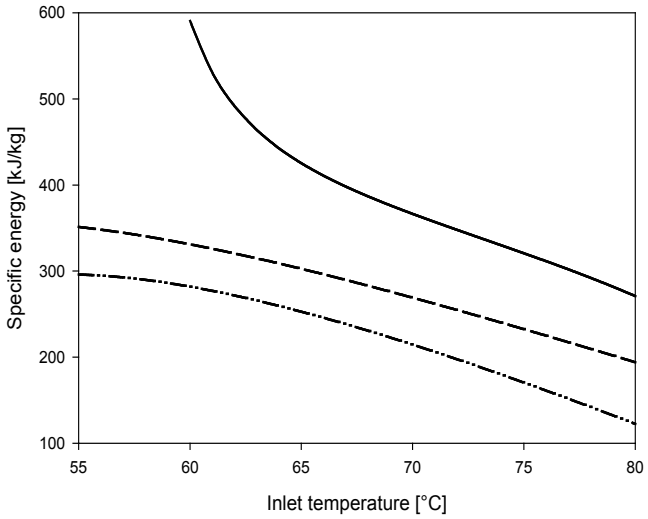

Figure 4-7: 3 log inactivation of *G. stearothermophilus* (—), *B. subtilis* (——) and *A. acidoterrestris* (—··—) in Ringer´s solution (4mS/cm, pH 7) at an electric field strength of 12.5 kV/cm as a function of specific energy and inlet temperature.

The *G. stearothermophilus* spores are the most resistant followed by *B. subtilis* and *A. acidoterrestris* spores. The different resistances can be explained by the differences in heat resistance. The results obtained in this study analyzing the heat resistance of each spore and corresponding values from literature (see chapter 2.1.6) indicate *G. stearothermophilus* spores with the highest heat resistance followed by *B. subtilis* and *A. acidoterrestris* spores. The differences in heat

resistance and energy required for inactivation demonstrates the importance of the temperature for the inactivation.

However, the impact of inlet temperature on the inactivation by PEF is already well known and studied for inactivation of vegetative microorganisms. By increasing the inlet temperature up to around 40 °C, less energy for a certain level of inactivation is required (Zhang et al. 1995; Wouters et al. 1999; Smith et al. 2002; Amiali et al. 2005, 2007; Fernández-Molina et al. 2005; Sampedro et al. 2006; Toepfl et al. 2007; Fox et al. 2008). The reason is based on structural changes at higher temperatures causing a higher sensitivity of the membrane. The phospholipid structure of the membrane is packed in a gel-like structure at low temperatures and shifts to a liquid crystalline structure at higher temperatures (Stanley et al. 1991). By further increase of temperature, the inactivation of bacterial endospores could be seen. Studies in literature analyzing the inactivation of spores by PEF at different inlet temperatures, showed a higher inactivation at higher inlet temperatures (Jin 2001; Marquez et al. 1997; Bermúdez-Aguirre et al. 2012). The studied inlet temperatures are ranging from 5 to 60 °C and the electric field strengths are much more higher (> 30 kV/cm) than the one used in this study. In the study of Uemura et al. (2003) an inlet temperature of 70 °C was used in a static PEF treatment chamber. An electric field strength of 16.3 kV/cm was used to achieve a 4 log reduction of *B. subtilis* spores in orange juice. Due to the treatment under pressurized conditions, temperatures above 100 °C gave the 4 log inactivation results. A final temperature of 121 °C was reached with a holding time of 1 s resulting in a successful inactivation of *B. subtilis* spores. Compared to this study, back pressure was used to achieve temperatures above 100 °C. A 3 log reduction of *B. subtilis* spores could be reached by applying 269 kJ/kg at an electric field strength of 12.5 kV/cm and at 70 °C inlet temperature. The difference in inactivation between the results from Uemura et al. (2003) and results obtained in this study can be explained by the different PEF systems used, namely the media and different electric field strengths. However, the results so far available in literature showed an inactivation of spores, if very higher electric field strengths were applied or the inlet temperature is increased.

At a specific energy level, which is different for each spore type and described by the shoulder length in the developed model, the inactivation starts. The occurrence of the shoulder indicates that a certain specific energy is required to start the inactivation. It can be assumed that the different layers around the spore core are protecting the spore until a specific energy level is achieved and therefore causing the shoulder. This might be in accordance with structural changes in the spores in order to increase the PEF sensitivity. It might be related to a temperature effect, because the energy causes a temperature increase (Joule heating).

A comparison of the effect of the electric field strength on the different spore types is shown in Figure 4-8 at an inlet temperature of 80 °C. The discussion of the results for each spore indicates a low impact of the electric field strength on the inactivation, which can be observed in Figure 4-8. The specific energy required for a 3 log reduction of all spore types is not influenced by the electric field strength. Only small differences can be detected in the range of 6 to 12 kV/cm, but they are quite insignificant, as a certain amount of energy is needed for inactivation.

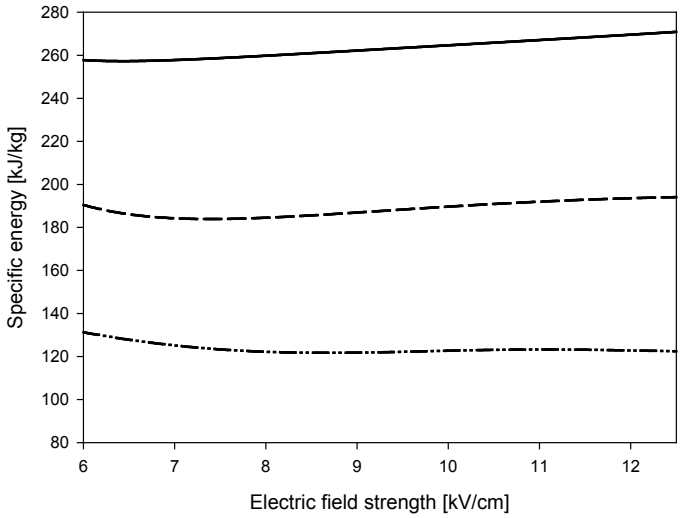

Figure 4-8: 3 log inactivation of *G. stearothermophilus* (—), *B. subtilis* (——) and *A. acidoterrestris* (—··—) at an inlet temperature of 80 °C as a function of specific energy and inlet temperature.

In literature, the electric field strength is very important for the inactivation of spores and also vegetative microorganisms. It has been reported that at low electric field strengths (< 30 kV/cm), which is traditionally used for inactivation of vegetative microorganism by PEF, no or almost no inactivation of bacterial spores can be achieved (Hamilton et al. 1967; Knorr et al. 1994). Knorr et al. (1994) reported no inactivation of *B. cereus* spores after application of 30 pulses with an electric field strength of 22.4 kV/cm. The difference between this study and the reference is the inlet temperature. The results obtained in this study indicate an inactivation of *B. subtilis* spores at an even lower electric field strengths than 22.4 kV/cm, because of a combination with thermal energy. Knorr et al. (1994) studied the effect of PEF at ambient temperature, whereas in this study higher temperatures in the range of 56 to 80 °C were analyzed. Other studies using higher inlet temperatures and higher electric field strengths of 20 to 50 kV/cm could also demonstrate an inactivation of bacterial endospores (Jin 2001; Uemura et al. 2003; Marquez et al. 2007; Bermúdez-Aguirre et al. 2012).

Generally, for inactivation of vegetative microorganisms, a so called critical field strength has to be exceed for an inactivation (Grahl et al. 1996). This critical field strength depends on the cell size and the cell orientation in the electric field (Heinz et al. 2002). The smaller the cell, the higher the critical electric field strength. *S. cerevisiae* is a large cell, and has a critical field strength of 2 to 4 kV/cm, whereas *E. coli* with a smaller cell size requires an electric field strength of 15 kV/cm (Grahl et al. 1996). As spores are much smaller than vegetative cells, the critical field strength should theoretically be much higher compared to vegetative bacteria. In fact, inactivation of spores could be achieved by using the same electric field strength range. In this study, the electric field strength ranges from 6 to 12.5 kV/cm. An inactivation could be shown for all spore types. Electric field strength of less than 6 kV/cm should be analyzed in order to determine the critical electric field strength. Within the section 4.2.4 lower electric field strength in a different medium is studied. As a result, a very high energy of 303 kJ/kg is required for a 4.2 log reduction of *B. subtilis* spores at 80 °C and 4 kV/cm. The resultant temperature is very high, so that a thermal inactivation can be assumed, which is not related to PEF. From this result, it can be assumed that the critical electric field strength is less than 6 and higher than 4 kV/cm. A more intense study should be performed to give more accurate critical electric field strength.

However, in the area of inactivation of vegetative microorganisms an increase in electric field strength leads to a decrease in required energy for a specific inactivation (Hülsheger et al. 1983; Heinz et al. 2000; McDonald et al. 2000; Álvarez et al. 2003a) limited to the dielectric strength of the food material (Ho et al. 2000). Álvarez et al. (2003a) studied the inactivation of *L. monocytogenes* by PEF at different electric field strengths. No inactivation was obtained by applying electric field strengths lower than 15 kV/cm at a maximum temperature of 32 °C. An increase in the electric field strength of up to 28 kV/cm and applying 200 kJ/kg leads to a 3.5 log reduction. The electric field strengths used in this study were lower, and the effect of increasing inactivation with increasing electric field strength at constant energy could not be observed. Higher electric field strengths should be analyzed in order to confirm this result.

4.2 Influence of production parameters on the inactivation of spores by PEF

4.2.1 Influence of fat on the inactivation of bacterial endospores by PEF

Especially in the area of soups, fat is added and therefore the effect of fat on resistance of spores and on the process efficiency becomes interesting. A higher heat resistance of spores and vegetative bacteria is already observed in fatty media, which is based on lower water activity (Senhaji et

al. 1977). As soups might be contaminated with bacterial endospores, the effect of fat on the inactivation could be important and has to be studied.

4.2.1.1 Inactivation of *B. subtilis* spores

To analyze the effect of fat on the inactivation of *B. subtilis* spores, sunflower oil and lecithin as emulsifier were added to the model solution to a final electric resistance of 4 mS/cm. The observed data showed a shoulder and a linear part. To describe these graphics, the model developed by Geeraerd et al. (2005) was applied. For the primary model the specific inactivation rate k_{max} and shoulder length *Sl* were estimated according to Equation 24 (Table 4-4).

Table 4-4: Specific inactivation rate k_{max} and shoulder length *Sl* at different electric field strengths (6, 9 and 12.5 kV/cm) and fat concentrations (0, 5 and 10 %) for *B. subtilis* spores. The r^2 value is showed in brackets.

		Electric field strength [kV/cm]		
Fat concentration [%]		6	9	12.5
k_{max} [(kJ/kg)$^{-1}$]	0	0.49 (0.953)	0.27 (0.973)	0.27 (0.989)
	5	0.16 (0.948)	0.15 (0.999)	0.15 (0.997)
	10	0.12 (0.981)	0.15 (0.982)	0.17 (0.999)
Sl [kJ/kg]	0	178.4 (0.953)	160.5 (0.973)	167.6 (0.989)
	5	171.0 (0.948)	159.7 (0.999)	154.0 (0.997)
	10	149.9 (0.981)	150.3 (0.982)	154.7 (0.999)

The specific inactivation rate k_{max} represents the slope of the linear inactivation part and indicates a fast inactivation at high k_{max} values. A decrease in k_{max} was observed with increasing fat concentration at an electric field strength of 6 kV/cm. But the difference between 5 and 10 % fat was smaller compared to the sample without fat. The decrease of k_{max} from 0.49 to 0.12 (kJ/kg)$^{-1}$ at 0 and 10 % fat indicated that the inactivation was faster in the linear part of the graphic in absence of fat in the sample, or in terms of energy, less specific energy was required to reduce the same log cycles compared to a sample with fat. At higher electric field strength the differences in k_{max} between different fat levels got smaller, which means the difference in linear part of the inactivation was similar at high electric field strengths. The initiation of the inactivation was given by the shoulder length *Sl*. The values calculated by the model showed a decrease of *Sl* with increasing fat concentration. At an electric field strength of 6 kV/cm the *Sl* was 178.4 kJ/kg and 149.9 kJ/kg at 0 % and10 % fat, respectively. With increasing electric field strength the same effect could be observed, but the difference was smaller. At 9 kV/cm the difference in *Sl* value between 0 and 10 % fat was 10.2 kJ/kg, whereas at 6 kV/cm a difference of 38.7 kJ/kg was obtained. Therefore, the addition of fat causes the initiation of inactivation of *B. subtilis* spores at lower energy inputs.

The values k_{max} and *Sl* were described by second order equations obtained by multiple regression. Those equations describe the influence of electric field strength (*E*) and fat concentration (*F*) in the k_{max} and *Sl* values.

The inclusion of the secondary models in the primary model results in the tertiary or final model, which allows calculation of inactivation at specific process and fat conditions.

$$k_{max} = \frac{(-0{,}119 + 0{,}021 \cdot F - 0{,}004 \cdot E - 0{,}002 \cdot E^2)}{(1 - 0{,}198 \cdot F + 0{,}023 \cdot F^2 - 0{,}233 \cdot E)}$$

r²=0.967

$$Sl = 183.869 + 1.155 \cdot F - \frac{475.209}{E} - 0.053 \cdot F^2 + \frac{2685.540}{E^2} - 19.690 \cdot \frac{F}{E}$$

r²=0.876

The validation of the model was performed by plotting the predicted inactivation against the observed data. Moreover, the accuracy (A_f) and bias (B_f) factor were calculated (Figure 4-9). The determination coefficient (r^2) and the root mean square error (*RMSE*) are in the range to evaluate the model as precise. A value of 1.341 for A_f is close to 1 indicating that the predicted values are similar to the observed ones. Also the B_f (1.090) is close to 1, which shows a good fit of the model, so that it can be used for further analysis.

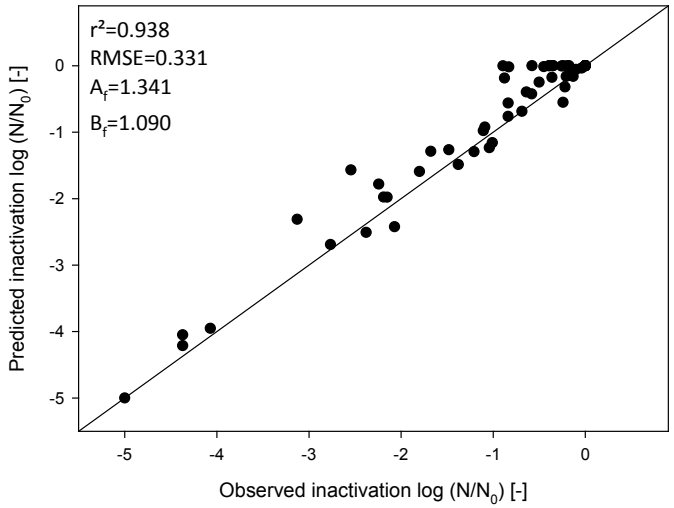

Figure 4-9: Correlation between the predicted, estimated by the tertiary model and observed inactivation of *B. subtilis* spores after PEF treatment (r^2 = determination coefficient, *RMSE* = root mean square error, A_f = accuracy factor and B_f = bias factor).

To analyze the effect of fat on the inactivation of *B. subtilis* spores isolog graphs were created showing the specific energy required to achieve a specific inactivation level at different fat concentrations (Figure 4-10a) as well as the specific energy required at different electric field

strengths to obtain 3 log cycles of inactivation on the spore population at different fat concentrations (Figure 4-10b).

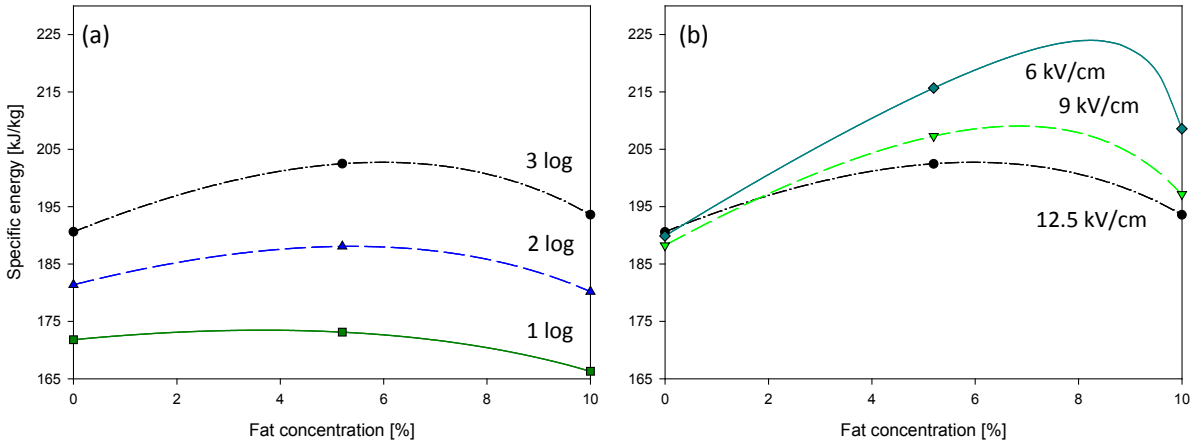

Figure 4-10: Influence of fat on the inactivation of *B. subtilis* spores by PEF at pH 7 and an inlet temperature of 80 °C, (a) inactivation of 3, 2 and 1 log cycle at an electric field strength of 12.5 kV/cm and (b) at different electric field strengths for a 3 log reduction; symbols in the graphs represents the experimental data.

Generally, a higher energy had to be applied for a higher inactivation. For a 3 log reduction with a fat concentration of 6 % an energy of 202.7 kJ/kg was required, although for a 1 log reduction only 172.6 kJ/kg was needed. Comparing the energy at different fat concentrations with a constant electric field strength of 12.5 kV/cm the difference in energy for a 1 log reduction was low. From 0 to 4 % fat the required specific energy increased slightly from 171.8 to 173.5 kJ/kg and decreased afterwards to 166.3 kJ/kg at 10 % fat. The same effect was observed at higher inactivation rates. The energy required for a 3 log reduction increased from 190.6 up to 202.7 kJ/kg from 0 to 6 % fat and decreased to 193.6 kJ/kg for 10 % fat. However, the changes in energy for a specific log reduction at different fat concentrations are very small, thus the fat concentration has no or minor influence on the PEF treatment efficiency at low inactivation rates. The effect of fat on energy required for a 3 log reduction at different electric field strengths is shown in Figure 4-10b. At 12.5 kV/cm the energy can be regarded as constant over the fat concentration range from 0 to 10 %. The slight increase and decrease might be based on statistical variations. With decreasing electric field strength, the energy increased from 0 to 5 % and decreased from 5 to 10 %. The model at 6 kV/cm showed an increase in energy up to 8.4 % followed by a decrease. Here, a validation of the model is required in order to confirm the curve shape. Moreover, the decrease in energy from 5 to 10 % for 6 and 9 kV/cm was less than 10 kJ/kg, which indicates only a slight difference.
However, it can be stated, that the effect of fat is increasing with decreasing electric field strength.

4.2.1.2 Inactivation of *A. acidoterrestris* spores

The same procedure of adding sunflower oil and lecithin as for the inactivation analysis for *B. subtilis* spores was used to allow comparison of data. The primary model developed by Geeraerd et al. (2005) (Equation 24) was used and the specific inactivation rate k_{max} as well as shoulder length *Sl* for each electric field strength and fat concentration was estimated.

Table 4-5: Shoulder length (*Sl*) and specific inactivation rate (k_{max}) for inactivation data of *A. acidoterrestris* spores at different electric field strengths and fat concentrations, r^2 is shown in brackets, nd - not detected.

		Electric field strength [kV/cm]		
Fat concentration [%]		6	9	12.5
k_{max} [(kJ/kg)$^{-1}$]	0	0.37 (0.980)	0.20 (0.953)	0.19 (0.984)
	5	0.32 (0.913)	0.26 (0.969)	nd
	10	0.59 (0.959)	0.30 (0.900)	0.24 (0.950)
Sl [kJ/kg]	0	107.8 (0.980)	90.2 (0.953)	88.7 (0.984)
	5	101.5 (0.913)	93.8 (0.969)	nd
	10	107.4 (0.959)	84.5 (0.900)	73.0 (0.950)

The specific inactivation rate k_{max} describes the linear part of the inactivation and therefore how much energy is necessary to obtain a certain level of inactivation. The observed data for k_{max} showed a decrease from 0 to 5 % fat and an increase of k_{max} at 10 % fat. At an electric field strength of 6 kV/cm, k_{max} was 0.37, 0.32 and 0.59 (kJ/kg)$^{-1}$ at a fat concentration of 0, 5 and 10 %, respectively. For 12.5 kV/cm and 5 % fat no k_{max} and *Sl* could be calculated, because only few values for the inactivation were obtained and therefore not included in the model. With increasing electric field strength, k_{max} was decreasing indicating a faster inactivation at high electric field strengths. The initiation of the inactivation was described by the shoulder length. With an increasing fat concentration, *Sl* decreased except for 10 % fat and 6 kV/cm. In this case, the *Sl* value was higher compared to 5 % fat and 6 kV/cm. The decrease in *Sl* indicated a lower energy requirement for the start of the inactivation at higher fat concentrations. But also the electric field strength influenced the shoulder length. An increase in electric field strength lead to a decrease in shoulder length. Therefore, the highest energy for the inactivation was observed for 0 % fat and 6 kV/cm, whereas the lowest energy was obtained at 10 % fat and an electric field strength of 12.5 kV/cm. Within the next step the values for k_{max} and *Sl* were fitted and integrated in the primary model to allow calculation of inactivation for different fat concentrations, electric field strengths and specific energies.

$$k_{max} = \frac{(0.154 - 0.076 \cdot F + 0.007 \cdot F^2 - 0.032 \cdot E)}{(1 - 0.260 \cdot F + 0.025 \cdot F^2 - 0.183 \cdot E)}$$

r^2=0.999

$$Sl = 154.544 + 1.507 \cdot F - 10.861 \cdot E - 0.006 \cdot F^2 + 0.445 \cdot E^2 - 0.237 \cdot F \cdot E$$

r^2=0.947

To validate the model statistical relevant values, determination coefficient (r^2), root mean square error (*RMSE*), accuracy factor (A_f) and bias factor (B_f) were calculated and illustrated in Figure 4-11. High values for r^2 of 92.3 % and *RMSE* of 0.496 indicate sufficient precision of the model. Moreover, a B_f of 1.000 was calculated, which even more confirms the goodness of the model. In summary, the validation is precise enough to allow use of the model.

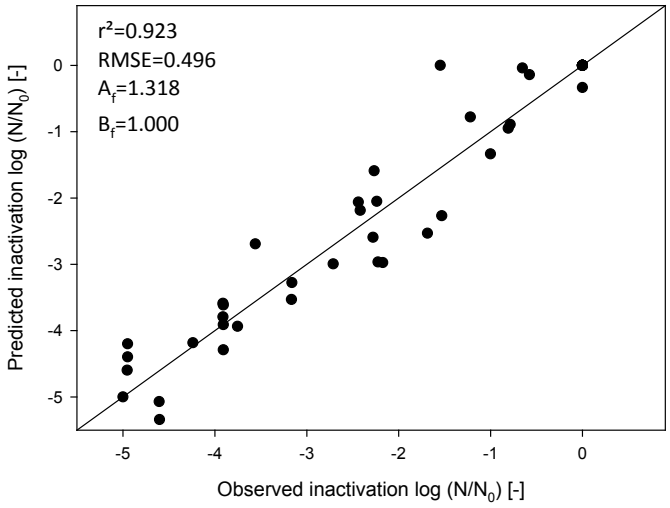

Figure 4-11: Correlation of experimental and predicted data by the estimated model for the inactivation of *A. acidoterrestris* by PEF (r^2 = determination coefficient, *RMSE* = root mean square error, A_f = accuracy factor and B_f = bias factor).

The inactivation of *A. acidoterrestris* spores depends on the energy applied, electric field strength and the fat concentration. Figure 4-12 shows the process conditions (fat concentration, electric field strength and specific energy) estimated by the model to obtain a certain level of inactivation. It could be observed that a higher inactivation rate required a higher energy input (Figure 4-12a). For a 3 log reduction an energy of 108.9 kJ/kg had to be applied in contrast to 88.8 kJ/kg for a 1 log reduction at an electric field strength of 12.5 kV/cm. Moreover, the energy required for inactivation depended on the fat content of the medium. With increasing fat concentration to a maximum of 10 % the energy input required for a given level of inactivation decreased. At 0 % fat the energy for a 2 log inactivation was 112.8 kJ/kg compared to 92.5 kJ/kg, when the medium contains 10 % fat. In general, the curve shape of energy input against fat concentration was linear and the lines for each inactivation rate were parallel. The change in energy to achieve a higher inactivation at a constant fat concentration was always around 10 kJ/kg. Thus, to increase the inactivation about 1 log an energy increase of 10 kJ/kg was necessary. Besides the impact of fat on energy

required for specific inactivation rates, the effect of electric field strength was analyzed (Figure 4-12b). The black straight line in both graphs in Figure 4-12 represents a 3 log reduction using an electric field strength of 12.5 kV/cm. By lowering the electric field strength more energy was required to reach a 3 log reduction. Furthermore, the curve shape changed with decreasing electric field strength. At 12.5 kV/cm a more or less linear curve shape was observed. Only at low fat concentrations a higher slope was shown compared to fat concentrations higher than 2 %. The same was also observed for 9 kV/cm, but the slope was smaller in contrast to 12.5 kV/cm. An even lower slope was detected for 6 kV/cm, especially until a fat concentration of 7 %. At higher fat concentrations a faster decrease in energy was observed. Thus, the differences in energy between 4 and 8 % fat was about 3.3 kJ/kg and increased up to 5.8 kJ/kg between 8 and 10 % fat.

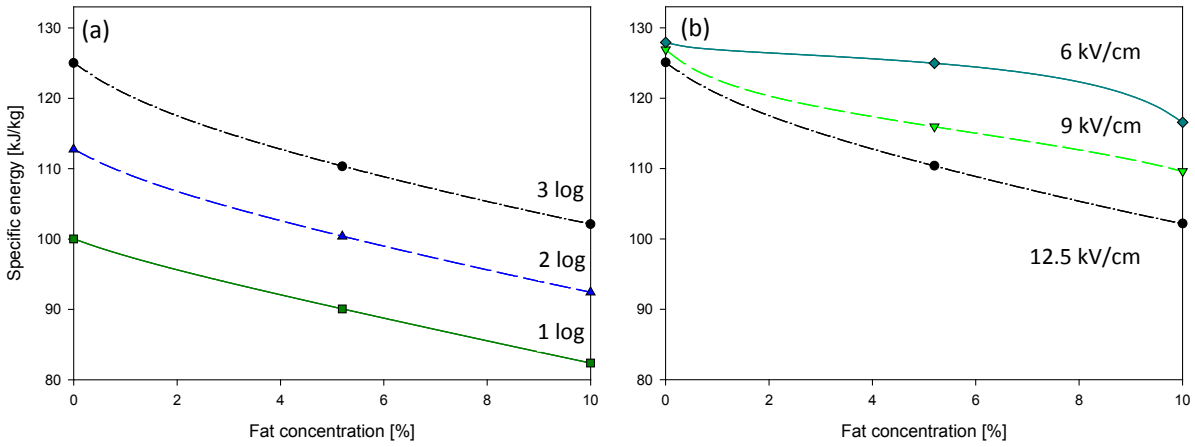

Figure 4-12: Influence of different fat concentrations from 0 to 10 % on the energy required to (a) achieve a 3, 2 and 1 log reduction of *A. acidoterrestris* spores at an electric field strength of 12.5 kV/cm and (b) to achieve a 3 log reduction at 6, 9 and 12.5 kV/cm by PEF using an inlet temperature of 80 °C at pH 7, symbols in the graphs represents the experimental data.

As the results show, the addition of fat influences the required energy to achieve a specific inactivation of *A. acidoterrestris* spores by PEF. An increase of fat concentrations leads to a reduction on the specific energy required to obtain a certain level of inactivation. A further decrease of energy could be achieved by applying a high electric field strength. Hence, the lowest energy for a 3 log reduction is about 88.8 kJ/kg using an electric field strength of 12.5 kV/cm and 10 % fat.

4.2.1.3 Inactivation of *G. stearothermophilus* spores

To analyze the effect of fat on the energy required for a specific inactivation, the model developed by Geeraerd et al. (2005) was used as the inactivation data indicates a shoulder and a linear part.

Both parts (linear and shoulder) for each analyzed electric field strength and fat concentration were fitted by the Equation 24 (Table 4-6).

The specific inactivation rate k_{max} describes the slope of the linear part of the inactivation data. With increasing fat concentration a decrease of k_{max} was observed (Table 4-6). At an electric field strength of 6 kV/cm the specific inactivation rate was 0.19 $(kJ/kg)^{-1}$ at 0 % fat and 0.11 $(kJ/kg)^{-1}$ at 10 % fat. Without any fat in the medium the energy increased for a higher inactivation is lower compared to fatty medium. With increasing electric field strength k_{max} was increasing except for 10 %, where k_{max} was slightly decreasing.

Table 4-6: Specific inactivation rate (k_{max}) and shoulder length (*Sl*) for different electric field strengths and fat concentrations, r^2 is shown in brackets.

		Electric field strength [kV/cm]		
Fat concentration [%]		6	9	12.5
k_{max} [$(kJ/kg)^{-1}$]	0	0.19 (0.972)	0.26 (0.999)	0.28 (0.993)
	5	0.12 (0.928)	0.17 (0.999)	0.19 (0.995)
	10	0.11 (0.999)	0.09 (0.980)	0.09 (0.983)
Sl [kJ/kg]	0	222.2 (0.972)	235.8 (0.999)	243.4 (0.993)
	5	198.9 (0.928)	215.6 (0.999)	231.8 (0.995)
	10	175.8 (0.999)	169.6 (0.980)	189.3 (0.983)

The initiation of the inactivation is given by the shoulder length. At higher fat concentrations lower *Sl* values were observed. For example at 6 and 9 kV/cm, the *Sl* values for 0 % fat were 222.2 kJ/kg and 235.8 kJ/kg and at 10 % fat 175.8 and 169.6 kJ/kg, respectively. Thus an increase in fat concentration lowered the energy to start the inactivation, but it has to be taken into account that the slopes of the inactivation data at 10 % fat were lower than 0 % fat, which means more energy had to be applied to achieve the same level of inactivation. Comparing *Sl* at different electric field strengths an increase was observed with increasing electric field strengths. Only at 10 % fat, the *Sl* value decreased from 6 (175.8 kJ/kg) to 9 kV/cm (169.6 kJ/kg) followed by an increase at 12.5 kV/cm (189.3 kJ/kg).

In the following procedure of developing the isolog graphics to analyze the influence of fat (*F*) and electric field strength (*E*) on the inactivation, k_{max} and *Sl* were fitted and included in the primary model.

$$k_{max} = 0.416 - 0.035 \cdot F - \frac{1.565}{E} + 0.0005 \cdot F^2 + \frac{1.099}{E^2} + 0.131 \cdot \frac{F}{E}$$

r^2=0.988

$$Sl = \frac{(209.972 - 3.408 \cdot F - 35.049 \cdot E + 1.918 \cdot E^2)}{(1 - 0.011 \cdot F - 0.168 \cdot E + 0.009 \cdot E^2)}$$

r^2=0.990

The use of the complete model allows the calculation of inactivation for specific energy inputs, electric field strengths and fat concentrations. To validate the model a correlation of the predicted and the experimental data was performed (Figure 4-13). The determination coefficient (r^2) was 97.5 % and the root means square error was 0.186 indicating a precise model. This is also visible in Figure 4-13, because all data points are close to the line of equivalence. Moreover, the accuracy (A_f) and the bias (B_f) factors were close to 1, which indicates a high precision of the model.

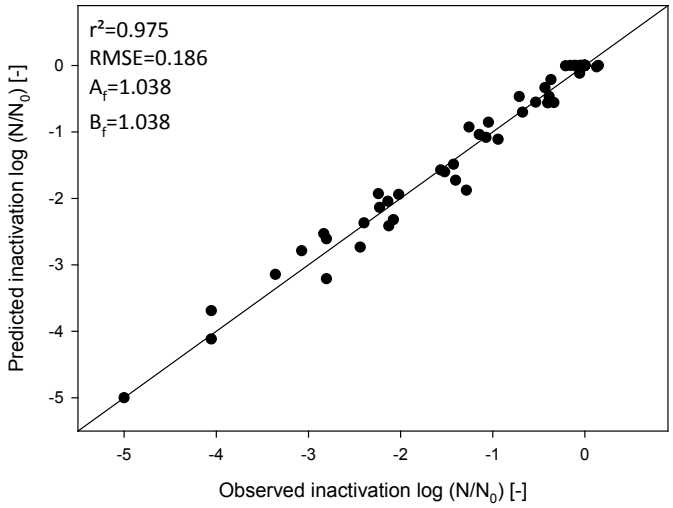

Figure 4-13: Regression of observed and by the developed model predicted inactivation data by PEF (r^2 = determination coefficient, *RMSE* = root mean square error, A_f = accuracy factor and B_f = bias factor).

The final model including the fitting of k_{max} and *SI* allows the calculation of the inactivation at different conditions. Figure 4-14a represents different inactivation levels as a function of energy input and fat concentration. Generally, a higher inactivation rate required a higher specific energy at constant electric field strength, which was in this case 12.5 kV/cm. At low inactivation level, a decrease in energy with increasing fat concentration was observed, but with increasing inactivation the opposite was detected. At a fat concentration of 4 % the energy to achieve a 1 and 3 log reduction was 244.1 and 266.8 kJ/kg. An increase of fat up to 10 % lead to an energy decrease to 231.1 kJ/kg for 1 log reduction and to an increase to 278.2 kJ/kg for a 3 log reduction. The results were obtained at a constant electric field strength of 12.5 kV/cm. By lowering the electric field strength, the required energy for a 3 log reduction decreased (Figure 4-14b). At an electric field strength of 6 kV/cm, the specific energy required for 3 log inactivation was nearly constant until a fat concentration of 7 %. A further increase in fat lead to a decrease of energy requirement. The same curve shape could be observed for 9 kV/cm, but in this case an increase of fat concentration lead to a decrease in the specific energy requirements even at low fat concentration. With further

increase in electric field strength the energy for a 3 log inactivation increased. An electric field strength of less than 11 kV/cm indicated a slight decrease in energy with increasing fat concentration. At 11 kV/cm, the energy was slightly decreasing with increasing fat concentration, but here it is more a linear relation. A further increase in electric field strength up to 12.5 kV/cm caused an increase in energy with increasing fat concentration. Consequently, the required energy for a 3 log inactivation depends on the fat concentration and the applied electric field strengths. The differences in energy at different electric field strengths were small at low fat concentrations. In all cases, the energy was nearly constant until a fat concentration of 6 %. The observed tendencies in this range can be explained by statistical variances. At fat concentration of more than 6 %, the required energy depends on the electric field strength. With increasing electric field strength the energy was increasing, but the difference in energy at 0 and 10 % wss 10 kJ/kg. Due to this small difference the effect of fat at this electric field strength is small and can be regarded as a tendency.

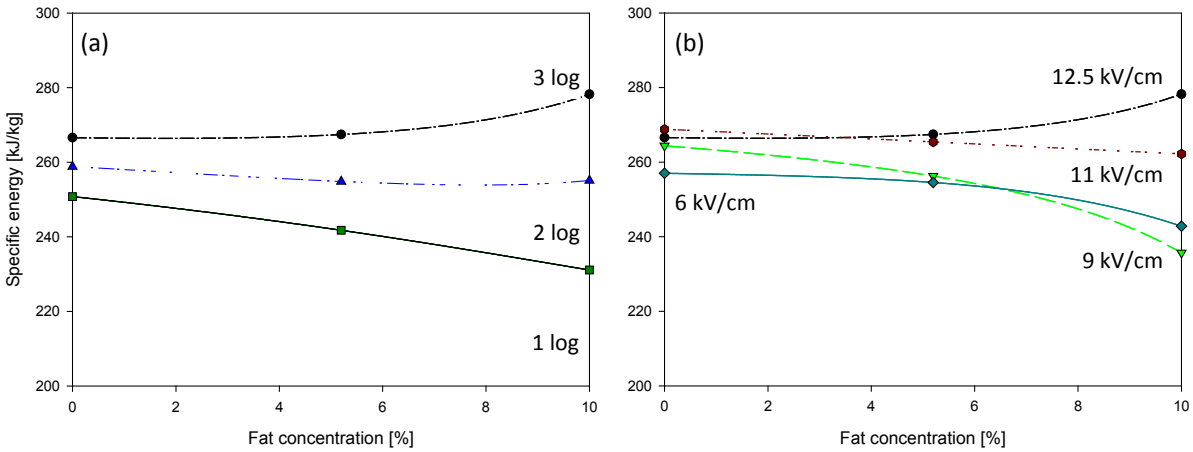

Figure 4-14: Influence of fat concentration on the inactivation of *G. stearothermophilus* spores by PEF using an inlet temperature of 80 °C at different energy values for (a) different log inactivation (3, 2 and 1 log) at an electric field strength of 12.5 kV/cm and (b) 3 log inactivation at different electric field strengths (pH 7); symbols in the graphs represents the experimental data.

4.2.1.4 Discussion and comparison of influence of fat on inactivation of different spore types by PEF

To study the effect of fat on the inactivation of *B. subtilis*, *A. acidoterrestris* and *G. stearothermophilus* spores, a mathematical model was performed to facilitate the comparison and discussion. The comparison of all spore types at different fat concentrations (Figure 4-15) indicated

G. stearothermophilus spores as most resistant spore for the applied PEF treatment followed by *B. subtilis* and *A. acidoterrestris* spores.

With increasing fat concentration different curve shapes were obtained for the spore types. The energy required to achieve a 3 log inactivation of *A. acidoterrestris* spores is decreasing with increasing fat concentration. The difference in energy between 0 and 10 % fat is about 20 kJ/kg and represent the highest difference in energy compared to the other two spore types. Therefore, the resistance of *A. acidoterrestris* spores is decreasing with increasing fat concentration. The inactivation of *B. subtilis* spores shows up to 4 % fat a slight increase in energy of about 10 kJ/kg and at more than 7 % fat a decrease down to the energy same as required at 0 % fat. The difference in energy of 10 kJ/kg is low and indicates only a slight effect of fat. This can be regarded as a tendency, because the statistical difference has to be considered. The same was observed for inactivation data of *G. stearothermophilus* spores, where until a fat concentration of 5 % no effect was observed. An increase up to 10 % fat causes an increase of energy about 10 kJ/kg. As the difference in energy is so small, the effect can be regarded as a tendency.

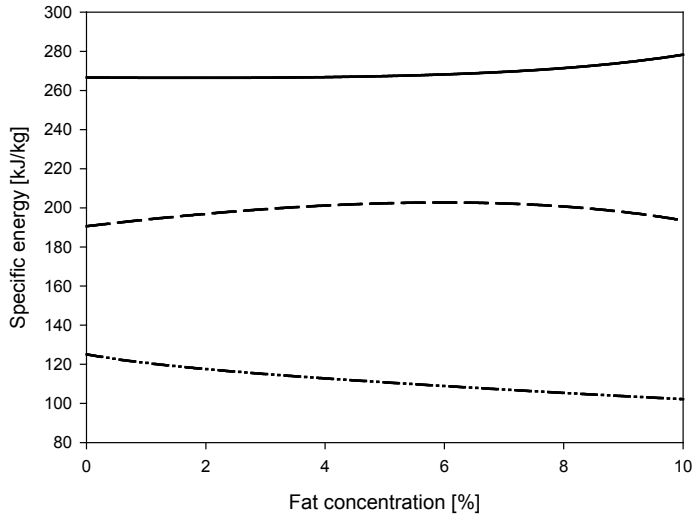

Figure 4-15: Specific energy required for a 3 log inactivation as a function of fat concentration for *G. stearothermophilus* (—), *B. subtilis* (— —) and *A. acidoterrestris* (—··—) at an electric field strength of 12.5 kV/cm and an inlet temperature of 80 °C.

Generally, a higher thermal resistance of spores is observed if suspended in pure fat (Molin et al. 1967; Senhaji et al. 1977; Ababouch et al. 1995). The study of Senhaji et al. (1977) showed the effect of fat on thermal inactivation of *B. subtilis* spores. An increase of D_{95} from 7.3 min in buffer to 166.7 min in soybean oil was reported. The higher thermal resistance is based on the lower

water activity of the medium (Jagannath et al. 2003). Studies analyzing the effect of fat in food products detected no or only limited effects on the inactivation (Senhaji 1977; Jagannath et al. 2003; Rodrigo et al. 1999). Rodrigo et al. (1999) reported no effect on the D_T value for *B. subtilis* spores in tomato sauce after adding 16 % soybean oil to the product. Whereas Jagannath et al. (2003) reported a D_{89} for *B. subtilis* spores of 35.09 min in UHT treated whole milk and 29.33 min in UHT skim milk. The increase in D_T value in medium with a higher fat content could be explained by the complexity of milk. In this case, it is not only an effect of fat, also of carbohydrates, proteins, salts and vitamins.

The results of this study were obtained at a maximum fat concentration of 10 %, which is lower than in the study of Rodrigo et al. (1999). Based on the results published, it can be assumed that the fat content analyzed in this study has no effect on the thermal resistance. The energy required for a 3 log inactivation indicates no or slight changes in energy at an electric field strength of 12.5 kV/cm for *G. stearothermophilus* and *B. subtilis* spores. The modeled inactivation data for *A. acidoterrestris* spores indicates a decrease in energy with increasing fat concentration. Therefore, at higher fat concentrations the outlet temperature is lower compared to low fat concentrations and the thermal effect on inactivation is reduced. Here, the changes in specific heat capacity c_p at higher fat concentrations have to be mentioned. With increasing fat concentration c_p is decreasing, which results in higher boiling point. The inactivation of *G. stearothermophilus* and *B. subtilis* spores indicates no influence of fat on the inactivation by PEF. Considering no effect of fat on thermal resistance in this fat concentration range, the temperature might be important for inactivation. The data in Figure 4-12 are calculated for a 3 log inactivation. At lower inactivation rates less energy is required resulting in a lower heat load of the product and therefore less impact of temperature on the inactivation.

The study performed by Bermúdez-Aguirre et al. (2012) focused on the inactivation of *B. subtilis* spores by PEF in combination with inlet temperatures of 65 °C and an electric field strength of 40 kV/cm in whole (4 % fat) and skim milk (0.3 % fat). The best result in terms of energy requirements for a high inactivation was observed in skim milk. The higher resistance in whole milk can be explained by the fat, but also other ingredients, such as proteins, which may influence the inactivation of PEF. Compared to this study, no or only slight influence of fat ranging from 0 to 10 % on inactivation of *B. subtilis* spores was observed. The different results can be explained by different treatment conditions and the medium characteristics.

Controversy results were obtained regarding the influence of fat on the resistance of vegetative bacteria to PEF treatment. Some studies indicate a higher resistance of cells, if the media contains fat (Grahl 1996), whereas other studies showed no effect of fat on the PEF resistance (Mañas et

al. 2001; Pol et al. 2001). Therefore, the influence of fat on PEF inactivation strongly depends on the microorganisms and the strain as well as other ingredients.

4.2.2 Influence of pH on the inactivation of spores by PEF

Another important product parameter is the pH value of the product. For acidic products, mainly acidophilic spore forming bacteria, such as *A. acidoterrestris* are of interest. A contamination with other spore types might occur depending on the origin of raw materials used for the product manufactured.

However, this study compares the inactivation of *B. subtilis*, *A. acdioterrestris* and *G. stearothermophilus* spores suspended in media with a pH of 4 and 7.

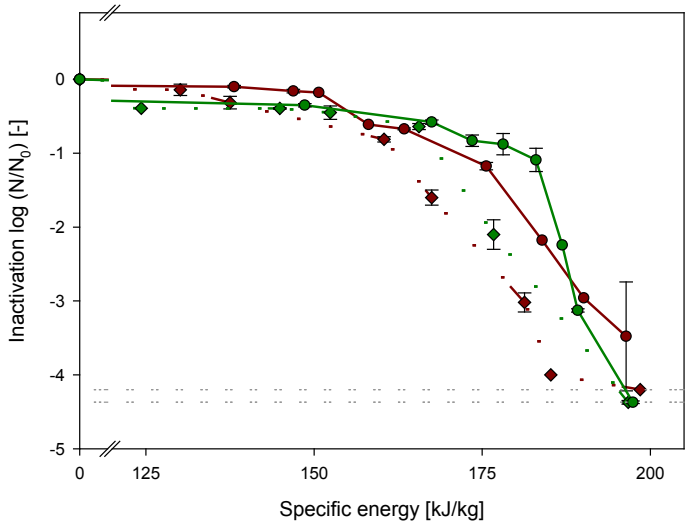

Figure 4-16: Inactivation of *B. subtilis* spores in Ringer´s solution (4mS/cm) at pH 4 at 6 kV/cm (●) and 9 kV/cm (◆) as well as pH 7 at 6 kV/cm (●) and 9 kV/cm (◆), detection limit (—·—).

Figure 4-16 represents the inactivation of *B. subtilis* spores at pH 4 and 7. Generally, the same curve shape of inactivation data for pH 4 is observed if compared to pH 7, which means the data can be separated in the shoulder part (initiation of inactivation) and linear part. At pH 4, a shorter shoulder for both analyzed electric field strengths is observed. Thus, less energy for the start of inactivation is required in an acidic media.

At an electric field strength of 6 kV/cm, the highest difference in energy, comparing medium with different pH, is observed at low inactivation rates. For an inactivation of 0.6 log an energy of 158.1 kJ/kg is required in acidic media compared to 178.5 kJ/kg at pH 7. The differences in energy between both pH values get smaller at high inactivation rates. Therefore, an energy of 184.9 kJ/kg has to be applied in acidic medium and 186.1 kJ/kg in neutral medium to achieve a 2 log spore

reduction. Although the shoulder at pH 7 is longer compared to pH 4, the inactivation in the linear part is faster, meaning that a certain increase in the specific energy applied at pH 7 results in a higher inactivation than at pH 4.

The inactivation data at an electric field strength of 9 kV/cm and pH 4 indicates a lower energy requirement for inactivation compared to the energy requirement at pH 7. For a 1.6, 3.0 and 4.0 log inactivation a specific energy of 167.5, 181.3 and 185.2 kJ/kg in acid media and 174.5, 186.9 and 195.5 kJ/kg in neutral media is required, respectively. Thus, the highest difference in energy between inactivation in acid and neutral media is about 10 kJ/kg. The detection limit and inactivation curves cross each other and the energy at pH 4 is slightly higher than at pH 7.

The impact of the pH on the inactivation by PEF is small, as the difference in the specific energy required for inactivation is small.

The influence of acid media on the inactivation of *A. acidoterrestris* spores is presented in Figure 4-17. At an electric field strength of 6 kV/cm, the energy required for an inactivation in acidic media is lower compared to the neutral media. For a 1.0 and 3.2 log inactivation, an energy of 113.6 and 125.8 kJ/kg at pH 4 and 118.7 and 132.2 kJ/kg at pH 7 is required. The differences in energy for a specific inactivation are small and getting smaller with increasing electric field strength up to 12.5 kV/cm. At this electric field strength, the energy required for a certain level of specific inactivation is lower at pH 4 compared to pH 7. The energy for a 2.4 log inactivation at pH 4 is 116 kJ/kg, compared to 123 kJ/kg for a 2.6 log inactivation at pH 7.

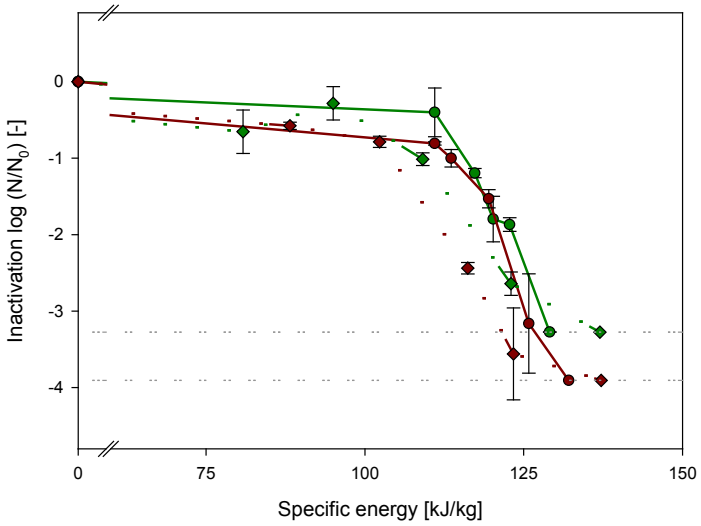

Figure 4-17: Inactivation of *A. acidoterrestris* spores at pH 4 at 6 kV/cm (●) and 9 kV/cm (◆); pH 7 at 6 kV/cm (●) and 9 kV/cm (◆), detection limit (—·—).

A limited effect of the pH on the inactivation of *G. stearothermophilus* spores is observed by applying an electric field strength of 12.5 kV/cm (Figure 4-18). The energy for an inactivation at acidic pH is lower than at pH 7, but the differences in energy are small, especially at high inactivation rates. For example, at pH of 4, a 3.0 and 4.4 log inactivation is achieved with an energy input of 260 and 277 kJ/kg and 267 and 278 kJ/kg is required at pH 7. Lowering the electric field strength to 6 kV/cm, a stronger influence of the pH on the inactivation is observed. Generally, the energy at 6 kV/cm is lower compared to 12.5 kV/cm and at pH 4 the differences in energy between those two electric field strengths is higher compared to pH 7. However, at 6 kV/cm the energy required for a specific inactivation in an acidic media is smaller compared to neutral media. At an energy of 233 kJ/kg an inactivation of 0.9 log was achieved at pH 7. Applying a similar energy level in acidic media, an inactivation of 3 log is observed.

At high electric field strengths the difference in energy required for a specific inactivation between pH 4 and 7 was small, showing that the effect of pH on inactivation is small. On the contrary, at low electric field strength an effect of pH in terms of lowering the energy was observed.

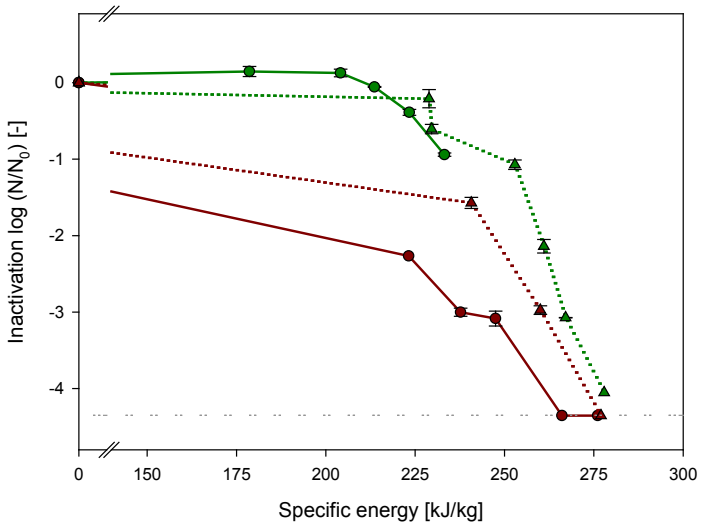

Figure 4-18: Inactivation of *G. stearothermophilus* spores at pH 4 at 6 kV/cm (●) and 12.5 kV/cm (▲) as well as pH 7 at 6 kV/cm (●) and 12.5 kV/cm (▲), detection limit (—·—).

As a result the effect of pH on the inactivation of *B. subtilis*, *A. acidoterrestris* and *G. stearothermophilus* spores by PEF in combination with thermal energy is low, because the energy required for a specific inactivation is just slightly lower in acidic media compared to neutral media. An exception is the inactivation of *G. stearothermophilus* spores applying lower electric field strength of 6 kV/cm. In this case, the difference in energy observed at pH 4 and 7 is high, so that an effect of the pH in order to allow energy reduction at pH 4 is shown.

In the area of inactivation of vegetative microorganisms by PEF, the influence of pH was studied by several researchers (see section 2.2.2.2) and controversy results were obtained. The studies, where the pH affected the inactivation by PEF (Wouters et al. 1999; Álvarez 2000; Aronsson et al. 2005), termed the strain of the microorganism as an important parameter. Therefore, gram positive bacteria are more resistant in neutral media compared to gram negative bacteria. The opposite relation was observed in acidic media (García et al. 2005b). The reason might be the ability of repairing sub lethal injury caused by the PEF treatment and the cell membrane/wall structure (Mackey 2000). From these results it shows, that the acidic environment acts on the cell surrounding elements of bacteria and therefore influences the efficiency of the treatment. Comparing bacteria and spores though, the structure of spores is much more complex in terms of layers around the core (see section 2.1.3). Due to this complexity, an effect of the acidic environment on the layers is small. Considering the effect of the pH on the spore layers, and also the temperature on the spore has to be reflected. A decrease in thermal resistance of spores was observed in acid media (Hutton et al. 1991; Ababouch et al. 1995; Palop et al. 1996; Mazas et al. 1998; Pontius et al. 1998; Silva et al. 1999; Fernández et al. 2002; Setlow et al. 2002; Bevilacqua et al. 2008). In very acidic media (pH 1), a very high inactivation of *B. subtilis* spores can be achieved within 1 min at 24 °C (Setlow et al. 2002), which is based on a rupture of spore layers. The study of López et al. (1996) focused on the thermal inactivation of *G. stearothermophilus* spores in McIlvaine buffer with a pH range from 4 to 7 and varying the temperature from 115 to 135 °C. At low temperature, the thermal resistance is decreasing with decreasing pH. The D_{115} at pH 4 is 1.39 min compared to 10.2 min at pH 7. With increasing temperature up to 135 °C the differences in D value are small; 0.015 min at pH 4 compared to 0.037 min at pH 7. The same effect of higher impact of pH value on thermal resistance at lower temperatures was observed for *A. acidoterrestris* (Pontius et al. 1998). Considering the inactivation of *G. stearothermophilus* spores by PEF, an energy of around 270 kJ/kg has to be applied. The calculated outlet temperature (Equation 13) is 147.5 °C, which is higher than the temperatures used in the study of López et al. (1996). Consequently, the effect of pH on thermal resistance is expected to be low. The discussion of the influence of temperature on the inactivation will be discussed in chapter 4.3. However, the slight effect of pH value in terms of reduced energy in acidic pH might be based on the lower thermal resistance. The acidic pH value affects the spore coat layers, which have an impact on the resistance of the PEF treatment. The effect of reduced resistance to PEF might be higher at pH values lower than 4.

4.2.3 Influence of sugar on the inactivation of spores by PEF

The addition of sugar influences the taste in order to affect the sweetness, but also physical parameters, such as water activity are influenced. The following results demonstrate the influence of increased soluble solids content on the inactivation of various spore types by PEF in combination with thermal energy. The sugar level varies from 0 to 10 %. It is not sensible to analyze higher sugar concentrations, because at very high concentrations the water activity represents another hurdle for spores (Jagannath et al. 2003).

4.2.3.1 Inactivation of *B. subtilis* spores

The observed inactivation data showed a shoulder representing the initiation of the inactivation and a linear part. To facilitate the demonstration of the effect of sugar content on the inactivation the model developed by Geereard et al. (2005) was used. The shoulder and linear part of the inactivation at different sugar concentrations and electric field strengths as a function of the specific energy are described by the shoulder length (*Sl*) and the specific inactivation rate (k_{max}) (Table 4-7).

Table 4-7: Specific inactivation rate (k_{max}) and shoulder length (*Sl*) of inactivation data of *B. subtilis* spores at different sugar concentrations (0, 5 and 10 %) and electric field strengths (6, 9 and 12.5 kV/cm) obtained using Equation 24, r^2 is shown in brackets, nd - not detected.

		Electric field strength [kV/cm]		
Sugar concentration [%]		6	9	12.5
k_{max} [(kJ/kg)$^{-1}$]	0	0.49 (0.953)	0.27 (0.975)	0.27 (0.989)
	5	0.17 (0.998)	0.12 (0.986)	nd
	10	0.11 (0.911)	0.24 (0.994)	nd
Sl [kJ/kg]	0	178.40 (0.953)	160.55 (0.975)	167.58 (0.989)
	5	114.46 (0.998)	110.00 (0.986)	nd
	10	100.14 (0.911)	116.90 (0.994)	nd

At an electric field strength of 6 kV/cm, k_{max} was decreasing with increasing sugar concentration. At 10 % sugar, a higher energy input was required to increase the inactivation compared to 0 % sugar. An increase in electric field strength up to 9 kV/cm indicated a decrease in k_{max} from 0.27 to 0.12 (kJ/kg)$^{-1}$ at 0 and 5 % sugar followed by an increase of up to 0.24 (kJ/kg)$^{-1}$at 10 % fat. No values for k_{max} were obtained at 12.5 kV/cm, because not enough inactivation data were available. Generally, the slope obtained in a medium without sugar was higher compared to k_{max} at high sugar concentration. Therefore, at higher sugar concentrations a higher energy increase was necessary to increase the inactivation. The initiation of the inactivation is described by the shoulder length. The highest values, which means longest shoulder, was observed at 6 kV/cm with no sugar

in the medium. The addition of sugar caused a shorter shoulder. Between the two different sugar levels, only slight differences between 6 and 9 kV/cm were observed. To sum up, the addition of sugar lead to a shorter shoulder, but a reduced slope indicating a higher energy increase to increase the inactivation.

For the secondary model, the obtained values for k_{max} and *Sl* were fitted and expressed by the following equations, where *S* indicates the sugar concentration and *E* the electric field strength.

$$k_{max} = 1.488 - 0.152 \cdot S - 0.231 \cdot E + 0.005 \cdot S^2 + 0.01 \cdot E^2 + 0.011 \cdot S \cdot E$$

r^2=0.998

$$Sl = 288.592 - 25.44 \cdot S - 26.174 \cdot E + 1.07 \cdot S^2 + 1.319 \cdot E^2 + 1.152 \cdot S \cdot E$$

r^2=0.999

The developed model was validated by correlation of experimental observed and calculated inactivation data as well as calculated the correlation coefficient, accuracy and bias factor.

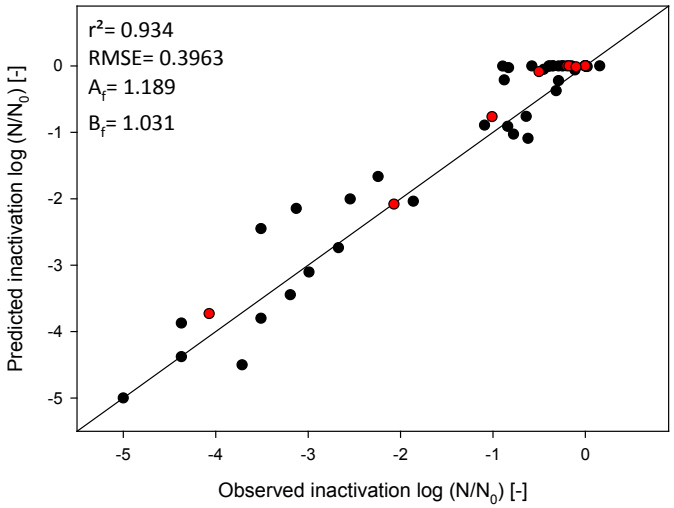

Figure 4-19: Correlation of predicted and observed inactivation of *B. subtilis* spores (● 6 and 9 kV/cm, ● 12.5 kV/cm) (r^2 = determination coefficient, *RMSE* = root mean square error, A_f = accuracy factor and B_f = bias factor).

The deviation in the prediction of inactivation compared to experimental data when the level of inactivation was lower than 0.5 log was neglected as this low level of inactivation was not relevant for the analysis. Due to the low inactivation at this level, it was not relevant for the analysis, and it was disregarded. The red symbols in Figure 4-19 represent the observed and calculated inactivation at 12.5 kV/cm. As stated previously, insufficient data were available for calculations of k_{max} and *Sl*. The model was extrapolated for this electric field strength. As the experimental and calculated inactivation rates fit quite well, the values were included for the creation of isographs.

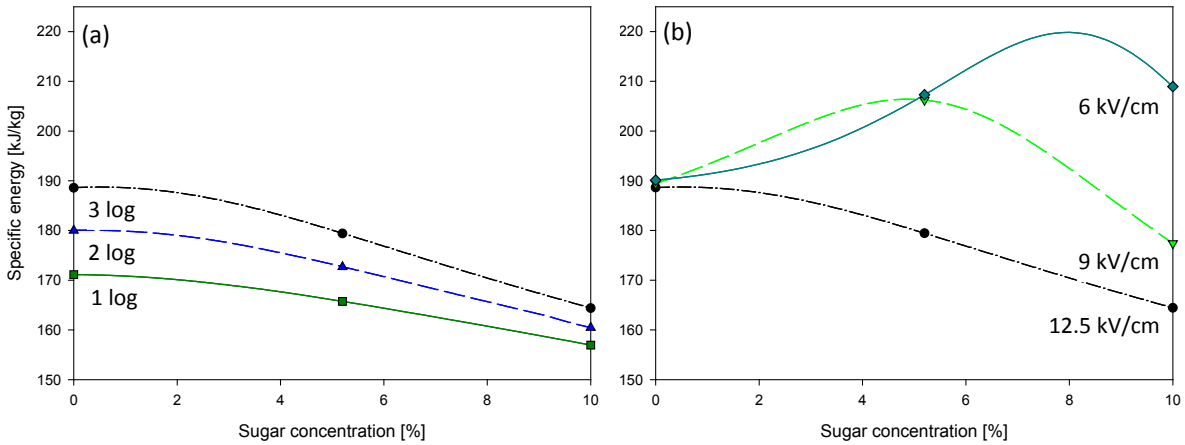

Figure 4-20: Inactivation of *B. subtilis* spores at (a) 12.5 kV/cm and different inactivation rates as well as (b) for a 3 log inactivation at different electric field strengths as a function of specific energy using an inlet temperature of 80 °C obtained in Ringer´s solution with a conductivity of 4 mS/cm and a pH of 7, symbols in the graphs represent the experimental data.

The isographs allow analysis of dependence of the inactivation on specific energy, electric field strength and sugar concentration. Figure 4-20a represents the energy required for a specific inactivation at an electric field strength of 12.5 kV/cm. The results show an increase of energy for high inactivation rates observed for all sugar concentrations. The difference in energy at low and high sugar concentration increased for higher inactivation rates. For a 1 log and 3 log reduction of *B. subtilis* spores, an energy of 170.2 and 187.6 kJ/kg was required at a sugar level of 2 % compared to 160.8 and 170.4 kJ/kg at 8 % sugar. Consequently, the energy difference for a 3 log inactivation was 17.5 kJ/kg in contrast to a difference of 9.6 kJ/kg for a 1 log inactivation. Thus, the energy for a 3 log reduction decreased from a maximum 188.5 kJ/kg at 1 % sugar to 164.4 kJ/kg at 10 % sugar, which is a difference of 24.1 kJ/kg, whereas the difference for a 1 log reduction is 13.2 kJ/kg.

However, the energy required for an inactivation is also dependent on the electric field strength (Figure 4-20b). In a medium without sugar, no difference in energy at different electric field strengths was evident, but with increasing sugar concentration the differences increased. The lowest energy for a 3 log reduction was required using an electric field strength of 12.5 kV/cm. Here, a slight decrease in energy was observed with increasing sugar concentration. Reducing the electric field strength to 9 kV/cm, an increase in energy was observed until a specific sugar concentration followed by a decrease in energy. The PEF treatment of a Ringer´s solution without sugar required 189.4 kJ/kg and increased up to 206.4 kJ/kg at 4.8 % sugar. A higher sugar concentration lead to a decrease in specific energy to 177.4 kJ/kg. This kind of turning point was also observed at 6 kV/cm, but the energy increase was higher (maximum of 219.8 kJ/kg) and the turning point to a decrease in energy was at 8 % sugar. It has to be note, that the energy at 8 % sugar was

calculated by the model. A better illustration of specific energy, sugar concentration and electric field strength for a 3 log reduction is shown in Figure 4-21.

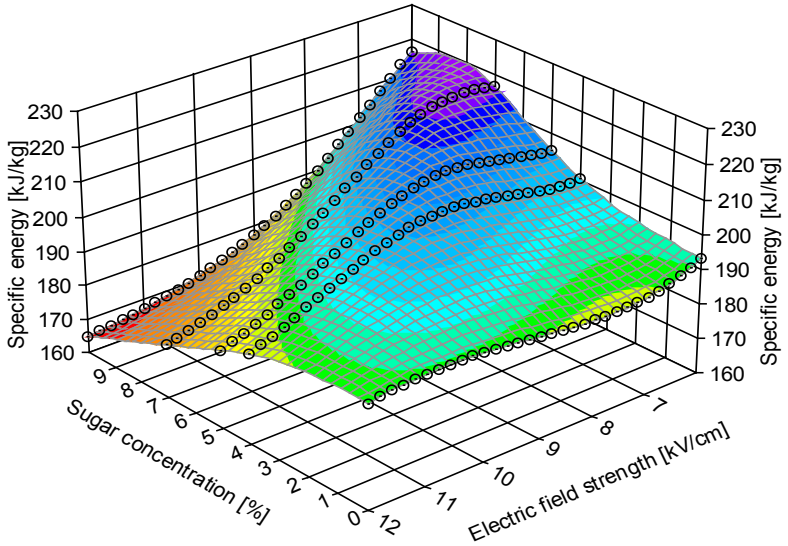

Figure 4-21: 3 log reduction of *B. subtilis* spores in Ringer´s solution with a conductivity of 4 mS/cm and pH 7 as a function of specific energy, electric field strength and sugar concentration.

As a result, the energy required for inactivation of *B. subtilis* spores depended on the sugar level. At high electric field strengths, a high sugar level was beneficial for the inactivation, because lower specific energy was required. On the contrary, it seems that the presence of sugar in the medium had a protective effect against PEF, at low electric fields.

4.2.3.2 Inactivation of *A. acidoterrestris* spores

To model the inactivation data of *A. acidoterrestris* spores by PEF the model describing linear inactivation preceeding a shoulder (Geeraerd et al. 2005) was used. By using Equation 24, the specific inactivation rate k_{max} and the shoulder length *Sl* were calculated (Table 4-8).

Table 4-8: Specific inactivation rate (k_{max}) and shoulder length (*Sl*) for *A. acidoterrestris* spores based on Equation 24, r^2 is shown in brackets, nd - not detected.

		Electric field strength [kV/cm]		
Sugar concentration [%]		6	9	12.5
k_{max} [(kJ/kg)$^{-1}$]	0	0.37 (0.980)	0.20 (0.953)	0.19 (0.984)
	5	0.25 (0.885)	0.17 (0.992)	nd
	10	0.34 (0.862)	0.18 (0.893)	0.19 (0.977)
Sl [kJ/kg]	0	107.80 (0.980)	90.24 (0.953)	88.65 (0.984)
	5	111.23 (0.885)	94.96 (0.992)	nd

	10	107.95 (0.862)	93.65 (0.893)	91.38 (0.977)

The slope of the linear inactivation part is described by the specific inactivation rate k_{max} and is represented in Table 4-8 for each sugar concentration and electric field strength. At an electric field strength of 6 kV/cm, k_{max} decreased from 0.37 to 0.25 (kJ/kg)$^{-1}$. With increasing sugar concentration from 0 to 5 % the k_{max} increased up to 0.34 (kJ/kg)$^{-1}$ at 10 % sugar. The differences in k_{max} at different sugar concentrations were small and became even smaller at higher electric field strengths. At 9 and 12.5 kV/cm, k_{max} was smaller compared to 6 kV/cm and in the same range of approximately 0.19 (kJ/kg)$^{-1}$. The initiation of the inactivation is described by the shoulder length. Only small differences at different sugar levels were observed, where *Sl* decreased with increasing electric field strengths. At high electric field strengths, the initiation of the inactivation was shorter compared to low electric field strengths, but the slope of the linear inactivation part was lower, which meant a higher energy increase had to be applied for an inactivation increase.
To describe these results in a complete model, the k_{max} and *Sl* values were fitted and the resultant equations were inserted into the primary model.

$$k_{max} = \frac{(0.088 + 0.004 \cdot S - 0.0004 \cdot S^2 - 0.02 \cdot E)}{(1 - 0.0004 \cdot S - 0.213 \cdot E + 0.005 \cdot E^2)}$$

r^2=0.996

$$Sl = \frac{(177.62 + 1.684 \cdot S - 17.473 \cdot E + 0.732 \cdot E^2)}{(1 + 0.004 \cdot S + 0{,}001 \cdot S^2 - 0{,}013 \cdot E)}$$

r^2=0.999

Before using the model, it has to be validated by estimating statistical relevant parameters, such as determination coefficient, root mean square error, accuracy and bias factor. The values displayed in Figure 4-22 show a high precision of the developed model. Especially the B_f value of 1 indicates a good fit of the model.

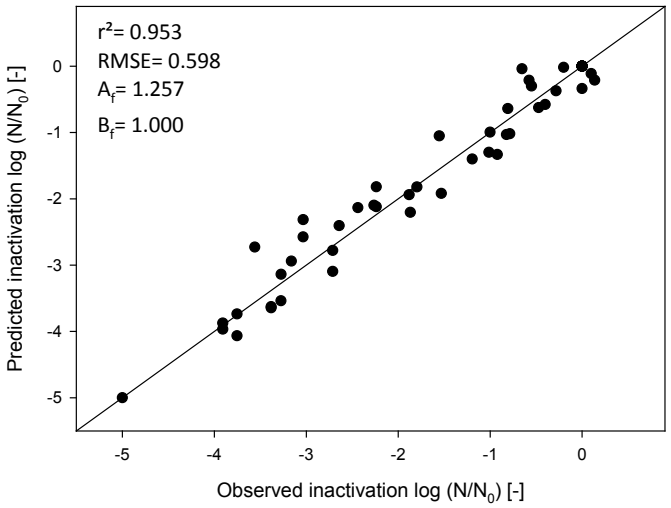

Figure 4-22: Correlation of observed and predicted inactivation of *A. acidoterrestris* spores (r^2 = determination coefficient, *RMSE* = root mean square error, A_f = accuracy factor and B_f = bias factor).

The good statistical fit of the model allows the use of the model to form isographs in order to demonstrate the dependency of energy required for a specific inactivation from sugar concentration and electric field strength. Figure 4-23a represents the energy required for 1, 2 and 3 log reduction as a function of sugar concentration. It is obvious, that a higher energy is required for a higher inactivation rate. For a 3 log reduction the energy had to be increased from 101.6 kJ/kg and for a 1 log inactivation up to 126.4 kJ/kg at a sugar concentration of 1.2 %. The energy increase necessary to increase the inactivation about 1 log was 12 kJ/kg independent from the sugar level. The energy showed a slight increase with increasing sugar concentration up to a maximum followed by a decrease in energy. For a 3 log reduction, the energy was 123.8 kJ/kg at 0 % sugar and increased up to 130.6 kJ/kg at 5.2 % followed by a decrease to 127.0 kJ/kg at 10 % sugar at an electric field strength of 12.5 kV/cm. Comparing the different energy values, the difference in energy is small. A decrease in electric field strength (Figure 4-23b) leads to an increase in energy difference. The maximum energy at 6 kV/cm was at 5.2 % sugar, as already observed at 12.5 kV/cm, but the difference in energy from 0 to 5.2 % was 11.4 kJ/kg compared to 6.8 kJ/kg at 12.5 kV/cm. As the differences in energy were quite small, only limited effects of sugar on the required energy were observed. Generally, a higher energy is required for a 3 log reduction, when low electric field strengths are applied.

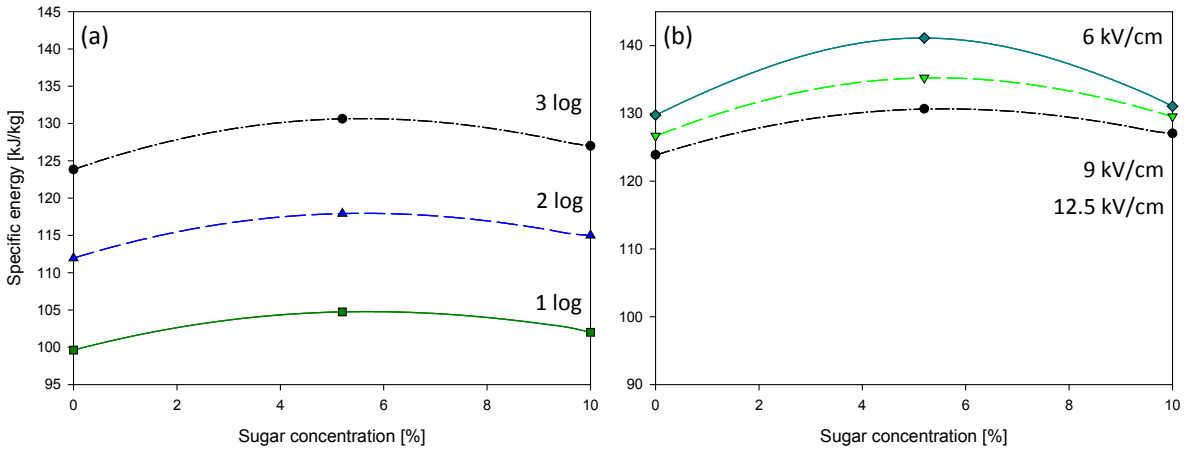

Figure 4-23: Specific energy as a function of sugar concentration for a (a) 3, 2 and 1 log reduction of *A. acidoterrestris* spores at an electric field strength of 12.5 kV/cm and for a (b) 3 log reduction at different electric field strengths of 6, 9 and 12.5 kV/cm, symbols in the graphs represent the experimental data.

4.2.3.3 Inactivation of *G. stearothermophilus* spores

For modeling the effect of sugar on the inactivation of *G. stearothermophilus* spores the proposed model by Geeraerd et al. (2005) was used. First stage of the mathematical modeling is calculation of specific inactivation rate k_{max} and shoulder length *Sl* by using Equation 24.

Table 4-9: Specific inactivation rate (k_{max}) and shoulder length (*Sl*) calculated by Equation 24 using the inactivation of *G. stearothermophilus* spores by PEF, r^2 is shown in brackets, nd - not detected.

		Electric field strength [kV/cm]		
Sugar concentration [%]		6	9	12.5
k_{max} [(kJ/kg)$^{-1}$]	0	0.19 (0.972)	0.26 (0.999)	0.28 (0.993)
	5	0.12 (0.963)	0.25 (0.990)	nd
	10	0.16 (0.996)	0.12 (0.999)	0.13 (0.997)
Sl [kJ/kg]	0	222.22 (0.972)	235.80 (0.999)	243.47 (0.993)
	5	227.17 (0.963)	226.17 (0.990)	nd
	10	220.12 (0.996)	204.43 (0.999)	198.05 (0.997)

The slope of the linear inactivation part represents the specific inactivation k_{max}. At an electric field strength of 6 kV/cm, k_{max} was decreasing from 0.19 to 0.12 (kJ/kg)$^{-1}$ with an increased sugar concentration from 0 to 5 % followed by an increase up to 0.16 (kJ/kg)$^{-1}$ at 10 % sugar (Table 4-9). With increasing electric field strength up to 9 and 12.5 kV/cm, an increase in k_{max} was observed indicating less energy increase for an inactivation increase. The initiation of the inactivation is given by the shoulder length (*Sl*). The shoulder length at different sugar concentrations was similar at an electric field strength of 6 kV/cm, whereas at 9 and 12.5 kV/cm higher differences were observed. With increasing sugar concentration, the *Sl* decreased from 235.8 and 243.5 kJ/kg to

204.4 and 198.1 kJ/kg for 9 and 12.5 kV/cm at sugar concentrations of 0 and 10 %, respectively. Thus, at higher electric field strengths and high sugar concentration (10 %) a shorter shoulder was observed.

To describe all the experimental data with one single equation, *Sl* and k_{max} were fitted in a second step by using TableCurve 3D resulting in a best fitting equation. Afterwards, the equations were inserted in the primary model and the inactivation of *G. stearothermophilus* spores was calculated. By comparing the experimental and predicted data, the model was statistically evaluated (Figure 4-24).

$$k_{max} = 0.187 - 0.03 \cdot S + \frac{2.482}{E} - 0.0001 \cdot S^2 - \frac{15.395}{E^2} + 0.167 \cdot \frac{S}{E}$$

r²=0.852

$$Sl = 277.318 - 7.112 \cdot S - \frac{436.699}{E} - 0.241 \cdot S^2 + \frac{629.095}{E^2} + 56.122 \cdot \frac{S}{E}$$

r²=0.999

Before mentioning the statistical factors for model validation, the coefficient of determination for the k_{max} equation has to be discussed. An r^2 of 0.852 was calculated for the best fitting model. As it was lower than 90 % it was not statistically significant. However, the equation was used for the prediction and as a result a lower r^2 for the final model was observed. As the r^2 of the final model was higher than 90 % the model is accurate enough for further analysis. This is supported by the valuable other statistical factors that were calculated such as *RMSE*, A_f or B_f.

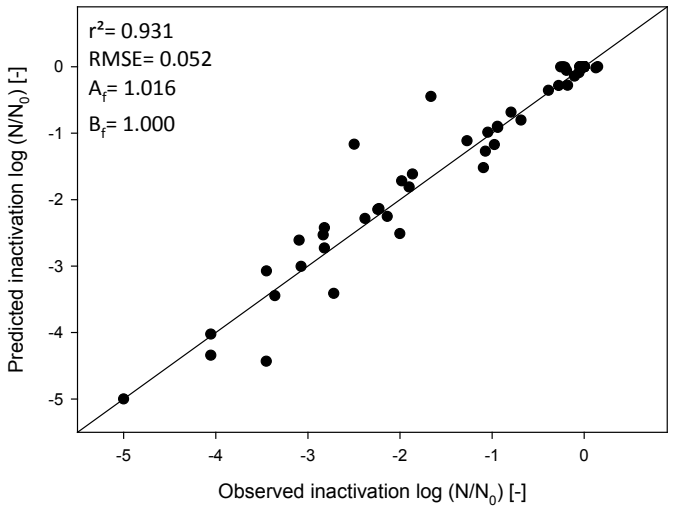

Figure 4-24: Statistical values for validation of the developed model calculation the inactivation of *G. steraothermophilus* spores at different conditions (r^2 = determination coefficient, *RMSE* = root mean square error, A_f = accuracy factor and B_f = bias factor).

The final model allows the prediction of inactivation under non analyzed conditions and therefore a facilitated comparison or analysis of the effect of sugar on inactivation. Figure 4-25 represents different inactivation rates of *G. steraothermophilus* spores in Ringer´s solution with different sugar concentrations at constant electric field strength of 12.5 kV/cm. To achieve a higher inactivation, a higher specific energy was required. At 5.2 % sugar an energy of 237.4, 249.6 and 261.4 kJ/kg was necessary for a 1, 2 and 3 log spore reduction. At low inactivation rates about 1 log, the energy was decreasing with increasing sugar level. The same effect of decreasing energy up to a sugar level of 7 % was observed at higher inactivation rates. At higher sugar concentrations the energy started to increase slightly.

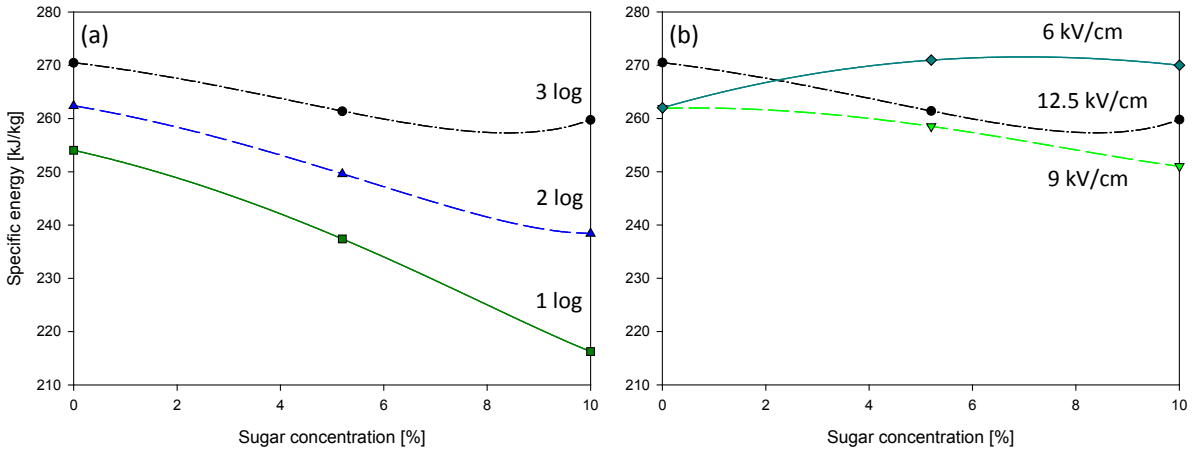

Figure 4-25: Specific energy for (a) 3, 2 and 1 log reduction of *G. stearothermophilus* spores at an electric field strength of 12.5 kV/cm and (b) different sugar concentration in Ringer´s solution with a conductivity of 4 mS/cm and pH 7, symbols in the graphs represent the experimental data.

To demonstrate the effect of electric field strength, specific energy and sugar concentration for a 3 log reduction a 3D plot was generated (Figure 4-26). The maximum energy for a 3 log reduction was 6 kV/cm. Only slight differences in energy at different sugar concentrations were observed. In this case, the energy ranges from 262 to 272 kJ/kg. At 0 % sugar and high electric field strengths of 12.5 kV/cm, a high energy of 269 kJ/kg was required. Based on these observations, the energy decreased with increasing sugar concentration and electric field strength. The minimum energy was at an electric field strength of 9 kV/cm. A further increase in electric field strength resulted in an increase in energy. However, the energy for a 3 log reduction varied about 20 kJ/kg from 250 to 270 kJ/kg, which not very high, so that the effect of sugar on the inactivation is low.

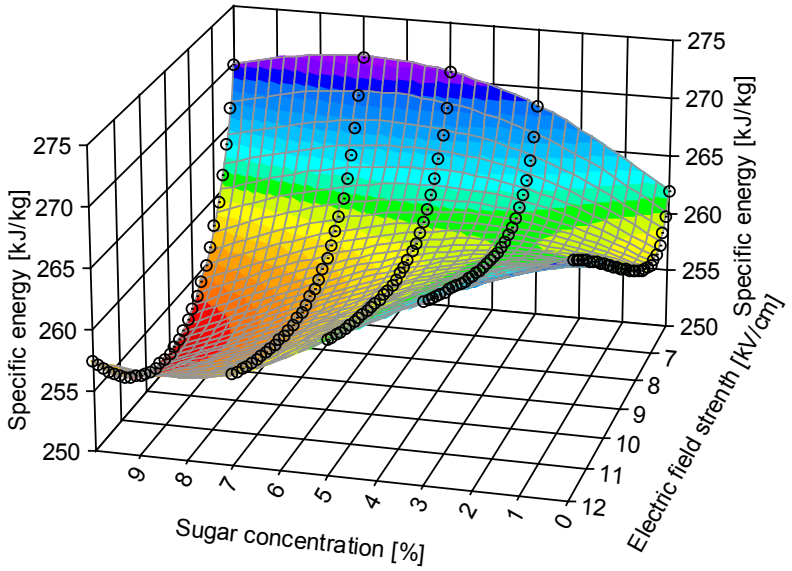

Figure 4-26: Dependence of specific energy on the electric field strength and sugar concentration for a 3 log reduction of *G. stearothermophilus* spores.

4.2.3.4 Comparison and discussion of influence of sugar on inactivation of endospores by PEF

The obtained results using mathematical modeling showed the influence of sugar for each spore type. A comparison between inactivation of *B. subtilis*, *A. acdioterrestris* and *G. stearothermophilus* spores at different sugar level is shown in Figure 4-27.

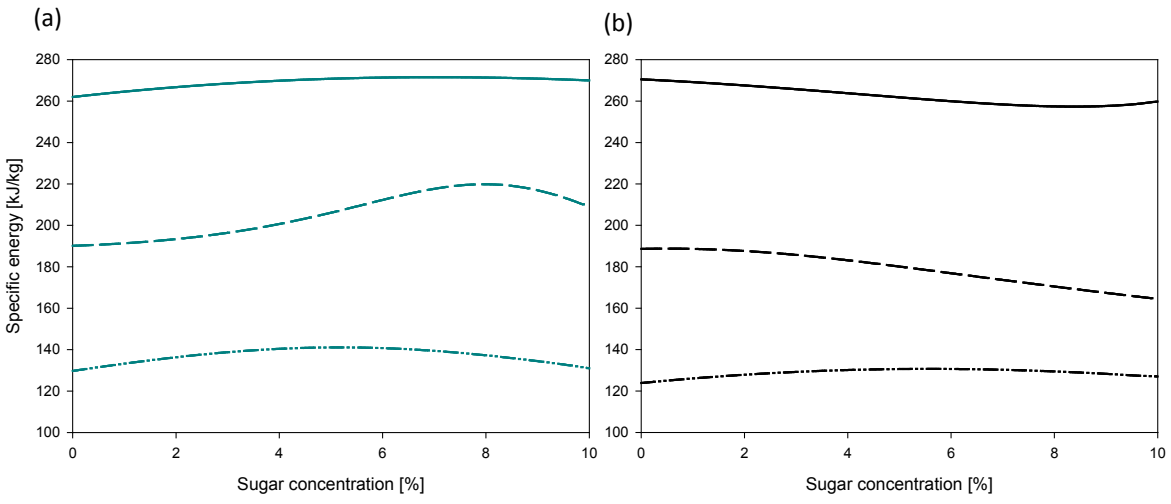

Figure 4-27 Specific energy required for a 3 log inactivation of *G. stearothermophilus* (—), *B. subtilis* (— —) and *A. acidoterrestris* (—··—)as a function of fat concentration at an electric field strength of (a) 6 kV/cm and (b) 12.5 kV/cm at an inlet temperature of 80 °C.

As observed in previous chapters, studying the influence of processing parameters or properties of the product, such as fat, *G. stearothermophilus* spores showed the highest resistance against the PEF treatment followed by *B. subtilis* and *A. acidoterrestris* spores.

The highest effect of sugar at an electric field strength of 12.5 kV/cm was observed for *B. subtilis*. In a medium without sugar, an energy of 188 kJ/kg was required to achieve a 3 log reduction. With increasing sugar contents of up to 10 %, the energy was reduced to 164 kJ/kg. Less difference in energy was obtained for *A. acidoterrestris* and *G. stearothermophilus* spores. For the inactivation of both spores, the energy at 10 % sugar was lower compared to 0 %, but around 5 % sugar, *A. acidoterrestris* spores showed a slight increase in energy. At an electric field strength of 6 kV/cm, the addition of sugar influenced the energy for inactivation of *B. subtilis*. In contrast to the results at 12.5 kV/cm, an increase is energy was observed up to a maximum of 219.8 kJ/kg followed by a decrease. For the inactivation of *G. stearothermophilus* spores slight differences in energy with increasing sugar concentration was observed.

The increase of sugar content causes an increase in osmotic pressure, which leads to an increase in heat resistance (Komitopoulou et al. 1999). The formation of osmotic pressure leads to a cross flow of ions out- and into the cell. Due to this cross flow, the spore core dehydrates and the spore shows a higher resistance against heat (Komitopoulou et al. 1999). In the case of *B. subtilis* spores at an electric field strength of 6 kV/cm, an increase in energy was observed with increasing sugar concentration. The energy increase may have been due to the effect of sugar on the heat resistance. At higher sugar concentration, the spores were more resistant to heat and because of the relation between PEF energy and outlet temperature, a higher energy was required. This stated that thermal inactivation was involved in the inactivation mechanism. At higher electric field strengths, a decrease in energy was observed. Going back to the fact, that sugar affects the heat resistance and the relation of energy and outlet temperature, it can be assumed, that the heat resistance played only a limited role. With less energy, the outlet temperature is lower and therefore the higher heat resistance might not have affected the inactivation of the spores by PEF. Regarding the other two spore types, only limited effects were observed. The *G. stearothermophilus* spores are highly temperature resistant and may not be affected by the analyzed sugar concentration. It might be that the sugar concentration used in this study was too low to affect the resistance. Also, the energy required for a 3 log reduction of *A. acidoterrestris* indicates only slight differences with increasing sugar concentration. In literature, it is found that at sugar concentration lower than 50° Bx, no effect of sugar on the heat resistance of *A. acidoterrestris* was observed (Maldonado et al. 2008). As the heat resistance is mainly increased due to changes in core dehydration caused by penetration of ions through the membrane (Setlow 2006), the studied sugar

level is too low to induce ion penetration. Therefore, no changes in spore structure could occur due to the studied sugar concentration affecting the spore resistance to PEF treatment.
High sugar concentrations are affecting the resistance of vegetative bacteria towards PEF (Aronsson et al. 2001; Álvarez et al. 2002). The effect is based on the effect of sugar on the membrane. Due to the sugar, the permeability and the fluidity of the membrane is affected in order to make it thicker and more complex, which results in a higher energy demand causing permeabilization (Neidhardt et al. 1990). Only at low electric field strengths, a higher PEF resistance of *B. subtilis* spores could be shown, which might be in relation to the thermal heat resistance. At 12.5 kV/cm, the energy decreases, which might be due to the effect on the spore permeability barrier. Nevertheless, the PEF effect of sugar on vegetative bacteria cannot be transferred to inactivation of spores by PEF. The main reason for this is the difference in structural composition.

4.2.4 Influence of ion concentration on the inactivation of spores by PEF

Low conductivity values are expected in the area of fruit juices or smoothies, whereas high conductivities are to be expected in the area of soups. As it is already explained in section 2.2.2.2, the influence of salt concentration should be evaluated as a process parameter and as an influencing parameter on inactivation of spores. The effect as process parameter is mainly an issue for the setup of the PEF system. At high conductivity levels the resistance is lower and therefore a higher current has to be applied in order to achieve the required voltage. Due to current limit of the PEF system no high electric field strength can be applied at high conductivities.
The following analyses focus on the effect of salt concentration on the inactivation of *B. subtilis*, *A. acdioterrestris* and *G. stearothermophilus* spores suspended in Ringer´s solution with different concentrations. The spore inactivation at 1, 4 and 15 mS/cm was analyzed at different electric field strengths. Because of the current limit, the maximum electric field strength at 15 mS/cm was 4 kV/cm. To allow comparison in spore inactivation between 15 mS/cm and other concentrations, the developed model in section 4.1 was used to calculate inactivation at 4 kV/cm and conductivities lower than 15 mS/cm. The developed model is based on experimental data ranging from 6 to 12.5 kV/cm. To obtain inactivation data at 4 kV/cm, the model has to be extrapolated.

The inactivation of *B. subtilis* spores suspended in Ringer´s solution with different conductivities in the range from 1 to 15 mS/cm is shown in Figure 4-28. At an electric field strength of 4 kV/cm, the energy required to achieve an inactivation of less than 1 log is higher for 4 mS/cm. A 0.6 log inactivation can be achieved applying an energy of 261.0 kJ/kg for 4 mS/cm and 243.8 kJ/kg for 15 mS/cm. But as it is demonstrated in section 4.1.1, where the inactivation of *B. subtilis* spores

is modeled as a function of inlet temperature, specific energy and electric field strength, the developed model is not accurate at low inactivation rates. Consequently, no exact comparison of energy requirement at 4 and 15 mS/cm applying 4 kV/cm could be done at inactivation levels lower than 1 log.

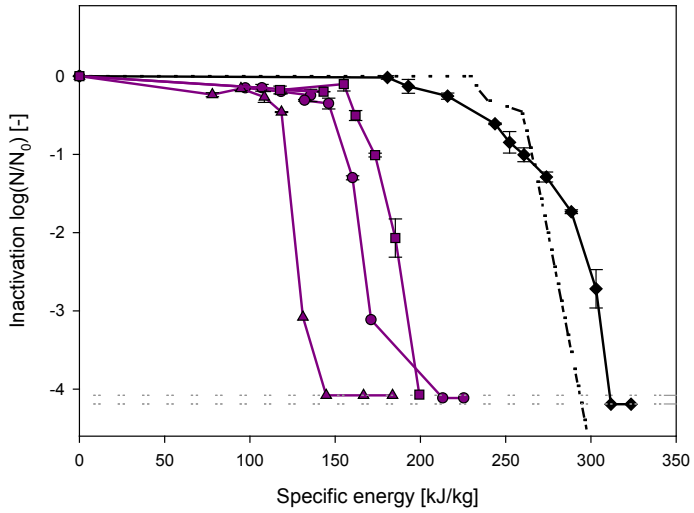

Figure 4-28: Inactivation of *B. subtilis* spores suspended in Ringer´s solution with a conductivity of 1 (▲), 2 (●), 4 (■, --) and 15 (◆) mS/cm at electric field strengths of 4 kV/cm (black lines and symbols) and 11 kV/cm (purple symbols); inactivation data at 4 mS/cm and 4 kV/cm (--) was modeled (see section 4.1.1); detection limit (—·—).

At higher inactivation levels of more than 1 log the required energy is higher at a conductivity of 15 mS/cm compared to 4 mS/cm. A 2.7 log spore reduction can be achieved applying an energy of 281.2 kJ/kg, if the spores are suspended in solution with 4 mS/cm and 303.0 kJ/kg at 15 mS/cm. Therefore, at higher salt concentrations a higher specific energy is required to achieve a specific inactivation. The same effect is observed using an electric field strength of 11 kV/cm. Here, no experimental data were available for 15 mS/cm due electrical limit of the used PEF unit. The comparison of energy at conductivities of 1, 2 and 4 mS/cm indicated a higher energy required for inactivation with increasing conductivity. For a 3 log inactivation the energy at 1 mS/cm is 131.1 kJ/kg. With increasing conductivity the energy for a 3 log spore reduction is increasing to 171.1 kJ/kg for 2 mS/cm and to 191.9 kJ/kg for 4 mS/cm. The difference in energy between 1 and 2 mS/cm is 40.0 kJ/kg for a 3.1 log inactivation, whereas the difference between 2 and 4 mS/cm is 20.8 kJ/kg. Therefore, the influence on the energy is getting smallerwith increasing salt concentration.

The inactivation of *A. acidoterrestris* spores suspended in media with different salt concentrations and different electric field strengths is shown in Figure 4-29.

At high salt concentrations with conductivity of 15 mS/cm, a high amount of energy was required to achieve a sufficient inactivation. For a 3 log inactivation an energy of 153.1 and 186.1 kJ/kg had to be applied in a medium with 4 and 15 mS/cm. With decreasing salt concentration to conductivity of 4 mS/cm and an increase in electric field strength up to 9 kV/cm, the effect of less energy at lower conductivities could not be observed. An energy of 104.1 and 106.5 kJ/kg is required for a 1.7 log spore reduction in media at 1 and 4 mS/cm. Comparing the inactivation data for 4 mS/cm at 4 and 9 kV/cm a difference in energy could be observed, indicating an influence of the electric field strength on the energy input required for inactivation.

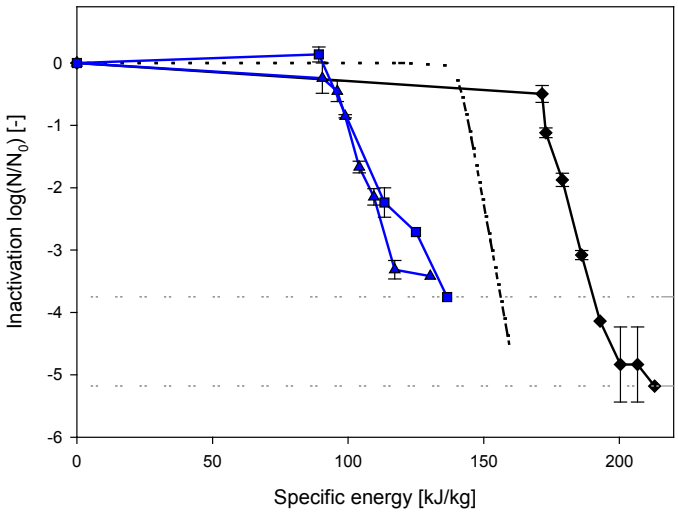

Figure 4-29: Inactivation of *A. acidoterrestris* spores suspended in Ringer´s solution with a conductivity of 1 (▲), 4 (■, --) and 15 (◆) mS/cm at electric field strengths of 4 kV/cm (black lines and symbols) and 9 kV/cm (blue symbols), inactivation data at 4 mS/cm and 4 kV/cm (--) were modeled (see section 4.1.2); detection limit (—·—).

Also, the inactivation data of *G. stearothermophilus* spores (Figure 4-30) in Ringer´s solution indicated an effect of electric field strength in the range from 4 to 12.5 kV/cm. At 4 kV/cm no inactivation was observed after PEF treatments up to more than 300 kJ/kg. Whereas a 3 log reduction was achieved using an electric field strength of 12.5 kV/cm and applying an energy of 279.8 kJ/kg. The experimental data of inactivation in media with 15 mS/cm indicated no inactivation applying a maximum energy of 300 kJ/kg. A decrease in salt concentration and increase in electric field strength enabled a spore reduction. Comparing the inactivation at 1 and 4 mS/cm, the energy required for a specific inactivation is higher at 1 mS/cm.

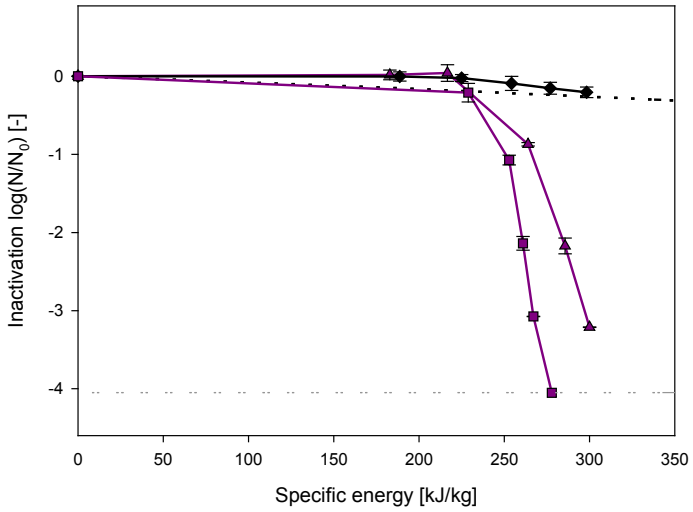

Figure 4-30: Inactivation of *G. stearothermophilus* spores suspended in Ringer´s solution with a conductivity of 1 (▲), 4 (■ ,--) and 15 (◆) mS/cm at electric field strengths of 4 (black lines and symbols) and 12.5 (purple symbols) kV/cm inactivation data at 4 mS/cm and 4 kV/cm (--) was modeled; detection limit (—·—).

As a result, the analyzed spores react partly different to different salt concentrations. At low electric field strength of 4 kV/cm the highest difference in energy comparing 4 and 15 mS/cm was observed for the inactivation of *G. stearothermophilus* spores, as no inactivation was achieved at 15 mS/cm. Less difference, but the same trend was obtained for *B. subtilis* and *A. acidoterrestris* spores.

Besides the salt concentration, also the electric field strength influences the achieved inactivation, especially for *G. stearothermophilus*. At constant conductivity of 4 mS/cm no inactivation was obtained at 4 kV/cm applying 300 kJ/kg, whereas a 3.2 log reduction was observed at 12.5 kV/cm. The same effect of less energy at higher electric field strength could also be observed for the other spore types. But it has to be note, that the inactivation data at 4 kV/cm were calculated extrapolating the developed model in section 4.1.

At higher electric field strengths different dependencies for the inactivation of the spores at different salt concentrations were observed. For *B. subtilis* spores a lower salt concentration resulted in lower energy requirement for an inactivation. In contrast, the energy applied for inactivation of *A. acidoterrestris* spores showed no difference comparing 1 and 4 mS/cm. For inactivation of *G. stearothermophilus* spores a higher energy had to be applied at 1 mS/cm compared to 4 mS/cm. The different inactivation behavior might be based on the different resistance to environmental parameters. This could be also demonstrated by the different amount of energy for inactivation. Inactivation of *A. acidoterrestris* spores requires the lowest energy followed by inactivation of *B. subtilis* and *G. stearothermophilus* spores.

In literature the effect of salt on the thermal resistance of spores was studied. Hutton et al. (1991) studied the thermal resistance of *B. subtilis* spores at ranging NaCl concentration from 0 to 2 % with the result of a lower heat resistance at higher salt concentration. The representative salt concentration used in this study is 0.23 % for 4 mS/cm and 0.92 % for 15 mS/cm. With regard to the results from Hutton el al. (1991), the heat resistance of the *B. subtilis* spores at conductivity of 15 mS/cm should be lower. Due to the Joule heating, the product is heated up according to the amount of energy applied by PEF (Equation 13). Therefore, at higher salt concentration, higher temperature is reached in the product compared to a medium of low salt concentration due to the higher energy applied for inactivation. If thermal inactivation is involved in the total inactivation, a decrease in specific energy should be observed at higher salt concentration for equivalent level of inactivation. This assumption cannot be confirmed by the experimental results (Figure 4-28), because a higher energy is required at higher salt concentrations. This can be also demonstrated by the results of inactivation of *G. stearothermophilus* spores (Figure 4-30). At 15 and 4 mS/cm no inactivation was observed at 300 kJ/kg applying an electric field strength of 4 kV/cm. If the spores would be more heat sensitive at 15 mS/cm an inactivation should be observed, but as it is not the case the thermal influence on the inactivation is low.

In this case, the influence of the electric field strength is high, because an increase in electric field strength from 4 up to 12.5 kV/cm enabled to an inactivation from 0 to 3.1 log at 4 mS/cm applying an energy of 267 kJ/kg. A high influence of electric field strength on inactivation of spores by PEF was also reported by other researchers, but the electric field strengths range from 30 to 60 kV/cm (Marquez et al. 1997; Jin et al. 2001; Bermúdez-Aguirre et al. 2012). As an example Marquez et al. (1997) used a batch PEF system applying 2 µs pulses in a treatment chamber with a parallel configuration under isothermal conditions. The comparison to the PEF conditions used in this study (20 µs pulses, colinear treatment chamber, and continuous operation) demonstrates the impact of different PEF systems and conditions on the inactivation.

However, studies using these high electric field strengths were obtained in low concentrated NaCl solution (0.15 %) (Marquez et al. 1997) or in food products, such as milk, with conductivities around 4 mS/cm (Bermúdez-Aguirre et al. 2012).

In the area of inactivation of vegetative microorganisms by PEF controversy results regarding influence of salt on inactivation were obtained. Some authors detected a higher sensitivity of the cells at high salt concentrations (Hülsheger et al. 1981; Vega-Mercado et al. 1996), because of a change of membrane due to the presence of salt. For inactivation of spores these results cannot be confirmed and the main reason for this is based on the structure difference between spores and bacteria.

4.3 Separation of total inactivation in PEF and thermal induced inactivation

4.3.1 Thermal inactivation kinetics of spore types

To characterize the thermal inactivation kinetics of spores, the D_T and z value was calculated. D_T (decimal reduction time) represents the time required at an adjusted temperature to inactivate 90 % of the studied spore type and is given by reciprocal slope of inactivation over time at a certain temperature (Fellows 2009). From the graph showing logarithm of D_T value versus corresponding temperature, the z value can be calculated taking the reciprocal slope. The z value reflects the temperature dependence of the inactivation and therefore it is defined as the temperature modification required changing D_T by factor 10 (Fellows 2009). The D_T and z value were calculated for *B. subtilis*, *A. acidoterretris* and *G. stearothermophilus*. The corresponding graphs can be found in the appendix (Figure 6-1, Figure 6-2 and Figure 6-3) and the results were summarized in Table 4-10.

Table 4-10: D_T and z values of *B. subtilis*, *A. acidoterrestris* and *G. stearothermophilus* at different temperatures and temperature holding times obtained in Ringer´s solution (4 mS/cm) at pH 4 and 7.

Spore type	pH value	Temperature [°C]	D_T [min]	z value [°C]
B. subtilis	4	100	14.08	7.69
		105	0.96	
		110	0.35	
		121	0.045	
	7	100	8.42	9.09
		105	0.74	
		110	0.26	
		121	0.037	
A. acidoterrestris	4	90	6.52	13.17
		100	0.35	
		110	0.06	
		121	0.027	
	7	90	5.21	13.70
		100	0.37	
		110	0.06	
		121	0.026	
G. stearothermophilus	4	121	2.03	12.19
		130	0.20	
		140	0.051	
	7	121	2.12	10.59
		130	0.17	
		140	0.033	

The decimal reduction time for *B. subtilis* at pH 4 ranged from 14.08 min at 100 °C to 0.045 min at 121.1 °C. A decrease in D_T value was observed with increasing temperature, which means at higher temperature less time was required for a 90 % inactivation. An increase in pH lead to lower D_T

compared to pH 4. At neutral pH, D_T ranged from 8.42 min at 100 °C to 0.037 min at 121.1 °C. In neutral pH medium, the spores were less resistant to heat. The z value at an acidic pH was 7.69 °C and at neutral pH was 9.09 °C. A lower temperature increase was required at pH 4 to cause a tenfold change of D_T. Sala et al. (1995) studied the influence of pH on the thermal resistance of *B. subtilis* spores. As a result, a lower D_{110} of 0.06 min was obtained in contrast to D_{110} of 0.26 min at pH 7. Compared to the results observed in this study, the D_{110} at pH 7 was in the same range and also the z-value was comparable. However, different results were obtained in acidic media, which could be explained by the different method for determining the thermal kinetics and also the strain. The D_T and z values in the study of Sala et al. (1994) were obtained by the injection method and the strain could have been different as it was not reported in the study. The same method of using capillaries was performed by Serp et al. (2002). The obtained D_{100} was in the range of 1.6 min in distilled water, which was lower compared to the results from this study (D_{100}=8.42 min, pH 7). Also the reported z value was lower as the one in this study. The reason for the different results could be the bacterial strain and also the media in which the spores were suspended. An overview of D_T and z values for *B. subtilis* is given in Table 2-2.

The thermal resistance of *A. acidoterrestris* was lower compared to *B. subtilis*, because lower D_T values at a certain temperature were observed. D_T for *A. acidoterretris* ranged from 6.52 min at 100 °C to 0.027 min at 121.1 °C in acid environment. An increase in pH up to 7 did not affect the thermal resistance. The observed values were in the same range as at pH 4. Also, the z value at pH 4 (13.2 °C) and 7 (13.7 °C) were in the same range. The thermal resistance of *A. acidoterrestris* was also studied by Oliver-Daumen et al. (2012). They analyzed the spores from the same strain as used in this study, but using injection method. The reported D_{95} was 10.7 min in Ringer´s solution with a pH of 7. Although the temperature of 95 °C was not studied here, the thermal resistance of *A. acidoterrestris* was lower, due to a lower D_{90} of 5.21 min. Also, the z value of 13.7 °C was lower as the one reported in the study of Oliver-Daumen et al. (2012). The different results could be explained by different sporulation techniques. It is already known that a higher thermal resistance was observed at higher sporulation temperatures (Condon et al. 1992; Sala et al. 1995). Moreover, Oliver-Daumen et al. (2012) used an injection method, which could give different results. Generally, a comparison of D_T and z values obtained in this study to compare with literature is difficult, as the strains, media and sporulation process varied (Table 2-2).

The obtained thermal resistance of *G. stearothermophilus* was higher compared to the other studied spore types. The D_T ranged from 2.03 min at 121 °C to 0.051 min at 140 °C at pH 4. At pH 7 the D_{130} and D_{140} were slightly lower. The z value at pH 4 was 12.19 °C compared to 10.59 °C at pH 7,

which means at pH 7 less temperature increase was necessary to cause a tenfold change in D_T. Comparable results in D_T were reported by Serp et al. (2009). Using the capillary method, they observed a D_{121} of 3.7 min in citrate buffer. The z value was 7.6 °C and therefore lower than the values observed in this study. The reason could be the difference in media (composition and pH) and spore strain, which was not reported in the study of Serp et al. (2009). The same strain, but different media (ACES buffer, pH 7) was used in the study of Mathys et al. (2008). The observed D_{121} and D_{130} were 2.1 and 0.21 min, which is in the same range as the values obtained in this study. An overview about D_T and z values for *G. stearothermophilus* spores in literature is given in Table 2-2.
The most resistant spore type is *G. stearothermophilus*. The D_{121} was 0.2 min in Ringer´s solution (4 mS/cm) at pH 4 compared to D_{121} of 0.045 min for *B. subtilis* spores and 0.027 min for *A. acidoterrestris* spores.

As already previously discussed, an effect of pH value on thermal resistance was only observed for *B. subtilis*. The effect of pH depends on the spore type as well as on the studied pH range. Setlow et al. (2002) indicated a decrease in thermal resistance at high acidities of about a pH of 1. At this pH range, the surrounding spore layers were affected causing a lower thermal resistance. Moreover, the temperature was important, as at lower temperatures (around 97 °C for *A. acidoterrestris*) the impact of pH was higher compared to higher temperatures (López et al. 1996; Pontius et al. 1998; Bahçeci et al. 2007). This statement can be confirmed by our results, because the highest difference in D_T value at pH 4 and 7 was observed at 100 °C. But in contrast to the studies referred to, D_T were smaller in neutral media, which might be explained by the strain and also the sporulation conditions.

The determination of thermal inactivation data was performed using capillary method. As it was already observed in Table 2-2 in chapter 2.1.6, different kinetic data were obtained, which might be partly explained by the method. In this study, glass capillaries were filled with spore suspension, sealed and kept in an oil bath for a specific time. Afterwards, the capillaries were cooled on ice. The description of this method is detailed in chapter 3.4.1. An advantage of this technique in comparison to the tube method, where the solution is heated in small batch, is the fast heat transfer. It allowed accurate measurements in the range of seconds (Jaeger et al. 2010). Nevertheless, there was a delay in time until the suspension in the capillaries reached the desired temperature. Especially at high temperature and holding times in the range of some seconds, the delay might influence the result. The experimental time was separated in t_0, which was the time to heat up and t, which was the real time, when the final temperature was reached. In this study, the delay

has to be considered for *G. stearothermophilus* spores, because at high temperatures of 140 °C only seconds were necessary to inactivate the spore. For the calculation of D_T, the whole experimental time was considered. Therefore, the assumed effect of temperature was higher than the real one. In terms of estimating the thermal inactivation during the PEF process, the consideration of a higher thermal effect was more valuable. However, it was stated that the highest difference in temperature was between 0 and 1 s (Mathys et al. 2008) and therefore the effect of delay in heating up was only interesting for high temperatures and short temperature holding times in the range of some seconds.

4.3.2 Temperature time profile of PEF process

For determining the thermal inactivation during the PEF process, the temperature time profile of the PEF process has to be developed. The complete process was divided in four parts: heating up, PEF treatment, temperature holding time and cooling phase (Figure 4-31).

In the pre heating phase, the spore suspension was pumped through a plate heat exchanger to achieve a final temperature of 80 °C. The PEF treatment indicated two temperature peaks demonstrating the temperature increase after each treatment chamber. The temperature inside the PEF unit was recorded by a fiber optic temperature measurement. It had to be taken under consideration that the element inside the treatment chamber was disturbing the electric field. For this trial, the effect on the spores was not important, so that the effect of the temperature element on the efficiency of the treatment could be neglected. During the measurement, no change in electric field strength was observed by the system. After a temperature holding time in the system, the suspension was cooled down by a plate heat exchanger. The calculation of temperature time development in the plate heat exchanger is described in the section 3.4.2. Due to different applied energies, the outlet temperatures varied and different factors for calculating the temperature time development had to be used. The different factors are listed in the appendix (Table 6-3). An example for the temperature time profile is presented in Figure 4-31.

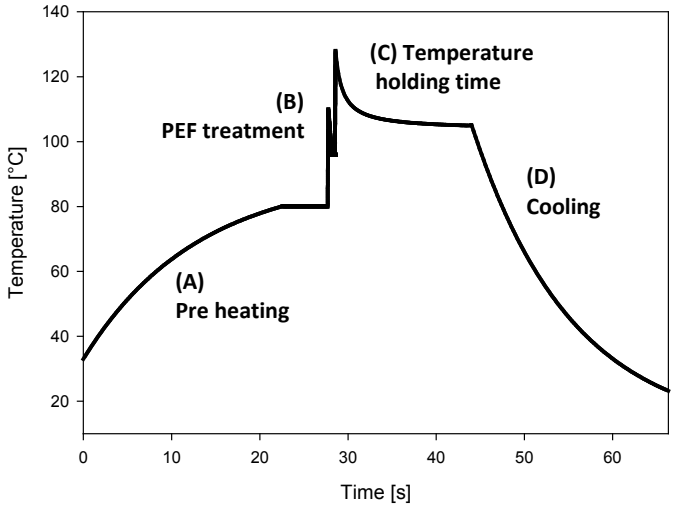

Figure 4-31: Temperature time profile of PEF process including (A) pre heating, (B) PEF treatment, (C) temperature in the pipe system and (D) cooling phase. Settings for PEF treatment: 177 ± 0.35 kJ/kg as specific energy, electric field strength of 8.6 ± 0.06 kV/cm at 20 µs pulse width, flow rate 30 l/h, Ringer´s solution with 4 mS/cm at pH 7, inactivation of *B. subtilis* -2.79 ± 0.014 log (N_0=2.4·10^5 ± 1.6·10^5 spo/ml).

According to the applied energy, different inactivation rates were achieved resulting in different outlet temperatures. The outlet temperature, directly after the treatment chambers was experimentally recorded by fiber optic measurement and calculated. Differences between both outlet temperatures were observed (Table 4-11). In general, the experimental outlet temperatures that were obtained were higher compared to the calculated temperatures. The reason for this might be based on the inhomogeneous electric field distribution within a colinear treatment chamber. Next to the edge of the insulator, there was a small area presenting high electric field intensity, called hot spot (Jaeger et al. 2009). This high electric field strength caused a high local increase in the temperature of the sample. At very high energy inputs used for inactivation of *G. stearothermophilus*, the calculated temperature was partly equal or even higher than the experimental one. This could be explained by the applied pressure. For *G. stearothermophilus* a back pressure of 4 bar was used to achieve temperatures of more than 100 °C. A further increase in pressure would result in higher temperatures similar or even higher than the calculated temperatures.

Table 4-11: Treatment conditions for PEF treatment of spores suspended in Ringer´s solution at 4 mS/cm (pH 4 and 7) and the induced temperature increase measured by fiber optic sensor in the system and calculated (Equation 13), T_{start}= 80 °C, electric field strength of 9 kV/cm, pulse width 20 µs, flow rate 30 l/h.

Spore type	pH	Specific energy [kJ/kg]	Inactivation log (N/N_0) [-]	$T_{experimental}$ [°C]	$T_{calculated}$ [°C]

B. subtilis	4	175	-1.3 ± 0.02	128	124
		180	-2.5 ± 0.12	130	125
		185	-2.9 ± 0.00	131	126
	7	164	-0.6 ± 0.04	123	121
		177	-2.8 ± 0.01	128	124
		195	-4.4 ± 0.02	133	129
A. acidoterrestris	4	116	-2.44 ± 0.07	123	109
		123	-3.56 ± 0.60	126	110
		137	-3.90 ± 0.00	127	114
	7	123	-2.6 ± 0.15	126	111
		137	-3.3 ± 0.00	127	114
G. stearothermophilus	4	226	-2.01 ± 0.88	140	137
		242	-2.78 ± 0.12	141	141
		252	-4.35 ± 0.00	143	143
	7	242	-1.15 ± 0.17	140	141
		252	-2.24 ± 0.13	141	143
		265	-3.38 ± 0.60	141	146

4.3.3 Modeling of thermal inactivation during PEF process

The combination of thermal inactivation kinetics of each spore and the temperature time profile of the PEF process allowed separation of total inactivation in thermal and PEF induced inactivation.

4.3.3.1 Thermal inactivation of *B. subtilis* spores during PEF process

From the developed model (chapter 3.4.2), the thermal inactivation during the PEF process depending on the specific energy could be calculated. The PEF induced inactivation was given as the difference between total and thermal inactivation. The results of the model are presented in Figure 4-32.

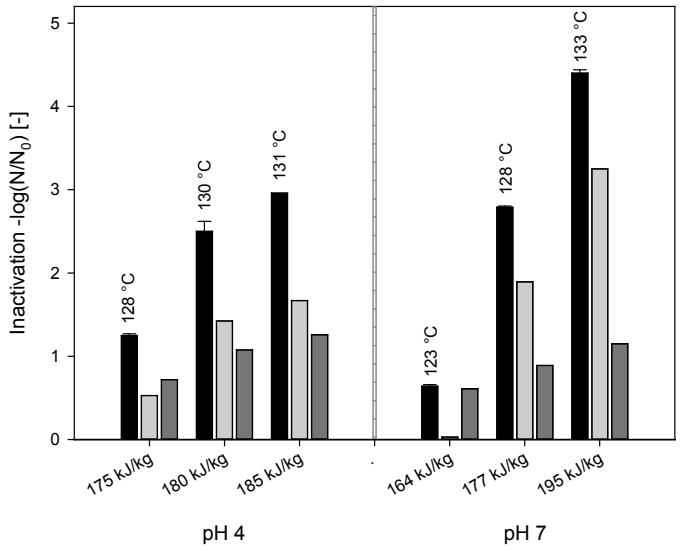

Figure 4-32: Separation of total inactivation (black bar) of *B. subtilis* spores after PEF treatment at an electric field strength of 9 kV/cm in PEF (light grey bar) and thermal (dark grey bar) induced inactivation in Ringer´s solution (4 mS/cm) at pH 4 and pH 7 indicating the outlet temperature to characterize the thermal tretament.

The separation of total inactivation in thermal and PEF induced inactivation was performed for different energy inputs at pH 4 and 7 (Figure 4-32). Independent from the pH, the total inactivation was increasing with increasing specific energy. Moreover, in both cases, the total inactivation consisted of a thermal and PEF induced inactivation rate. At low energy inputs, the thermal inactivation was higher compared to the PEF induced inactivation. With increasing specific energy the PEF induced inactivation showed a higher increase compared to thermal inactivation. After PEF treatment applying an energy of 164 kJ/kg in Ringer´s solution (4 mS/cm) with pH of 7, an inactivation of 0.6 log was observed, which was based on thermal inactivation. By increasing the energy up to 177 kJ/kg, a total inactivation of 2.8 log was achieved, which could be separated in 0.9 log thermal and 1.8 log PEF induced inactivation. The same effect is observed in medium with a pH of 4. Comparing the quatification of the thermal effect at different pH values, a higher thermal inactivation part was observed in acidic media, especially at inactivation of more than 2 log cycles. After PEF treatment in acid medium with a specific energy of 180 kJ/kg, a 2.5 log inactivation was observed including a 1.1 log thermal induced inactivation. A slightly higher total inactivation of 2.8 log at pH 7 applying 177 kJ/kg with a thermal induced inactivation of 0.9 log was achieved. As a result, the total inactivation of *B. subtilis* spores by PEF included a thermal and PEF induced inactivation.

4.3.3.2 Separation of total inactivation of *A. acidoterrestris* spores into thermal and PEF induced inactivation

The same procedure of separating the total inactivation in thermal and PEF induced inactivation was performed for inactivation study of *A. acidoterrestris* spores and presented in Figure 4-33.

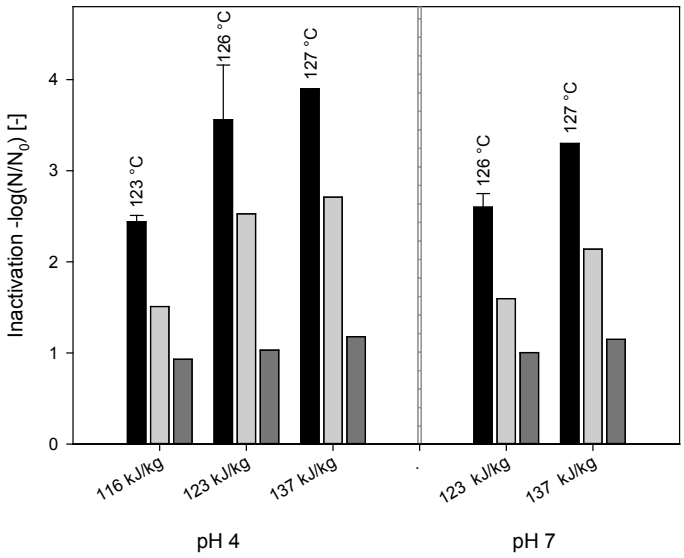

Figure 4-33: Separation of total inactivation (black bar) of *A. acidoterrestris* spores in PEF (light grey bar) and thermal (dark grey bar) induced inactivation at pH 4 and pH 7 at an electric field strength of 9 kV/cm in Ringer´s solution with a conductivity of 4 mS/cm indicating the outlet temperature for characterization of thermal treatment.

The model showed a quatification of thermal and PEF induced inactivation of *A. acidoterrestris*. With increasing specific energy, the total inactivation was increasing and therefore also the PEF and thermal induced inactivation was increasing. Comparing the contribution of thermal and PEF related inactivation, the PEF inactivation was in all cases higher than the thermal inactivation. Thus, the PEF inactivation increased more with increasing energy than the thermal inactivation. Applying an energy of 116 and 123 kJ/kg at pH 4, the achieved total inactivation was 2.4 and 3.6 log with a thermal inactivation rate of 0.9 and 1.0 log, respectively. The comparison of thermally induced inactivation at pH 4 and 7 showed similar inactivation levels. After PEF treatment with a specific energy of 123 kJ/kg an inactivation of 3.6 log at pH 4 and 2.6 log at pH 7 was achieved. In both cases, a thermal induced inactivation of approx. 1 log was calculated. As a result, the inactivation of *A. acidoterrestris* after PEF treatment indicated contribution of thermal effects on the total inactivation.

4.3.3.3 Thermal inactivation of *G. stearothermophilus* spores during PEF process

The developed model for evaluating the thermal effect on the total inactivation was applied to the experimental inactivation data of *G. stearothermophilus* spores.

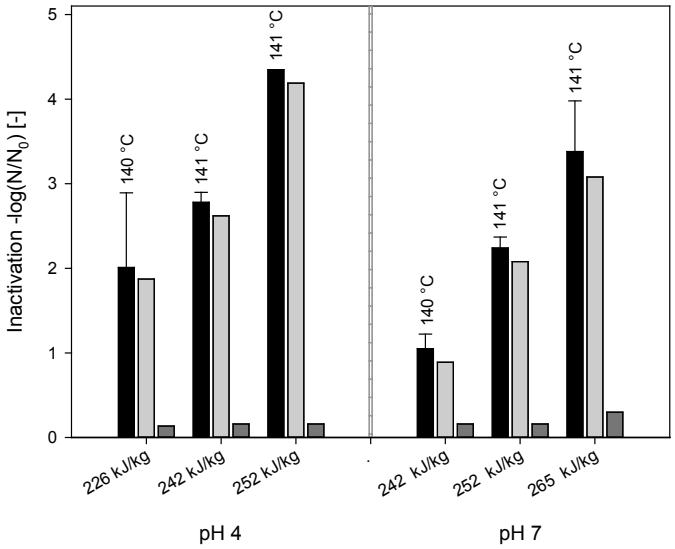

Figure 4-34: Separation of total inactivation (black bar) in PEF (light grey bar) and thermal (dark grey bar) induced inactivation of *G. stearothermophilus* spores at pH 4 and pH 7 after PEF treatment with an electric field strength of 9 kV/cm in Ringer´s solution with a conductivity of 4 mS/cm indicating the outlet temperature to characterize the thermal treatment.

The experimental inactivation data indicated an increase in inactivation with increasing energy. The thermal inactivation of this spore was lower than 0.3 log for all the cases, as it was predicted by the mathematical model previously developed for this inactivation mechanism. The maximum thermal inactivation was 0.3 log after PEF treatment with an energy of 265 kJ/kg at pH 7. Comparing the inactivation at pH 4 and 7, only differences in total inactivation were observed. In acidic media, a higher inactivation at equal specific energies was achieved. In conclusion, the inactivation of *G. stearothermophilus* spores was not based on a pure thermal inactivation.

4.3.3.4 Discussion of quantification of thermal inactivation effects during the PEF process

The developed mathematical model combined the thermal inactivation kinetics of each spore type with the temperature time profile of the PEF process. As a result, the inactivation caused by temperature and PEF process could be calculated. The results for *B. subtilis* (Figure 4-32) and *A. acidoterrestris* (Figure 4-33) indicated a thermal inactivation part, which was based on the temperature increase caused by the energy input (Joule heating). However, the PEF induced inactivation

was higher compared to the thermal inactivation part. The model for *G. stearothermophilus* (Figure 4-34) demonstrated a very low thermal inactivation. Consequently, the highest part of inactivation was caused by PEF treatment. At this point it has to be noted, that the principle of spore inactivation by PEF is unclear. It can be stated, that this inactivation part was not based on thermal inactivation, but how the temperature was involved was vague. It might be that the induced temperature made the spores more sensitive to the treatment. Generally, the treatment of spores at moderate temperatures induced an activation (Vary et al. 1965, Uehara et al. 1967), which might have affected the resistance of spores to PEF treatment. The D_{140} of *G. stearothermophilus* was 1.98 s at pH 7, which means that 90 % of the spore population was inactivated in 1.98 s at 140 °C. The maximum measured temperature within the PEF process (265 kJ/kg, pH 7) of 143 °C was achieved in the second treatment chamber, which can be seen in Figure 4-35. The residence time in the area was 0.094 s, which was much lower than the D_{140}. Therefore, the thermal inactivation during the PEF process was low. However, the temperature in the first treatment chamber and the temperature until the cooling was reached had to be taken into consideration. The temperature in the first treatment chamber was 134 °C, which was held for 0.094 s. Again, the thermal inactivation due to the temperature was quite low (Figure 4-35), because the residence time was lower than the D_{140} or even D_{130}. The time until the plate heat exchanger was reached was 14.8 s with a temperature of 125 °C after the treatment chamber and 119 °C before the plate heat exchanger. The D_T value for this range was 12 s for 130 °C and 127 s for 121 °C. As the times for thermal reduction were higher compared to the temperature holding time in the pipe, the thermal inactivation was low. In conclusion, the low thermal inactivation of *G. stearothermophilus* during the PEF process could be explained comparing the temperature holding times and the related D_T values.

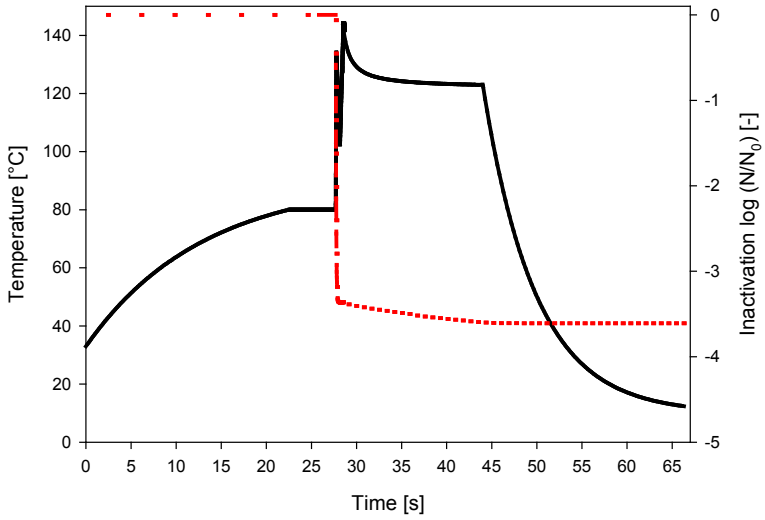

Figure 4-35: Temperature time profile of PEF process (265 kJ/kg, 9 kV/cm) (—) and inactivation of *G. stearothermphilus* separated in PEF (——) and thermal induced (···) inactivation.

Within the study of Reineke et al. (2013a), the thermal inactivation of *G. stearothermophilus* during lab scale PEF process was analyzed, using the same model as in this study. The thermal inactivation kinetics were with a D_{121} of 1.88 min and D_{130} of 0.77 min similar to the values obtained in this study. Saline solution with a conductivity of 5.3 mS/cm was treated by PEF in 2 colinear treatment chambers with a voltage of 6 and 10 kV, an energy of 118 and 144 kJ/kg and an inlet temperature of 95 °C. The resulted total inactivation of 3.29 and 2.97 log was separated in 3.19 and 2.95 log PEF induced inactivation and 0.10 and 0.02 log thermal inactivation. These results were in accordance with the results obtained in this study, where the modeled thermal inactivation of *G. stearothermophilus* was low. After PEF treatment applying a voltage of 9 kV, an energy of 265 kJ/kg and an inlet temperature of 80 °C a total inactivation of 3.38 log could be achieved, from which 0.3 log inactivation was based on thermal effects. The difference in energy between both studies was due to the inlet temperature. At higher inlet temperature, the energy for a specific inactivation could be decreased. This has already been discovered for the inactivation of vegetative microorganisms by PEF (Toepfl et al. 2007).

As already stated before, the total inactivation of *B. subtilis* and *A. acidoterrestris* consisted of a thermal and PEF induced part. Comparing the thermally induced inactivation of *B. subtilis* at pH 4 and 7, a higher thermal inactivation was observed at pH 4. In section 4.3.1 the thermal inactivation kinetics of *B. subtilis* spores are listed, where a higher thermal resistance at pH 7 was observed. The analyzed temperature range was from 100 to 121 °C, but the maximum temperature after PEF process (Table 4-11) was for all settings higher than 121 °C. As the D_T value at higher temperature was not available, a comparison was difficult. The differences in D_T between acid and neutral media were decreasing with increasing temperature. Therefore, it can be assumed that at temperatures of more than 121 °C, the thermal resistance in acid media was the same as in neutral

media or even slightly higher. Moreover, the high temperature in the second treatment chamber explains the modeled thermal inactivation. Applying an energy of 164 and 195 kJ/kg at pH 7, the temperature increased up to 123 and 133 °C in the second treatment chamber. The D_{121} for *B. subtilis* was 2.24 s at pH 7. Taking the residence time of 0.094 s into account, it could be assumed that the thermal inactivation was low at 164 kJ/kg and increasing for 133 °C (Figure 4-36). Consequently, the calculated thermal inactivation of *B. subtilis* (Figure 4-32) could be explained by the temperature holding time and temperature within the PEF process and the related D_T values. The same result of thermal inactivation during the PEF process was observed by Reineke et al. (2013a). They treated *B. subtilis* spores from the same strain as used in this study by PEF using an inlet temperature of 70 °C. The thermal inactivation kinetics, which were obtained by using the glass capillary method, were partly similar. The z value in the study of Reineke et al. (2013a) was 7.69 °C compared to 7.41 °C obtained in this study at a pH of 4. The D_{100} and D_{110} were different in both studies. Whereas Reineke et al. (2013a) observed a D_{100} of 3.69 min and D_{110} of 0.21 min, the values from this study were 14.08 and 0.045 min, respectively. However, after applying a voltage of 8 kV/cm and an energy of 188 and 227 kJ/kg, the resulted total inactivation of 3.18 and 4.66 log was separated in 2.99 and 3.71 log thermal inactivation. Compared to the obtained results from this study, the calculated thermal inactivation was higher. Applying an energy of 180 kJ/kg, the thermal inactivation was 1.06 log from 2.5 log total inactivation. These differences could be explained by the difference in the sporulation method, treatment medium, total inactivation and different temperature time profiles due to the different PEF systems.

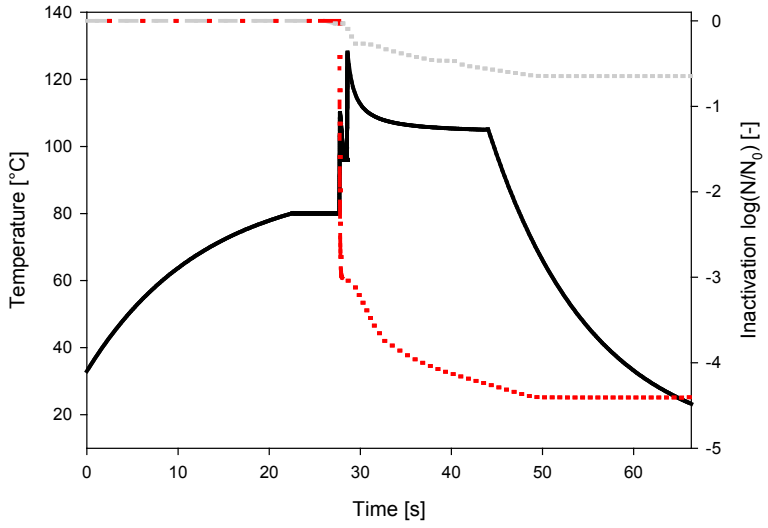

Figure 4-36: Temperature time profile (—) of PEF treatment at 164 kJ/kg and 9 kV/cm of Ringer´s solution (4 mS/cm, pH 7) and inactivation of *B. subtilis* spores separated in PEF (164 kJ/kg: — —; 195 kJ/kg: — —) and thermal (164 kJ/kg: ···; 195 kJ/kg: ···) induced inactivation.

The total inactivation of *A. acidoterrestris* spores indicated a thermal inactivation during the PEF process. The calculated thermal inactivation at pH 4 and 7 applying the same amount of energy resulted in 1 log spore reduction. This demonstrated that the thermal resistance was not influenced by the pH. Considering the thermal inactivation kinetics (section 4.3.1), the result could be confirmed, because no difference in D_T values at pH 4 and 7 were observed.

With increasing energy, the inactivation of *A. acidoterrestris* spores was increasing. The analysis of quantification of thermal inactivation during PEF process showed a high increase in PEF induced inactivation with increasing energy, whereas the thermal inactivation remained low. The measured temperatures in the second treatment chamber were 123 °C at 116 kJ/kg and 127 °C at 137 kJ/kg. The difference in temperature of 5 °C was quite low, which explained the low increase in thermal inactivation. The D_{121} for *A. acidoterrestris* spores was 1.56 s at pH 4. Taking the temperature in the second treatment chamber and the residence time of 0.094 s into account, the thermal inactivation was expected to be low (Figure 4-37). Here, the temperature and holding time after the treatment chambers until the plate heat exchanger for cooling was important. The measured temperatures were for all applied energies between 100 and 110 °C. Considering the related D_{100} and D_{110} of 22.2 and 3.88 s as well as a residence time of 14.8 s, a thermal inactivation could be assumed, which was shown in the results of the mathematical model (Figure 4-33).

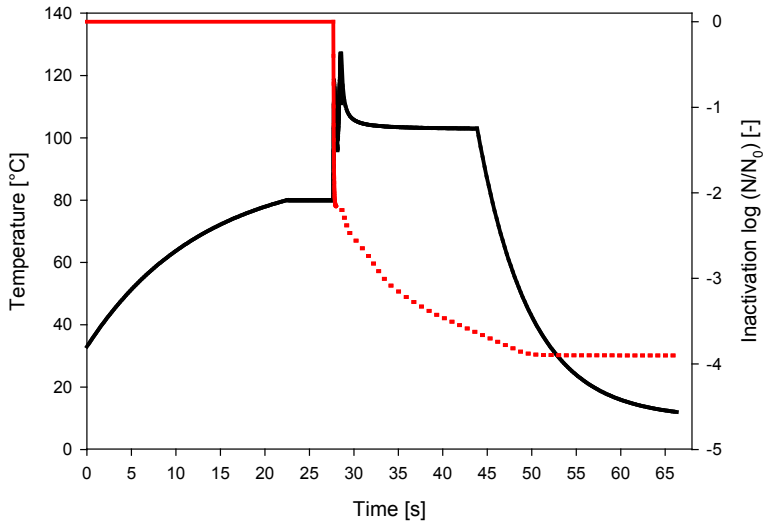

Figure 4-37: Temperature time profile (—) of PEF treatment at 137 kJ/kg and 9 kV/cm of Ringer´s solution (4 mS/cm, pH 4) and inactivation of *A. acidoterrestris* spores separated in PEF (— —) and thermal incuded (···) inactivation.

4.3.4 Thermal death time (F value) of thermal and PEF process

A thermal sterilization process is characterized by a specific temperature time profile. The integration of temperature time area results in the sterilization value and allows an evaluation of the designed preservation process. As the studied PEF process was combined with thermal energy, the F value could be calculated and compared to the thermal process. For calculation of $F_{thermal}$, Equation 23 was used with the correspnding D_{121}. The results of the F value for PEF and the thermal process are listed in Table 4-12.

With increasing specific energy, the F value was increasing based on the higher outlet temperature. The F_{PEF} values for the inactivation of *B. subtilis* and *A. acidoterrestris* were in the range of 0.6 to 2.4 s. Compared to the $F_{thermal}$ value, which gives the time required at 121 °C to achieve a specific inactivation rate, the F_{PEF} was lower. As an example, the F_{PEF} at pH 4, was 0.93 s for an inactivation of 2.44 log of *A. acidoterrestris* spores compared to $F_{thermal}$ of 3.95 s. Therefore, the heat load of the PEF processed product was lower than the thermally treated one. Due to the higher resistance of *G. stearothermophilus,* higher energies and therefore higher outlet temperatures result. The higher outlet temperatures lead to higher F_{PEF} values in the range of 16.3 s for 2 log reduction at pH 4 and 42.4 s for 3.4 log reduction at pH 7. Although, the F values for PEF process for inactivation of *G. stearothermophilus* spores were higher compared to the ones obtained for the other spore types, the values were still much lower compared to the thermal process. The $F_{thermal}$ values with the same inactivation rate as achieved by PEF ranged from 244.82 s for 2 log reduction to 529.83 s for a 4.4 log reduction at pH 7.

Table 4-12: F values for PEF process (F_{PEF})with different specific energies and at pH 4 and 7 calculated by Equation 22, $T_{Reference}$=121 °C, z values listed in Table 4-10 and corresponding F value for thermal process ($F_{thermal}$) calculated by Equation 23 including D_{121}.

Spore type	pH value	Specific energy [kJ/kg]	Inactivation log (N/N_0) [-]	F_{PEF} [s]	$F_{thermal}$ [s]
B. subtilis	4	175	-1.3 ± 0.02	0.84	3.51
		180	-2.5 ± 0.12	1.49	6.75
		185	-2.9 ± 0.00	1.66	7.83
	7	164	-0.6 ± 0.04	0.61	1.33
		177	-2.8 ± 0.01	1.27	6.22
		195	-4.4 ± 0.02	2.43	9.77
A. acidoterrestris	4	116	-2.44 ± 0.07	0.93	3.95
		123	-3.56 ± 0.60	1.03	5.77
		137	-3.90 ± 0.00	1.18	6.32
	7	123	-2.6 ± 0.15	1.00	4.06
		137	-3.3 ± 0.00	1.15	5.15
G. stearothermophilus	4	226	-2.01 ± 0.88	16.34	244.82
		242	-2.78 ± 0.12	20.35	338.60
		252	-4.35 ± 0.00	20.55	529.83
	7	242	-1.15 ± 0.17	23.53	146.28
		252	-2.24 ± 0.13	23.82	284.93

	265	-3.38 ± 0.60	42.44	429.94

The F value allowed comparison of different decontamination processes, where temperature was involved. Moreover, it enabled an optimization of the thermal process regarding cost and energy saving. For sterilization of canned foods, the so called 12D concept is applied. It states a 12 log reduction of the most resistant spore in the food. Mostly, *C. botulinum* spores are chosen as target spores with a D_{121} of 0.2275 min (Brown 1997). Following the 12D concept the F value should be 2.73 min, which means the food product should be exposed for 2.73 min to 121 °C to guarantee 12 log inactivation of *C. botulinum*. This treatment is taken as a standard process for food safety, but the product characteristics, such as water activity and pH, have to be taken into consideration. As a consequence of origin and food characteristics, the initial spore concentration is important (Beckmann et al. 1998). To apply the PEF process for 12D concept, the F_{PEF} for 12 log inactivation of each spore type was estimated. Therefore, the model developed in section 4.1 was extrapolated to calculate the required energy input for 12 log inactivation at 9 kV/cm. According to the specific energy, the temperature time profile was created and the F value based on Equation 22 calculated. The corresponding $F_{thermal}$ was obtained by using Equation 23 with a D_{121}. The F values for 12 log reduction of *B. subtilis* spores by the PEF and thermal process were exemplarily shown in Figure 4-48.

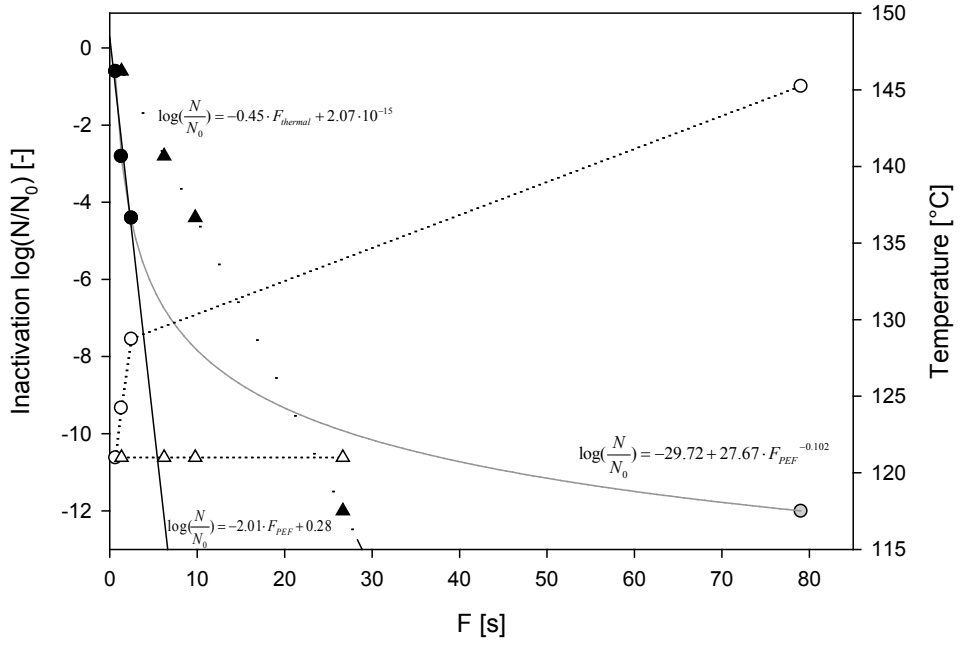

Figure 4-38: F values for PEF calculated by Equation 22 (F_{PEF} (●, based on experimental results; ●, based on energy demand for 12 log reduction obtained by the developed model (section 4.1.1)) and thermal calculated by Equation 23 ($F_{thermal}$ (▲)) process for inactivation of *B. subtilis* spores and the corresponding temperatures (PEF: ○, thermal: △).

The $F_{thermal}$ indicated a linear correlation between the F value and the inactivation (Figure 4-38). For the PEF process a linear relation can be assumed, when only the experimental obtained F_{PEF} were considered. The calculated F_{PEF} for 12 log reduction was 79.0 s and results in non linear relation (Figure 4-38). As the F_{PEF} at this stage is higher (79.0 s) than $F_{thermal}$ (26.6 s), the lines in the graph for F_{PEF} and $F_{thermal}$ indicated an intersection. Therefore, at a certain inactivation level, the heat load of PEF process is higher compared to the thermal process. The F_{PEF} for a 12 log reduction assuming the linear relation, which was obtained by the experimental results, was 6.12 s and therefore lower compared to the $F_{thermal}$ (26.6 s). In this case, the heat load of the PEF treated product would be lower. However, the F_{PEF} values for a 12 log reduction were calculated by an extrapolation of the developed model and by assuming a linear relation. An experimental validation is required to allow a concrete comparison of F value for a 12 log reduction between PEF and thermal process. Moreover, the temperature has to be considered for this comparison. As the temperature is constant for a thermal process, neglecting the pre heating and cooling, the temperature for PEF process is increasing depending on the energy input.

4.3.5 DPA Measurement after PEF treatment

Dipicolinic acid (DPA) is an important component in spores responsible for the dehydration of the spore core and therefore important for the high resistance to heat or chemicals (see section 2.1.3). During germination, DPA is released followed by a rehydration of the core. As a next step, the spore can germinate and start with its vegetative cell cycle. Moreover, DPA release can be measured after treatment with wet heat. The mechanism of spore inactivation by wet heat is based on protein and enzyme denaturation. As a result of protein denaturation, DPA is released (Coleman 2007).

The results of the mathematical modeling of thermal inactivation during PEF process (section 4.3.3) indicated an impact of temperature on the spores, especially *B. subtilis* and *A. acidoterrestris*. The analysis of DPA after PEF treatment in comparison to thermally treated spores allowed an evaluation of temperature impact (Table 4-13).

The DPA concentration after thermal treatment (121 °C, 15 min) was set to 100 %, because it indicated the maximum DPA release. Due to the thermal treatment, the spore concentration was reduced to the detection limit. The lowest DPA concentration was obtained for untreated spores. The low amount of DPA could be explained by the unpurified spore suspension. During the spore production or storage, some of the spores might be germinated and inactivated by the heat step. The DPA was resistant to heat and was not degraded during the process in order to induce germination. Therefore, the DPA present in the sample could be due to spores, which were germinated

before this step. After PEF treatment, a lower DPA concentration compared to thermal process and higher compared to untreated sample was obtained. This demonstrated that thermal inactivation was part of the inactivation by the PEF process. Moreover, with increasing energy an increase in DPA concentration was observed, which indicated a higher thermal inactivation rate with increasing energy.

Table 4-13 Dipicolinic acid (DPA) content after untreated, PEF treated and thermal treated of *B. subtilis* and *A. acidoterrestris* spores.

Spore type	Treatment	DPA [µM]	DPA [%]
B. subtilis	thermal*	1.86 ± 0.859	100.00
	untreated	0.25 ± 0.059	13.45
	PEF (180 kJ/kg)	0.36 ± 0.051	19.31
	PEF (200 kJ/kg)	0.66 ± 0.171	35.82
A. acidoterrestris	thermal*	5.89 ± 0.086	100.00
	untreated	0.25 ± 0.010	4.24
	PEF (123 kJ/kg)	0.35 ± 0.007	5.88
	PEF (137 kJ/kg)	0.42 ± 0.010	7.18

*Thermal treatmet conditions: 121 °C, 15 min

After PEF treatment applying an energy of 180 kJ/kg, a 2.8 log inactivation of *B. subtilis* was achieved. The calculated thermally induced inactivation was about 0.9 log. By increasing the energy up to 195 kJ/kg, the total inactivation increased up to 4.4 log spore reduction including 1.2 log inactivation based on thermal inactivation (Figure 4-32). The increase in thermal induced inactivation could also be observed by analyzing the DPA concentration (Table 4-13). The DPA concentration increased from 19.3 % at 180 kJ/kg to 35.8 % at 200 kJ/kg. This increase demonstrated an increase in thermal inactivation, which confirmed the results of the thermally induced inactivation model. The release of DPA after PEF treatment was also observed in literature. Jin et al. (2001) detected a release of 0.118 mM per 10 mg dry weight spores after PEF treatment at 35 kV/cm 2000 µs as treatment time for a 1 log reduction of *B. subtilis* spores. The different DPA concentrations obtained in literature and in this study could not be compared, because of different PEF treatment conditions and different *B. subtilis* strains. But the fact of DPA release after PEF treatment could be confirmed.

The modeling of quantification of thermal inactivation of *A. acidoterrestris* spores showed the influence of temperature during the PEF process (Figure 4-33). With increasing energy, the total inactivation was increasing. The separation of inactivation into thermal and PEF parts indicated a higher PEF induced inactivation compared to thermal inactivation. Table 4-13 showed the DPA concentration after PEF and thermal inactivation. The DPA concentration after PEF treatment was

much lower compared to thermal treatment. Applying an energy of 137 kJ/kg, an inactivation of 3.9 log could be achieved with a DPA concentration of 0.42 µM. After thermal treatment at 121 °C for 15 min, the DPA concentration was 5.89 µM. These results demonstrated a lower impact of temperature on the inactivation during PEF treatment and therefore a lower inactivation exclusively induced by heat. This result could be confirmed by the mathematical modeling for evaluating the thermal inactivation. A PEF treatment applying an energy of 137 kJ/kg and an electric field strength of 9 kV/cm resulted in a total inactivation of 3.9 log, which could be separated in 2.2 log PEF induced and 1.7 log thermal inactivation.

4.4 Inactivation mechanism of bacterial endospores by PEF

The principle of action for inactivating vegetative bacteria by PEF is based on electroporation. The membrane is affected by the electric field resulting in a higher permeability and cell death (section 2.2.2.1). The investigations published to date do not offer any explanation for the possible inactivation mechanism of spores by PEF. The results of this study demonstrate that an inactivation of spores can be achieved. The following chapter analyzes the role of germination in the inactivation mechanism and studies microscopic pictures before and after PEF in comparison to thermal treatment.

4.4.1 Analysis of germination after PEF treatment

During germination, spores convert from dormant to vegetative states. The exact description of germination is explained in section 2.1.2. To estimate the number of germinated spores after PEF treatment, each sample was analyzed twice. Within the first analysis the sample was treated by 80 °C for 10 min after PEF and before plating in order to inactivate the germinated spores. The second analysis was performed without the heating step. The number of germinated spores was given by the difference microbial concentration with and without heating step. Experimental data are shown in Table 4-14.

Table 4-14: *B. subtilis* spore concentration after PEF treatment in Ringer´s solution at pH 4 and different conductivities with and without heat treatment before microbial analysis.

Conductivity [mS/cm]	Electric field strength [kV/cm]	Specific energy [kJ/kg]	Without 80 °C, 10 min treatment [spo/ml]	With 80 °C, 10 min treatment [spo/ml]
1	Spore suspension[a]		$1.10 \cdot 10^5 \pm 1.22 \cdot 10^4$	$9.21 \cdot 10^4 \pm 1.53 \cdot 10^3$
	0	0[b]	$1.30 \cdot 10^5 \pm 1.41 \cdot 10^4$	$7.30 \cdot 10^4 \pm 2.83 \cdot 10^3$
	9	166	$1.15 \cdot 10^2 \pm 2.19 \cdot 10^1$	$1.00 \cdot 10^2 \pm 0$
	14	157	$1.23 \cdot 10^3 \pm 3.89 \cdot 10^2$	$7.15 \cdot 10^2 \pm 1.20 \cdot 10^2$

4	Spore suspension[a]		$1.00 \cdot 10^5 \pm 0.51 \cdot 10^3$	$9.95 \cdot 10^4 \pm 1.55 \cdot 10^3$
	0	0[b]	$9.90 \cdot 10^4 \pm 1.41 \cdot 10^3$	$7.15 \cdot 10^4 \pm 2.12 \cdot 10^3$
	9	177	$2.05 \cdot 10^2 \pm 3.54 \cdot 10^1$	$2.50 \cdot 10^2 \pm 2.12 \cdot 10^2$
	9	199	$5.40 \cdot 10^1 \pm 6.36 \cdot 10^1$	$9.00 \cdot 10^0 \pm 0$
15	Spore suspension[a]		$1.05 \cdot 10^5 \pm 1.05 \cdot 10^3$	$9.50 \cdot 10^4 \pm 5.27 \cdot 10^4$
	0	0[b]	$1.15 \cdot 10^5 \pm 7.07 \cdot 10^3$	$8.50 \cdot 10^4 \pm 1.41 \cdot 10^4$
	4	252	$3.75 \cdot 10^3 \pm 1.06 \cdot 10^3$	$1.40 \cdot 10^3 \pm 1.41 \cdot 10^2$
	4	265	$5.40 \cdot 10^2 \pm 1.27 \cdot 10^2$	$1.50 \cdot 10^2 \pm 7.14 \cdot 10^1$

a: Spore concentration without pre heating, PEF and cooling; b: Spore concentration after pre heating and cooling without PEF treatment.

The spore concentration including the heat step was lower than the concentration without the heat step. As a result, the spore suspension contained an amount of vegetative bacteria. The difference in spore concentration between both analyses was small (< 1 log) and therefore only a small amount of vegetative or germinated spores were present in the solution. In only a few cases, the spore concentration with heat treatment was slightly higher than the concentration without heat treatment, which can be explained by statistical variations.

The differences in spore concentration between analysis with and without heat treatment were found in PEF and untreated sample. The analysis of untreated sample demonstrated the effect of preheating on the spores. The difference was smaller than 1 log in each case, which was regarded as a low difference. Moreover, this low difference in spore concentration between with and without heating was found in the initial spore suspension. Consequently, the preheating procedure within the process induced no germination. The comparison of the difference in spore concentration of the untreated and PEF treated sample indicated similar ratios. Because the decrease in spore concentration after heat treatment of 80 °C for 10 min was not that high, no germination was induced by PEF. These results were confirmed by the studies performed by other researchers (Knorr et al. 1994, Jin et al. 2001). Jin et al. (2001) studied the effect of PEF on *B. subtilis* spores and detected an inactivation. As the maximum inactivation occurred at 36 °C, the germination at that temperature with and without PEF was analyzed with the result of no induced germination. Although the initial temperature and the temperature increase in this study were higher compared to the results of Jin et al. (2001), the same result of no germination induction by PEF was obtained. Moreover, the temperature used in this study was too high for germination, as the optimum germination temperature is between 30 to 36 °C (Levinson et al. 1970) and inhibited at more than 49 °C (Wax et al. 1968).

4.4.2 Microscopic analysis of PEF treated *B. subtilis* spores

A good technique to visualize the inactivation of spores by thermal or PEF process is microscopic analysis. Scanning electron microscopic pictures of untreated, thermal and PEF treated spores were prepared. For the analyses, the spores were first suspended in Ringer´s solution and treated by the different processes. After centrifugation, the spores were suspended in sterile distilled water. This procedure was performed in order to avoid the interference of salts in the microscopic picture.

The pictures of the spores are illustrated in Figure 4-39. Micrograph (a) shows the untreated spore, which are rod shaped with a length of about 1.1 µm and a smooth surface. After thermal treatment at 121 °C for 15 min, the spores appeared shrunken (Figure 4-39b). The surface and the size remained, but they look wrinkled. Due to the heat treatment, the proteins and enzymes playing a key role in outgrowth process were denatured (Coleman et al. 2007, 2010; Tabit et al. 2010; Zhang et al. 2010). The spore was not able to germinate or even survive the heat treatment.

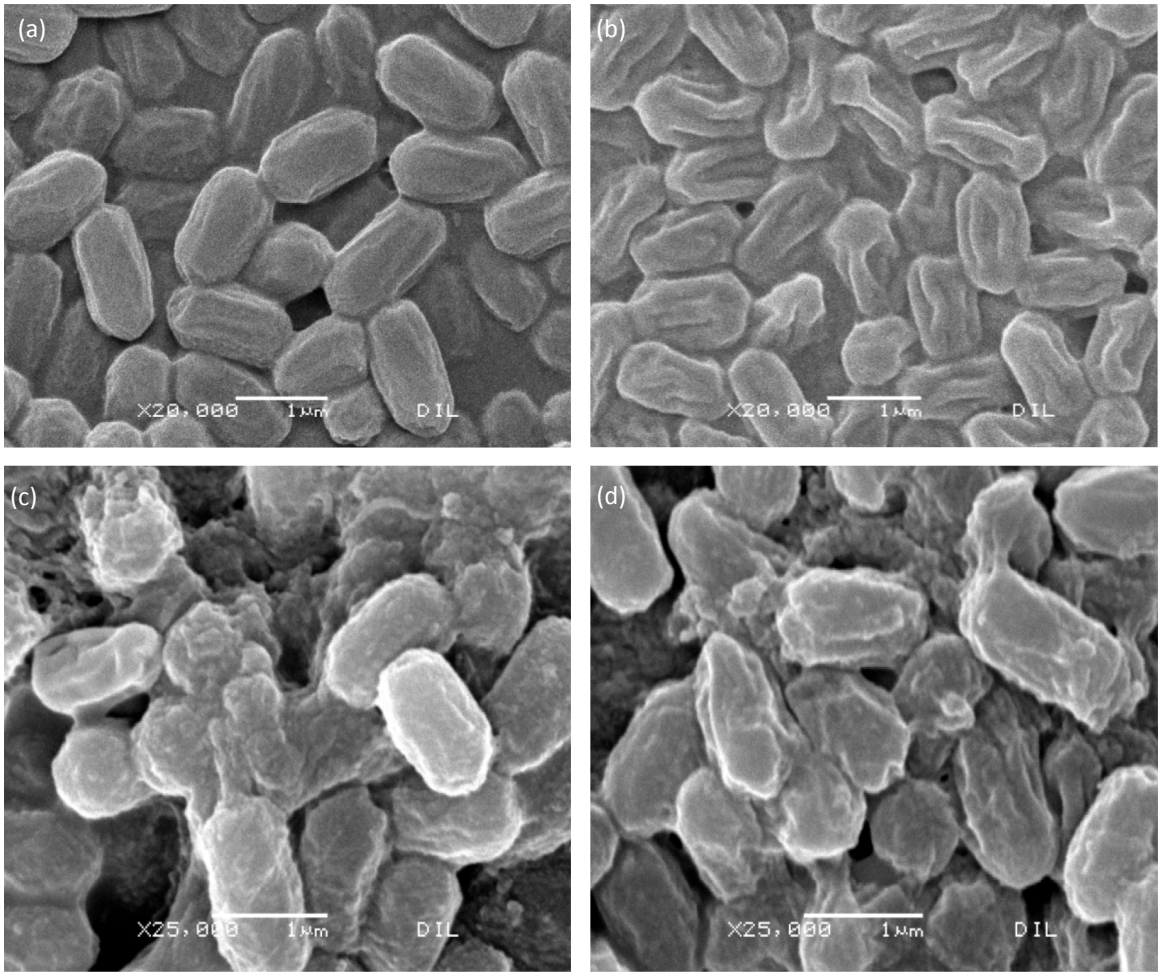

Figure 4-39: SEM micrographs of (a) untreated, (b) autoclaved at 121 °C for 15 min, (c) PEF treated (5.3 log, 9.1 kV/cm, 200.8 kJ/kg) without heat treatment at 80 °C for 10 min and (d) PEF treated (5.3 log, 9.1 kV/cm, 200.8 kJ/kg) and heat treatment (80 °C, 10 min) (N_0=3.0·10^7 ± 3.5·10^6 spo/ml) *B. subtilis* spores.

Comparing the micrographs of untreated and thermally treated spores with PEF treated spores (Figure 4-39c and d), distinguishable differences were observed. The size and the shape of the spores were comparable to the untreated spores. Only a few could be found on the picture having a similar appearance as thermal treated spores. On the contrary, PEF treated spores seemed to form groups of aggregates. As the principle of thermal inactivation is based on protein denaturation and the PEF treated spores appears completely different, the inactivation mechanism of PEF treatment is likely not based on protein denaturation. It might be due to the heat shock and the electric field that the layer structure is affected leading to leakage of the spore content. Similar SEM micrographs were obtained by Jin et al. (2001) (Figure 2-9). They also detected structural differences after thermal and PEF treatment. The PEF treated *B. subtilis* spores were also shrunken and had wrinkles compared to thermal treated spores. The presence of a layer of an unknown substance was observed. Some spores were surrounded by the substance, like it is obtained in this study. Also other researchers' detected cracks, holes and wrinkles on the surface of PEF treated spores (Yonemoto et al. 1993; Marquez et al. 1997; Dunn 2001).

To analyze the substance in the surrounding layer of the spores after PEF treatment, CLSM micrographs were obtained (Figure 4-40).

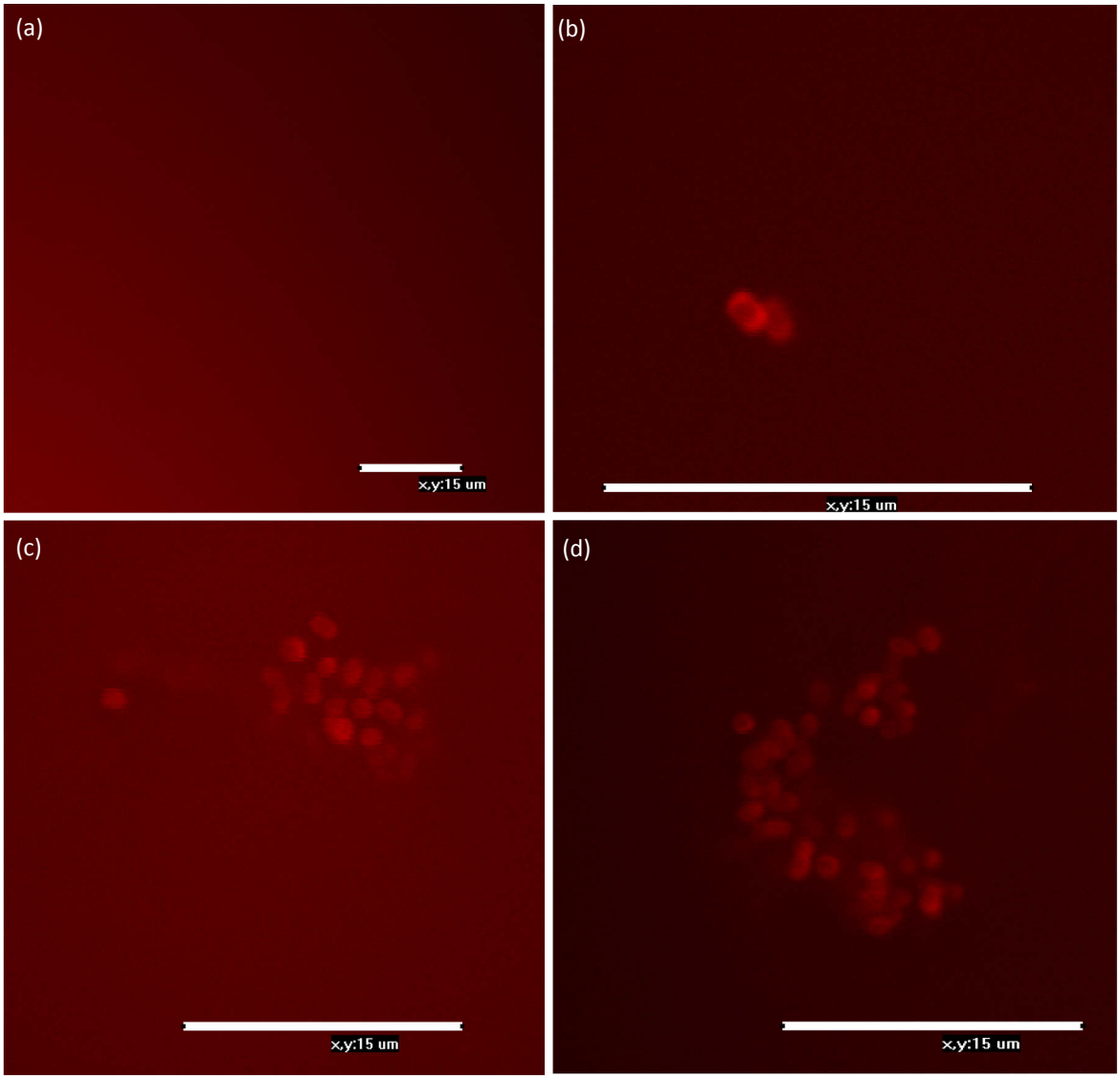

Figure 4-40: CLSM of *B. subtilis* spores in sterile water, fluorescence dye: rhodamin to mark the proteins (a) untreated, (b) autoclaved at 121 °C for 15 min, (c) and (d) PEF treated (4.8 log, 9.1 kV/cm, 189.0 kJ/kg); $N_0=3.0\cdot10^7 \pm 3.5\cdot10^6$ spo/ml.

Each sample was dyed with rhodamin red in order to see the proteins in the solution. In the untreated sample no red spores were visible, which meant no proteins were detected. The result demonstrated that the spores were intact, because no proteins were covering the spore. After thermal treatment only very few rod shaped cells were visible. After PEF treatment many cells were visible (Figure 4-40c and d). Based on the results obtained from the SEM micrographs (Figure 4-39), it was assumed that the layer, which was surrounding the spores, was visible on the CLSM micrograph. As a consequence the layers contained proteins, which could have been released from the core or maybe layer elements containing protein channels.

4.4.3 PEF treatment at isothermal conditions and treatment of decoated *B. subtilis* spores

For performance of PEF treatment under mostly isothermal conditions, the batch PEF system was used. As described in section 3.2.3, the ground electrode was tempered by circulating fluid at the desired temperature. The contact of the sample with the ground electrode surface keeps the temperature of the sample almost constant by dissipating the heat produced during PEF by Joule effect. For the PEF treatment using batch system, different inlet temperatures from 75 to 90 °C were analyzed. The achieved experimental data were compared to inactivation achieved with the continuous PEF system. The developed model (section 4.1) was used to calculate the inactivation at these inlet temperatures.

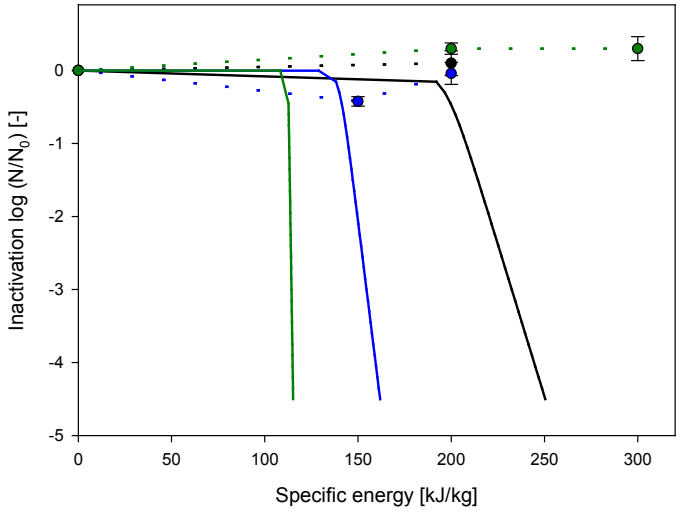

Figure 4-41: Inactivation of *B. subtilis* spores in after PEF treatment in batch system under isothermal conditions at 75 °C (●), 85 °C (●) and 90 °C (●) at an electric field strength of 25 kV/cm and a pulse repetition rate of 4 Hz in comparison to calculated inactivation (see section 4.1) after continuous PEF treatment at an electric field strength of 12.5 kV/cm and an inlet temperature of 75 °C (—), 85 °C (—) and 90 °C (—).

As a result, after PEF treatment under isothermal conditions no inactivation of *B. subtilis* spores was observed. Regarding the continuous PEF treatment, where the temperature of the product increased depending on the specific energy, an inactivation of 4 log cycles was achieved. The energy for an inactivation was reduced by increasing the inlet temperature for a continuous treatment. The treatment in the batch PEF system showed no dependence on inlet temperature or specific energy.

The results using batch operating system indicated no inactivation even at the highest temperature of 90 °C. As the temperature in the continuous treatment is higher than 100 °C, further analysis with the batch PEF unit at higher inlet temperatures should be tested in order to confirm the results.

The results obtained from trials with the batch PEF system were in accordance with some literature. No inactivation was obtained at low electric field strengths (Hamilton et al. 1967; Knorr et al. 1994; Pol et al. 2001; Shin et al. 2008), or at even high electric field strengths about 60 kV/cm and inlet temperature of 60 °C (Pagán et al. 1998). Most of the studies were also performed at isothermal conditions. In continuous treatment, where the temperature increased, an inactivation could be observed (Dunn 2001; Uemura et al. 2003; Bermúdez-Aguirre et al. 2012). Consequently, the fast increase in temperature in combination with electric field causes a damage of spores.

The high resistance of spores is partly caused by the surrounding layers protecting the spore. To evaluate the influence of the coat on the resistance to PEF, analysis with *B. subtilis* mutant (mutant ΔcotE::tet), where the coat is missing, were performed. To evaluate the influence of temperature increase by PEF, the inactivation was studied using batch and continuous PEF treatment. A markedly characteristic of decoated spores is the higher sensitivity to lysozyme (Driks 1999; Nicholson et al. 2000). To demonstrate that the mutant spore has no coat, the spore suspension was treated by lysozyme.

Table 4-15: Survival fraction of *B. subtilis* wild type and mutant after lysozyme treatment (0.5 mg/ml lysozyme for 10 min at 37 °C).

	Survival fraction [%]
Wild type	113.2 ± 10.4
Mutant ΔcotE::tet	15.0 ± 5.7

After the lysozyme treatment, the number of decoated spores was significantly reduced (Table 4-15), while the suspension with wild type spores showed no reduction in spore concentration. As a result, the mutant used for this study had no coat.

For the next step, the decoated spores were treated by PEF with continuous and batch system. To evaluate the influence of the coat on the resistance to PEF treatment, the results were compared to the inactivation of wild type spores (Figure 4-42).

The inactivation at isothermal conditions at 80 °C with electric field strength of 25 kV/cm was about 0.8 log cycles for decoated spores and no inactivation for wild type spores applying an energy of 200 kJ/kg. Due to the nonexistent coat, a higher inactivation was achieved which demonstrated the influence of the coat on the PEF resistance. But it has to be noted, that the achieved inactivation was not very high. The reduction in energy for certain inactivation of decoated spores

was also observed after treatment using the continuous PEF system. Generally, a higher inactivation compared to batch treatment could be obtained, but less energy was required for inactivation of decoated spores. For the wild type, no inactivation was observed until a specific energy of 150 kJ/kg, whereas the inactivation of decoated spores started applying higher energies than 95 kJ/kg. This indicated a shorter shoulder for decoated spores compared to wild type. However, due to the additional temperature increase during continuous treatment, a higher inactivation was observed and higher inactivation rates were obtained for decoated spores at constant PEF conditions. The coat is the first barrier and therefore defense against chemicals, such as peptidoglycan lytic enzymes (section 2.1.3). From the results obtained in this study, it can be concluded that the coat plays a role in resistance to PEF. The absence of the first barrier caused an increase in sensitivity and therefore a better action of PEF treatment in order to inactivate the spores. It might be that the electric field affected the other layers.

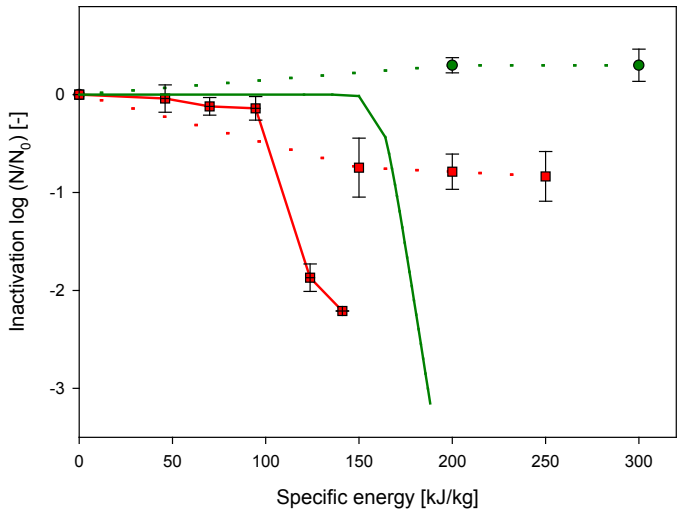

Figure 4-42: Inactivation of wild type (●) and decoated (▪) *B. subtilis* spores after PEF treatment in a batch system (dashed lines) under isothermal conditions at a temperature of 90 °C, pulse repetition rate of 4 Hz and electric field strength of 25 kV/cm compared to continuous PEF treatment with an inlet temperature of 80 °C and an electric field strength of 9 kV/cm (solid line).

The results did not explain the exact principle of action for inactivation of spores by PEF in combination with thermal energy, but some assumptions could be made. The micrographs indicated a shrunken surface with wrinkles. Compared to thermally treated spores a material release was visible on the pictures (Figure 4-39), which contained proteins (Figure 4-40). It is assumed that the substance came from the spores and was a result of spore leakage. Moreover, no inactivation was shown under isothermal conditions applying an electric field strength of 25 kV/cm, specific energy of maximum 300 kJ/kg and an inlet temperature of 90 °C. Using a PEF system without isothermal

conditions, a certain inactivation was achieved. This indicated that the fast increase in temperature was necessary for the inactivation, at least under the applied conditions. It seems that the fast increase in temperature activated the spores to make them more sensitive for a PEF treatment. Furthermore, the coat played an important role. Without the first barrier, the spores were more sensitive to the PEF treatment. This indicated a possible action of PEF on the inner layers.
To sum up, the temperature plays a key role in the inactivation mechanism. It might be that the spore was activated by the rapid temperature increase, which then makes them more sensitive for the PEF treatment.

4.4.4 Sequential PEF treatment

The results presented, demonstrated a possible inactivation of bacterial spores by PEF, but according to the analysis of separation of total inactivation (section 4.3.2), the resulted outlet temperature was high. A possibility to reduce the temperature might be a sequential PEF treatment using lower inlet temperatures. Therefore, the spore suspension was treated twice by PEF. For the first PEF treatment three different settings were used and the resulting spore concentrations are listed in Table 4-16.

Table 4-16: *B. subtilis* spore concentration in Ringer´s solution (4 mS/cm, pH 7) before and after 1st PEF treatment as well as after 1 h incubation (before the 2nd PEF treatment) at different conditions.

	Spore concentration [spo/ml]		
	PEF 1	PEF 2	PEF 3
Initial concentration	$1.5 \cdot 10^5$	$1.1 \cdot 10^5$	$2.2 \cdot 10^5$
After 1st PEF treatment	$1.6 \cdot 10^5$	$8.4 \cdot 10^4$	$6.0 \cdot 10^4$
After 1 h, before 2nd PEF treatment	$1.6 \cdot 10^5$	$6.7 \cdot 10^4$	$6.5 \cdot 10^4$

PEF 1: 10 kV/cm, 241 kJ/kg, 34 °C inlet; PEF 2: 9 kV/cm, 276 kJ/kg, 45 °C inlet; PEF 3: 9 kV/cm, 385 kJ/kg, 34 °C inlet

No effect of the PEF treatment on spore concentration was observed after setting 1 and a slight reduction in spore concentration was obtained after applying setting 2 and 3. The delay in time before starting the 2nd PEF treatment was based on the cleaning procedure. However, no changes in spore concentrations were observed after 1 h storage. For the second treatment, different specific energies were tested applying an electric field strength of 9 kV/cm at an inlet temperature of 45 °C (Figure 4-43). A reduction in spore concentration was obtained for spore suspension, which was previously treated by setting 2 and 3 with an energy higher than 300 kJ/kg. Applying an energy of 326 and 332 kJ/kg, an inactivation of 1.7 and 1.1 log was achieved for previous PEF treatment setting 2 and 3. Approx. the same specific energy (321 kJ/kg) was applied to spore suspension previously treated by setting 1 and no inactivation was observed. Comparing these results with

achieved spore concentration after 1st PEF treatment, no effect of setting 1 was obtained. Consequently, PEF setting 2 and 3 affected the spores in order to make them sensitive for the 2nd PEF treatment.

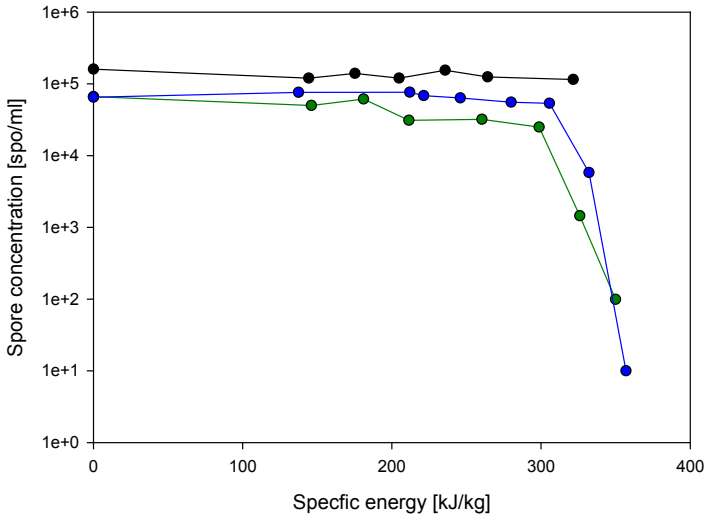

Figure 4-43: Inactivation of *B. subtilis* spores in Ringer´s solution (4 mS/cm, pH 7) after 2nd PEF treatment at different specific energy input at an electric field strength of 9 kV/cm and an inlet temperature of 45 °C, after PEF treatment at 10 kV/cm, 241 kJ/kg and 34 °C inlet temperature (PEF 1, •);9 kV/cm, 276 kJ/kg and 45 °C inlet temperature (PEF 2, •) and 9 kV/cm, 385 kJ/kg and 35 °C inlet temperature (PEF 3, •).

At this stage, the amount of energy used for both PEF settings had to be taken under consideration. The maximum inactivation was observed after PEF pretreatment 2 and 357 kJ/kg. If this energy level was applied in a continuous PE treatment, according to Equation 13, the temperature would increase to a final temperature of 134 °C. The main objective of this section was to reduce the temperature in order to allow a more gentle treatment of heat sensitive food products. The results of single PEF treatment using higher inlet temperatures obtained similar inactivation with lower energy inputs. A 2.1 log inactivation of *B. subtilis* spores was observed after PEF treatment at 9 kV/cm and 177 kJ/kg. Comparing the applied energy values in sequential and single PEF trial, a lower energy input can be used, if only one PEF treatment is applied. The lower energy results in lower operating costs, although a higher inlet temperature is used. Bermúdez-Aguirre et al. (2012) studied the inactivation of *B. cereus* spore in milk using a sequential PEF treatment. The inlet temperature for each run was constant at 65 °C applying 250 exponential decay pulses and an electric field strength of 30 kV/cm. No difference in inactivation was observed, which confirms the results obtained in this study.

4.5 Influence of sporulation temperature on PEF resistance of spores

The *B. subtilis* spores prepared for the study were sporulated at 37 °C. Very little information is available about sporulation in natural habitants (Nicholson et al. 2000). It can be assumed that the sporulation in nature is not happening at a constant temperature. It is stated that the sporulation temperature has an effect on the heat resistance of spores (Sala et al. 1995, Condon et al.1992; Palop et al. 1996). Therefore, spores of natural origin in food products have different heat resistances and require a higher thermal treatment to ensure the inactivation. In this study *B. subtilis* spores were sporulated at 37 and 45 °C to analyze the effect of sporulation temperature on the PEF resistance.

Figure 4-44 shows the inactivation of *B. subtilis* spores sporulated at 37 and 45 °C. As a result the inactivation of spores, which were sporulated at 45 °C required a higher energy. For a 2.1 log inactivation, an energy of 177 kJ/kg was required for the spores sporulated at 37 °C compared to 184 kJ/kg to the spores sporulated at 45 °C. In conclusion, the *B. subtilis* spores cultivated at higher temperature indicated a slightly higher PEF resistance.

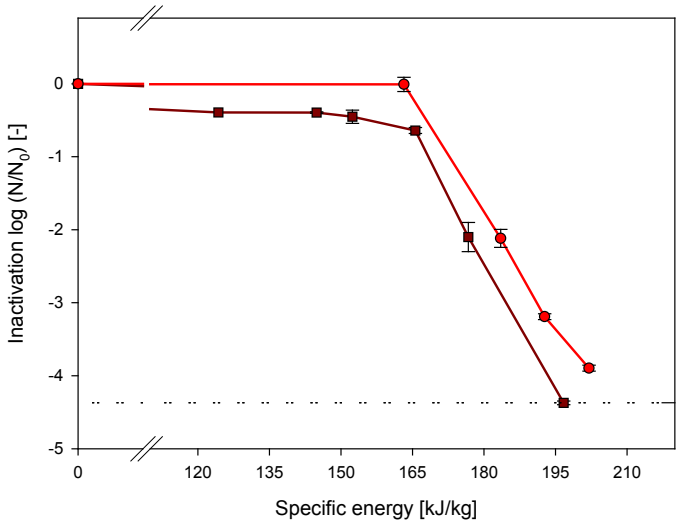

Figure 4-44: Inactivation of B. subtilis spores sporulated at 37 °C (■, $N_{0,37\,°C}$= 2.4·10^5 ± 1.6·10^5 spo/ml) and 45 °C (●,$N_{0,45\,°C}$= 2.7·10^5 ± 1.2·10^5 spo/ml) by PEF at an electric field strength of 9 kV/cm in Ringer´s solution with a conductivity of 4 mS/cm and a pH of 7 with an inlet temperature of 80 °C; detection limit (—·—).

A higher sporulation temperature affects the heat resistance of spores in order to increase the heat resistance (Condon et al. 1992; Raso et al. 1995). After PEF treatment, a higher resistance of spores that sporulated at 45 °C was observed. Due to the fact of Joule heating, the temperature of the product increased during the PEF treatment. In section 4.3.3 it was shown, that some parts of the spore population was inactivated by temperature. If the spores have a higher heat resistance due to the higher sporulation temperature, the energy and therefore the temperature

increase have to be higher to achieve inactivation. For determination of thermal heat resistance of spores that sporulated at 37 and 45 °C, the D_T and z values were calculated (Table 4-17).

Table 4-17: Thermal inactivation kinetics (D_T and z value) of *B. subtilis* spores that sporulated at 37 and 45 °C in Ringer´s solution with a conductivity of 4 mS/cm and pH 7.

Spore type	Temperature [°C]	D_T value [s]	z value [°C]
B. subtilis (sporulated at 37 °C)	100	505.05	9.09
	110	15.72	
	121	2.24	
B. subtilis (sporulated at 45 °C)	100	322.58	10.42
	110	11.53	
	121	2.90	

The D_{100} and D_{110} for *B. subtilis* spores that sporulated at 45 °C were lower compared to sporulation at 37 °C. But the difference in D_T value between 37 and 45 °C decreased with increasing temperature. This indicated a higher heat resistance of *B. subtilis* spores sporulated at 37 °C. At 100 °C the difference was 182.45 s in comparison to 4.19 s at 110 °C. A further increase in temperature resulted in a higher D_{121} for spores that sporulated at 45 °C and therefore in a higher heat resistance at 121 °C. Compared to literature, different results were detected. Condon et al. (1992) detected a 7.8 fold increase in D_{108} with a sporulation temperature ranging from 37 to 52 °C. The different results can be explained by the different strains, different treatment media and a higher sporulation temperature.

Moreover, the z value for *B. subtilis* spores that sporulated at 45 °C was 1.3 °C higher, which means a higher temperature increase was required to cause a tenfold change in D_T value.

In conclusion, a higher heat resistance of spores that sporulated at higher temperatures was observed for high treatment temperatures. Less inactivation can be expected treating spores that sporulated at 45 °C compared to spores that sporulated at 37 °C applying the same temperature time regime.

The developed method for quantification of thermal inactivation during PEF treatment (section 3.4.2) was used to analyze the difference in thermal induced inactivation between spores that sporulated at different temperatures.

The total inactivation of both spore types that sporulated at 37 and 45 °C can be separated in thermal and PEF induced inactivation (Figure 4-45). It was already observed in Figure 4-43, that a higher inactivation of spores that sporulated at 37 °C was obtained compared to spores at 45 °C applying the same amount of energy. After a PEF treatment using 196 kJ/kg the total inactivation of spores at 37 °C was 4.4 log, which can be separated in 1.2 log thermal inactivation and 3.2 log PEF induced inactivation. In contrast, applying the same energy a total inactivation of 3.2 log,

where 0.5 log is thermal induced, was observed for spores that sporulated at 45 °C. The percentage of thermal inactivation was 26 % for log spore count that sporulated at 37 °C and 17 % for log spore count that sporulated at 45 °C. These results showed a lower thermal inactivation, if spores were sporulated at higher temperatures, which could be explained by the higher heat resistance. The lower thermal inactivation, which was based on a mathematical calculation, could be confirmed by the DPA measurement after the PEF treatment (Table 4-18). The DPA concentration after the PEF treatment of spores that sporulated at 37 °C was 35.8 % compared to 30.0 % at sporulation temperature of 45 °C. The lower DPA concentration indicated a lower thermal impact and demonstrated a higher heat resistance of the spores that sporulated at 45 °C.

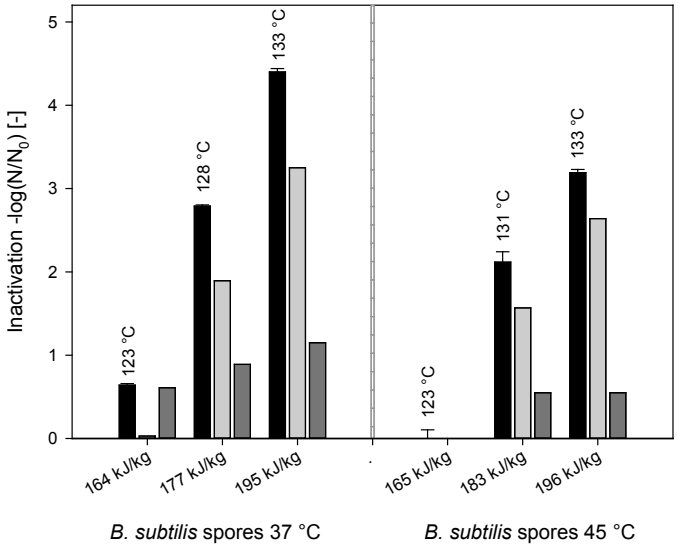

Figure 4-45: Separation of total inactivation (black bar) in PEF (light grey bar) and thermal (dark grey bar) induced inactivation of *B. subtilis* spores that sporulated at 37 °C (left side) and 45 °C (right side) indicating the outlet temperature to characterize the thermal treatment. For PEF treatment an electric field strength of 9 kV/cm was used in Ringer´s solution with a conductivity of 4 mS/cm and pH 7.

The results of analysis of thermal inactivation kinetics (Table 4-17) indicated a higher heat resistance of spores that sporulated at 45 °C at a treatment temperature of 121 °C. The highest temperature after PEF processing was 133 °C, which was experimentally obtained, and therefore a direct comparison to the thermal inactivation kinetics was not possible. Nevertheless, a higher heat resistance of spores that sporulated at higher temperature can be assumed.

Thus, the difference in total inactivation between *B. subtilis* spores sporulated at 37 and 45 °C applying the same energy might be explained by the lower thermal inactivation at higher sporulation temperature. The difference in thermal induced inactivation between both spores was 0.7 log inactivation, whereas the difference in total inactivation was 1.2 log. The PEF induced inactivation was 0.5 log higher at a sporulation temperature of 37 °C. In conclusion, the spores that

sporulated at higher temperatures had a higher heat resistance and showed a higher resistance to the PEF treatment. However, the mechanism of higher heat resistance at high sporulation temperature is not clear (Palop et al. 1996). The optimum sporulation temperature is usually higher than the optimum growth temperature and an increase in sporulation temperature leads to higher heat resistance, which is based on dehydration, mineralization and thermal adaption (Condon et al. 1992). This explains the lower obtained thermal induced inactivation during the PEF treatment. The lower PEF induced inactivation cannot be elucidated, because the mechanism of spore inactivation is unclear. Probably, the higher heat resistance is somehow involved, as the process is combined with thermal energy.

Table 4-18: Comparison of DPA concentration after thermal and PEF treatment at 9 kV/cm and 80 °C inlet temperature of *B. subtilis* spores that sporulated at 37 and 45 °C.

Spore type	Treatment	DPA [µM]	DPA [%]
B. subtilis (37 °C)	thermal	1.86 ± 0.859	100.00
	untreated	0.25 ± 0.059	13.45
	PEF (177 kJ/kg)	0.36 ± 0.051	19.31
	PEF (195 kJ/kg)	0.66 ± 0.171	35.82
B. subtilis (45 °C)	thermal	1.33 ± 0.008	100.00
	untreated	0.24 ± 0.009	18.26
	PEF (183 kJ/kg)	0.34 ± 0.009	25.60
	PEF (196 kJ/kg)	0.40 ± 0.012	30.04

In summary, the sporulation temperature has an impact on the inactivation of bacterial spores by PEF. The results demonstrate that the effect of sporulation temperature on heat resistance is a major part in resistance of spores to PEF. For comparing studies, this might be a reason for controversial results, because different resistances lead to different results. Moreover, these results are important for industrial application of the presented process. It demonstrates that the resistance of spores, if it is a natural contamination is not comparable to resistance observed in lab or pilot tests. Therefore, microbial testing after production is necessary to ensure the safety of the food and harsher processing conditions should be used to ensure spore inactivation.

4.6 Case studies

4.6.1 PEF treatment of yeast extract in combination with thermal energy

Yeast extract is considered as low acid product that can be contaminated with bacterial endospores, mainly with *B. subtilis* spores. To date, industry uses thermal processes to inactivate spores

and to achieve the desired shelf life. In this case study, yeast extract is chosen as an example to demonstrate the use of PEF in combination with thermal energy instead of high temperature thermal processes.

4.6.1.1 Inactivation of *B. subtilis* spores in yeast extract by PEF

In the first part, yeast extract was inoculated with *B. subtilis* spores and treated by PEF using different energy levels at an electric field strength of 4.5 kV/cm (Table 4-19).

Table 4-19: *B. subtilis* spore count and inactivation after PEF treatment at different energy levels at an electric field strength of 4.5 kV/cm. Initial spore count: $2.6 \cdot 10^5 \pm 2.3 \cdot 10^4$ spo/ml, pre heating up to 80 °C.

Specific energy [kJ/kg]	*B. subtilis* count [spo/ml]	Inactivation ($\log(N/N_0)$) [-]
184.4	$2.1 \cdot 10^5 \pm 4.9 \cdot 10^4$	-0.11 ± 0.113
195.6	$1.2 \cdot 10^5 \pm 2.5 \cdot 10^4$	-0.34 ± 0.088
208.0	$1.4 \cdot 10^5 \pm 2.6 \cdot 10^4$	-0.28 ± 0.087
220.5	$9.1 \cdot 10^4 \pm 1.7 \cdot 10^4$	-0.47 ± 0.076
232.5	$3.2 \cdot 10^4 \pm 5.3 \cdot 10^3$	-0.92 ± 0.070
244.1	$2.3 \cdot 10^3 \pm 1.0 \cdot 10^2$	-2.06 ± 0.019
257.0	$6.9 \cdot 10^2 \pm 5.2 \cdot 10^1$	-3.77 ± 0.601

A higher reduction of spore counts was achieved by PEF treatment with increasing energy level. At 244.1 kJ/kg, an inactivation of 2.06 log was obtained. This was increased to 3.77 log at an energy input of 257.0 kJ/kg. To exclude the germination of the spores by the pre heating step, a sample was taken without PEF treatment. The spore count in this sample was $2.6 \cdot 10^5 \pm 2.5 \cdot 10^4$ spo/ml, so no germination occurred due to the heat treatment. For further studies aimed at shelf life analysis of non-inoculated products, the energy values resulting in highest spore inactivation were chosen. For a comparison to a thermal treatment, a sample was taken from untreated inoculated product and treated at 121 °C for 20 min. The microbial analysis showed a maximum spore reduction, which meant the spore count was under $9.0 \cdot 10^0 \pm 0$ spo/ml.

4.6.1.2 Shelf life of PEF treated yeast extract

After the inactivation of spores, the shelf life of yeast extract was determined. The shelf life was presented by showing the comparison in total plate count of the PEF treated yeast extract to the thermal treatment.

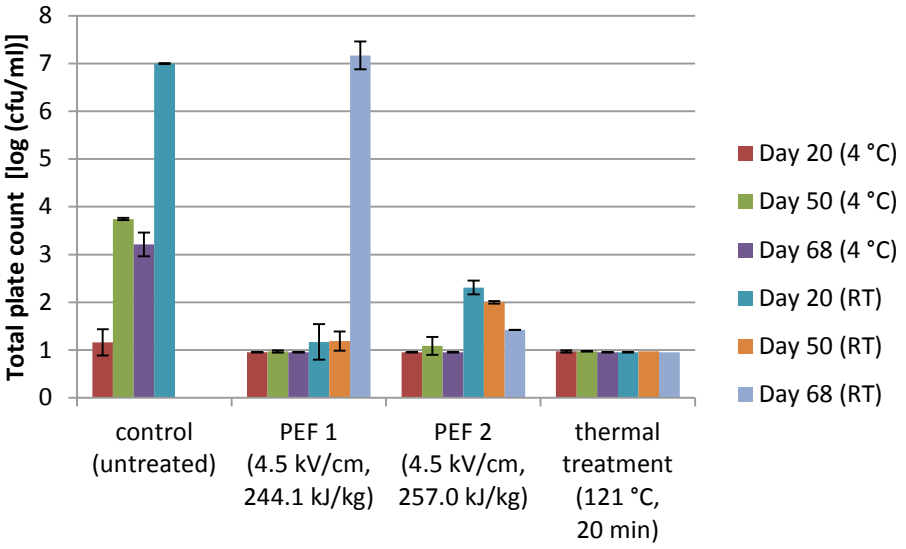

Figure 4-46: Comparison total plate count (TPC) of yeast extract analyzed over 68 days stored at room temperature (RT) and 4 °C after no treatment, PEF and thermal treatment.

The thermal and PEF treatment extended the shelf life of yeast extract (Figure 4-46). The storage temperature also had a major impact on shelf life. A higher storage temperature resulted in a faster growth of microorganisms. This is related to the optimum temperature for microorganism growth. For example, the optimum temperature for *E.coli* growth is at 37 °C. By lowering the temperature, the growth is slowed down. However, yeast extract stored at 4 °C had a shelf life of more than 68 days. Due to the higher concentration of $1.3 \cdot 10^{3} \pm 3.2 \cdot 10^{1}$ cfu/ml in the control sample compared to the PEF treated samples at day 68, the shelf life of the PEF treated sample was most likely longer. The untreated product stored at room temperature had a shelf life of less than 20 days. After thermal treatment, the shelf life of yeast extract was more than 68 days (TPC in each case < 10 cfu/ml). When the yeast extract was treated with PEF, the influence of applied energy was obvious. At higher energy levels, a longer shelf life was obtained. Using an energy input of 244.1 kJ/kg, the TPC at day 68 was $1.5 \cdot 10^{7} \pm 4.2 \cdot 10^{7}$ cfu/ml, compared to $2.6 \cdot 10^{1} \pm 2.9 \cdot 10^{1}$ cfu/ml. Regarding the TPC development during shelf life of the PEF treated sample at 257.0 kJ/kg, higher values were detected at day 20 ($2.0 \cdot 10^{2} \pm 1.5 \cdot 10^{2}$ cfu/ml) compared to day 68 ($2.6 \cdot 10^{1} \pm 2.9 \cdot 10^{1}$ cfu/ml). Nevertheless, this difference was too low to be considered significant and might have been due to difference in the plotting or sampling.

Microbiological analysis on the untreated, PEF and thermally treated yeast extract was performed over the shelf life of the product. Standard industry microbiological testing was performed, namely total plate count and yeast and mold. The shelf analysis of untreated, room temperature product was stopped at day 20 due to the high TPC. Hence, no further analysis was performed on day 50 or day 68 on the untreated samples for comparison. The mold count in each analyzed

sample was lower than 10 cfu/ml (Table 6-1, appendix). The yeast count was lower than 10 cfu/ml, except for the PEF treated (244.1 kJ/kg) product analyzed at day 68 stored under ambient conditions (Table 6-2, appendix). The yeast concentration in the samples was $4.7 \cdot 10^7 \pm 4.0 \cdot 10^6$ cfu/ml, which correlated to the high TPC. As a result, the treatment intensity of 244.1 kJ/kg is not enough to achieve a long shelf life.

4.6.1.3 Quality analysis

To evaluate the quality of the yeast extract, the color and pH over shelf life was measured. The pH value indicated a slight increase in each case (Table 4-20). In total, the pH varied from 5.50 to maximum 5.96, which was not significant.

Table 4-20: Comparison of pH value of untreated, PEF and thermal treated yeast extract stored at 4 °C and room temperature (RT); n.a. – not analyzed.

		Storage 4 °C		Storage RT	
	Day 0	Day 20	Day 68	Day 20	Day 68
Control	5.79	5.94	5.86	5.80	n.a.
PEF 1 (244.1 kJ/kg)	5.64	5.73	5.96	5.81	5.78
PEF 2 (257.0 kJ/kg)	5.65	5.80	5.94	5.68	5.78
Thermal (121 °C, 20 min)	5.50	5.67	5.72	5.50	5.58

The color of yeast extract was measured directly after the treatment, at day 20 and at day 68 of storage under different conditions. As the picture (Figure 4-47) and the color values (Table 4-21) indicated, the untreated samples differ very much from the treated products. During storage, a separation was visible and after mixing a cloudy and light solution appeared. After the treatment, the product appeared dark and transparent. The visible differences were supported by the measured color values (Table 4-21). Compared to the treated product, the L* value was higher, which indicated a lighter color, and the b* value was also higher, resulting in a more yellow color.

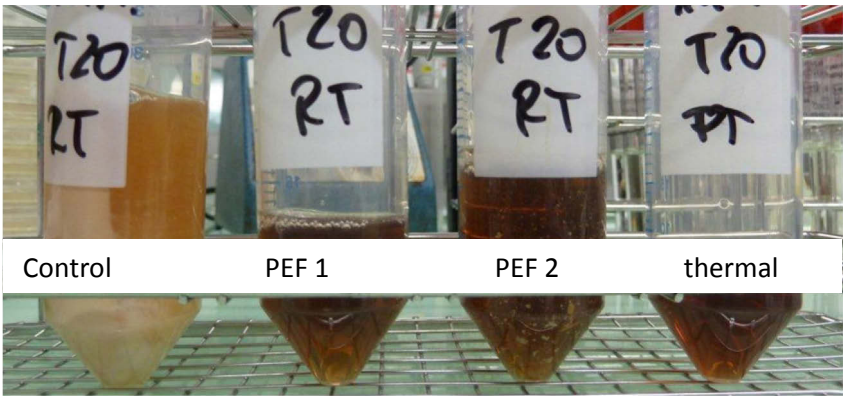

Figure 4-47: Yeast extract after 20 days storage at room temperature (RT).

The color of the PEF and thermally treated samples showed only slight differences. The biggest differences were observed for the b* values. For example, directly after the treatment the b* value, describing the blue-yellowness of the sample, was lower (0.01 ± 0.1) for the thermally treated sample than for the PEF sample at 244.1 kJ/kg (0.79 ± 0.2). This indicated that the thermally treated yeast extract was less yellow than the PEF treated sample.

Table 4-21: Color values (L*, a* and b*) of untreated, PEF and thermal treated yeast extract stored over 68 days at 4 °C and room temperature (RT); n.a. – not analyzed.

	Storage 4 °C			Storage at RT	
	Day 0	Day 20	Day 68	Day 20	Day 68
			L*		
Control	51.66 ± 1.27	48.61 ± 0.30	48.30 ± 0.31	41.89 ± 0.06	n.a.
PEF 1 (244.1 kJ/kg)	25.43 ± 0.15	25.10 ± 0.07	24.98 ± 0.10	24.19 ± 0.06	24.08 ± 0.05
PEF 2 (257.0 kJ/kg)	25.27 ±0.15	24.89 ± 0.09	25.42 ± 0.21	24.13 ± 0.05	24.64 ± 0.28
Thermal (121 °C, 20 min)	25.05 ± 0.22	24.08 ± 0.32	24.19 ± 0.05	24.04 ± 0.05	24.02 ± 0.09
			a*		
Control	0.52 ± 0.13	0.82 ± 0.02	1.43 ± 0.04	1.53 ± 0.05	n.a.
PEF 1 (244.1 kJ/kg)	1.09 ± 0.16	0.42 ± 0.07	0.33 ± 0.07	0.28 ± 0.07	0.11 ± 0.09
PEF 2 (257.0 kJ/kg)	0.87 ± 0.21	0.10 ± 0.09	0.58 ± 0.07	0.08 ± 0.06	0.31 ± 0.07
Thermal (121 °C, 20 min)	1.15 ± 0.14	0.33 ± 0.14	0.12 ± 0.07	0.07 ± 0.03	-0.01 ± 0.02
			b*		
Control	11.89 ± 0.75	13.10 ± 0.33	13.03 ± 0.26	11.89 ± 0.07	n.a.
PEF 1 (244.1 kJ/kg)	0.79 ± 0.20	0.30 ± 0.09	0.10 ± 0.08	-0.40 ± 0.05	-0.65 ± 0.05
PEF 2 (257.0 kJ/kg)	0.35 ± 0.25	-0.07 ± 0.07	0.68 ± 0.31	-0.54 ± 0.03	-0.30 ± 0.19
Thermal (121 °C, 20 min)	0.01 ± 0.10	-0.35 ± 0.12	-0.46 ± 0.04	-0.60 ± 0.02	-0.69 ± 0.03

To analyze the stability of the color, the color values of untreated, PEF treated and thermally treated sample were measured over shelf life. The net color difference (ΔE-value) was calculated (Equation 29) to allow an evaluation of color difference (Table 4-22). After 20 and 68 days of storage at 4 °C, the ΔE-value of untreated yeast extract was more than 4. This can be classified as an intensive color change according to the DIN 53230. At higher storage temperatures, the color difference was even higher (ΔE=10.20). The high color change might be related to the microbiological results. At 4 °C the TPC was higher than 10^3 cfu/ml and at ambient conditions more than 10^7 cfu/ml. In terms of color values, the L* value decreased over time, which means the yeast extract became darker and the a* value is increased, which resulted in a higher redness. The b* value only showed slight changes.

Table 4-22: Net color difference (ΔE-value) of untreated, PEF and thermal yeast extract color directly after treatment and different storage time (Day 20 and 68); n.a. – not analyzed.

	4 °C		RT	
	Day 20	Day 68	Day 20	Day 68
Control	4.53	4.78	10.20	n.a.
PEF 1 (244.1 kJ/kg)	0.99	1.36	4.13	5.14
PEF 2 (257.0 kJ/kg)	1.04	0.44	1.80	0.95
Thermal (121 °C, 20 min)	1.40	1.56	1.16	1.31

In contrast to the untreated yeast extract, the color change of PEF and thermal treated product was smaller. As it was observed for PEF treatment applying a lower energy input, a shelf life of more than 68 days could not be achieved. Therefore, the color change might be due to microbial growth. Although the microbial results (Figure 4-46) indicated a TPC lower than 10^2 cfu/ml, the color change was very intensive (ΔE >4). A higher applied energy caused a longer shelf life and a more stable color of yeast extract. At higher storage temperatures, the color difference was slightly higher, especially at day 20, but nevertheless the color difference was smaller than 2 indicating a color difference, which is only visible for a trained eye (Table 3-3). The color difference of thermally treated yeast extract was also lower than 2. Comparing the color differences at different time stages, it was obvious that color change decreased with increasing time in the case of PEF treatment. After thermal processing, the color difference was increasing with increased storage time. Because of these small color changes compared to control or PEF treatment at low energy input, the differences are small. More data are required to prove if the trend showing less color change during storage of PEF treated product is true.

4.6.2 Shelf life extension of carrot juice by PEF

Consumer demand for vegetable juices is increasing, because of the increasing health consciousness of the consumer. Due to carrot juice being a good source of carotenoids, fiber, vitamins, minerals and bioactive compounds, the juice can be considered as a healthy juice providing many benefits for human health (Qin et al. 2005). As carrot juice has an almost neutral pH, and the origin of the carrot is from the soil, the risk of contamination with bacterial endospores is high. Therefore, a severe thermal treatment has to be used to ensure the microbiological safety (Zhou et al. 2009; Gonçalves et al. 2010). This thermal treatment causes the quality of the product to be damaged. The results of this study demonstrate an inactivation of spores by PEF at lower temperatures. Thus, the developed process was used to extend the shelf life of a vegetable juice. The quality and the microbial concentration of the PEF treated carrot juice were analyzed over the shelf life and compared to traditionally sterilized product.

Carrot juice was chosen for this study, because its consumption has grown in popularity (Zhou et al. 2009). The health promoting characteristics are based on the secondary metabolites, dietary fibers and minerals (Gonçalves et al. 2010). Moreover, carrot juice is a natural source of vitamins and is low in fat and acid (Zhou et al. 2009).

4.6.2.1 Inactivation of relevant target spores in carrot juice by PEF

The first part of the case study on carrot juice was aimed at inactivating target spores by the developed process. Target spores, *A. acidoterrestris* and *B. subtilis*, were chosen. Due to the pH of 5, *A. acidotrestris* was chosen due to its capability to grow in acidic environment (Yamazaki et al. 1996).

The results of spore reduction by PEF are shown in Figure 4-48 indicating an inactivation of both spore types. Comparing the required energy for inactivation, a lower specific energy had to be applied for inactivation of *A. acidoterrestris* compared to *B. subtilis* spores. At energy of 153 and 162 kJ/kg, an inactivation of 4.8 log of *A. acidoterrestris* and 0.2 log of *B. subtilis* spores was achieved. Therefore, *A. acidoterrestris* spores were more sensitive to the PEF treatment than *B. subtilis* spores. This result is in accordance with the previous results analyzing the inactivation of these spores in Ringer´s solution (see section 4.1.4). The highest inactivation of 4.8 log for *A. acidoterrestris* spores and 2.5 log for *B. subtilis* spores was achieved by applying an energy of 153 and 198 kJ/kg. From these results, the PEF setting using an electric field strength of 9 kV/cm and a specific energy of 198 kJ/kg was used for the further experiments, where the quality and the microbiological stability was analyzed over shelf life.

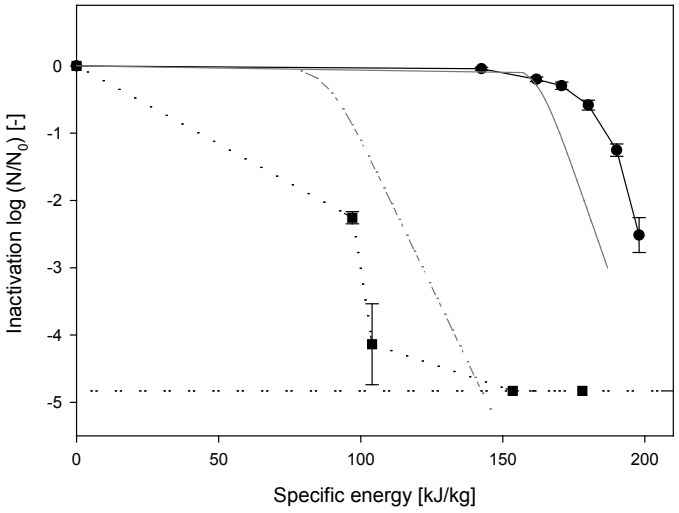

Figure 4-48: Experimental determined inactivation of *B. subtilis* spores (●, N_0=1.3·10^5 ± 7.1·10^3 spo/ml) and *A. acidoterrestris* spores (■, N_0=6.1·10^5 ± 6.9·10^5 spo/ml) in carrot juice and modeled inactivation of *B. subtilis* (—) and *A. acidoterrestris* (--) in Ringer´s solution by PEF at an electric field strength of 9 kV/cm and an inlet temperature of 80 °C.

The inactivation of these spore types was first analyzed in Ringer´s solution, which acted as a model solution to allow analysis of influencing parameters. For a 2.5 log inactivation of *B. subtilis* spores, energy of 182.8 kJ/kg at 9 kV/cm was required. Compared to the inactivation in carrot juice, a higher energy of 198.0 kJ/kg was required. A slight difference in energy between modeled and experimental energy was also observed for inactivation of *A. acidoterrestris* spores. The inactivation of 4.8 log was achieved by applying 141.8 kJ/kg in Ringer´s solution compared to 153 kJ/kg in carrot juice. The difference in energy required for the inactivation between model solution and carrot juice can be explained by the different media. The conductivity of the Ringer´s solution was 4 mS/cm with a pH of 7, compared to 6 mS/cm and a pH of 5 for carrot juice. Furthermore, carrot juice contains natural ingredients, such as vitamins or polyphenols and is therefore more complex and might require a more severe treatment.

4.6.2.2 Shelf life study of PEF treated carrot juice

For studying the shelf life of PEF and thermal treated carrot juice, the aerobic total plate count (TPC) as well as yeast and mold concentration was estimated over a time period of 9 weeks. The initial TPC concentration was reduced by PEF and thermal treatments from $5 \cdot 10^2$ to $<10 \cdot 10^0$ cfu/ml, which is the detection limit. The initial yeast concentration of $5 \cdot 10^2$ cfu/ml was decreased to $<10 \cdot 10^0$ cfu/ml by PEF and thermal treatment. No changes in mold concentration were observed, because the initial concentration was already $<10 \cdot 10^0$ cfu/ml. The development of microbiological concentration over 9 weeks storage at ambient temperature after PEF and thermal treatment is shown in Figure 4-49.

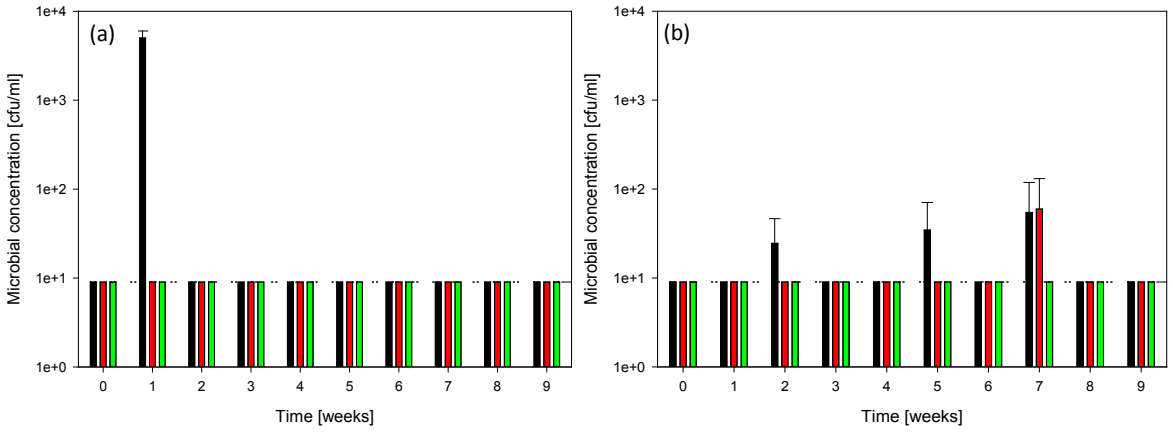

Figure 4-49: Microbial concentration (■ total plate count, ■ yeast and ■ molds) in carrot juice after (a) PEF treatment at an electric field strength of 9 kV/cm, an energy input of 198 kJ/kg and inlet temperature of 80 °C, and (b) thermal treatment 121 °C for 45 s over storage of 9 weeks at ambient temperature.

After PEF treatment, the analyzed microbial concentrations were reduced to the detection limit of $<10 \cdot 10^0$ cfu/ml. During the storage of 9 weeks at ambient temperature, the microbial concentration for TPC, yeast and mold was constant. Only after storage of 1 week, an increase of TPC up to $5.0 \cdot 10^3$ cfu/ml was detected. Due to the fact, that this high concentration was observed only one time, it can be assumed that this sample was contaminated by an external source. As a result the shelf life of the PEF treated carrot juice is more than 9 weeks.

The shelf life of the thermally treated carrot juice is also more than 9 weeks. The TPC, yeast and mold concentration was almost constant over the analyzed period of time. After week 2, 5 and 7 a slightly higher TPC concentration was observed, which can be explained by statistical variance. As the concentration was below 10^2 cfu/ml, the microbial safety is guaranteed.

4.6.2.3 Quality analysis of carrot juice after thermal and PEF treatment

The samples after PEF and thermal treatment were stored for 9 weeks at ambient temperature. Each week the quality parameters pH, Brix, total phenol content and antioxidant capacity were estimated and evaluated.

The pH and Brix values are presented in Table 4-23. Slightly different values for pH and Brix were observed for the control samples. The untreated sample before the PEF treatment had a pH of 5.05 and a soluble solid content of 10.25. In comparison, the control before thermal treatment had a pH of 4.85 and a Brix of 10.21. The differences can be explained by the procedure. The first step in the case study was the safety validation, which focused on inactivation of target spores. Due to the delay in time because of the incubation time required for analysis, the trials for quality and shelf life analysis could not be performed at the same time. Therefore, the carrot juice was

frozen for 6 days in order to avoid spoilage of the juice. The pH and soluble solid content of the thermal control sample was analyzed on the same day that the thermal treatment was performed. Consequently, the difference in pH and Brix could have been due to the freezing and thawing step. However, after thermal and PEF treatment, no significant differences in soluble solid content and pH were observed over a storage time of 9 weeks at ambient temperature. Other studies analyzing carrot or orange carrot juice indicated no effect of PEF on soluble solids and pH (Rivas et al. 2006; Caminiti et al. 2012). Akin et al. (2009) studied carrot juice mixed with 0.47 % demineralized whey, 0.47 % inulin, 0.47 % oligofructose, 2.98 % sugar and 0.19 % citric acid. The carrot juice is comparable to the one used in this study, because of the citric acid and the sugar. The carrot juice used in this study contained citric acid and honey to increase the sweetness of the juice. Moreover, the conductivity of both studied juices was 6 mS/cm. The pH of 4.7 and soluble solid of 13.2 did not change after continuous PEF treatment with an electric field strength of 13 kV/cm in the study of Akin et al. (2009). The study of Liao et al. (2007) focused on analysis of quality parameters after thermal sterilization. The soluble solid content and pH after pressing were 5.5 and 4.69. After sterilization at 110 °C for 5 min, the values showed no significant change (°Brix: 5.5 and pH of 4.64). After storage of 8 weeks at 25 °C, soluble solid content and pH showed no changes (Liao et al. 2009). Although the sterilization conditions and PEF settings from literature were different than the ones used in this study, the same effect of constant pH and soluble solid content was observed.

Table 4-23: pH and °Brix value before and after thermal (121 °C, 45 s) and PEF (198 kJ/kg, 9 kV/cm) treatment over storage time of 9 weeks at ambient temperature.

Time [weeks]	Thermal treatment		PEF treatment	
	°Brix	pH	°Brix	pH
Before treatment	10.21 ± 0.007	4.85 ± 0.071	10.25 ± 0.010	5.05 ± 0.071
0	10.24 ± 0.030	4.90 ± 0.071	10.15 ± 0.010	5.03 ± 0.042
1	10.16 ± 0.020	4.95 ± 0.071	10.31 ± 0.020	4.94 ± 0.092
2	10.23 ± 0.020	4.85 ± 0.141	10.20 ± 0.030	4.89 ± 0.127
3	10.20 ± 0.030	4.90 ± 0.071	10.31 ± 0.030	4.80 ± 0.064
4	10.08 ± 0.020	4.96 ± 0.085	10.28 ± 0.010	4.88 ± 0.113
5	10.20 ± 0.020	4.93 ± 0.042	10.30 ± 0.020	4.90 ± 0.049
6	10.15 ± 0.010	4.82 ± 0.028	10.32 ± 0.030	4.93 ± 0.042
7	10.19 ± 0.030	4.89 ± 0.127	10.30 ± 0.020	5.05 ± 0.071
8	10.14 ± 0.020	4.91 ± 0.085	10.34 ± 0.020	5.05 ± 0.042
9	10.20 ± 0.010	4.85 ± 0.071	10.26 ± 0.010	4.92 ± 0.028

Besides pH and soluble solid content, the color of the juice is an important quality parameter. The consumer expects a juice with a light and fresh orange color. Due to high temperatures and oxygen, the color has changed. Phenols form brown pigments and aroma compounds (Beldman et al. 2002). However, the results of color measurement are shown in Table 4-24. Directly after each treatment the color change was low. The calculated color difference for thermal treatment was 0.81 and for PEF treatment 0.75. According to the evaluation of color difference (Table 3-3), the obtained color difference is not detectable. This result is in accordance with results from other researchers (Rivas et al. 2006; Akin et al. 2009; Quitão-Teixeira et al. 2008). Quitão-Teixeira et al. (2008) analyzed the color retention after PEF treatment in carrot juice. After PEF treatment, with an electric field strength of 35 kV/cm at a frequency of 200 Hz for 1000 µs applying bipolar 6 µs pulses, no color change before or after the treatment was observed. Although the temperature in the study never exceeded a temperature of 35 °C and was therefore lower than the temperature range in this study, the result of no color difference directly after PEF treatment was the same.
During storage, a color change was observed after both processes. After week 2 of thermal treatment, the color change increased up to 2.76. All color values indicated a decrease, which means the juice was darker (lower L* value), less red (lower a* value) and more brown (lower b* value). With increasing storage time, the color values decreased more, which resulted in a higher color difference to the control. For week 5 and 6 smaller color differences were observed, which could be explained by statistical differences. After 9 weeks of storage, a color change of 8.20 was calculated, which was a significant color change. The highest difference between the control and the thermally treated juice in week 9 was the a* and b* values indicating a less red/orange and browner color of the carrot juice. Liao et al. (2009) studied the color difference of carrot juice after thermal sterilization at 110 °C for 15 min. After 4 weeks storage at 25 °C, the color difference was 2.31 and increased to 4.94 after 8 weeks. Compared to the values obtained in this study, higher color differences were obtained. This could be attributed to the type of carrot juice that was used. In the study of Liao et al. (2009) the pure carrot juice was analyzed, whereas in this study commercial juice containing sugar and citric acid was used. Moreover, the conditions for thermal treatment were both in the range of a sterilization, but not the same, which could have influenced the color.

Table 4-24: Color measurement (L*, a* and b* values) and color difference (ΔE) of thermal (121 °C, 45 s) and PEF (198 kJ/kg, 9 kV/cm) treated in comparison to untreated carrot juice.

Sample	Time [weeks]	L*	a*	b*	ΔE
Control		41.81 ± 0.30	17.30 ± 0.16	21.25 ± 0.33	
	0	41.37 ± 0.40	16.62 ± 0.17	21.16 ± 0.35	0.81

Thermal treatment	1	41.67	±	0.04	16.55	±	0.02	20.75	±	0.06	0.91
	2	40.21	±	0.03	16.28	±	0.01	19.23	±	0.03	2.76
	3	38.73	±	0.21	16.05	±	0.30	19.65	±	0.60	3.68
	4	40.45	±	0.30	14.90	±	0.10	17.99	±	0.20	4.26
	5	41.00	±	0.03	15.31	±	0.03	19.31	±	0.09	2.89
	6	40.28	±	0.09	16.43	±	0.06	19.37	±	0.15	2.57
	7	39.75	±	0.03	14.63	±	0.09	18.02	±	0.07	4.66
	8	39.10	±	0.12	13.53	±	0.19	17.18	±	0.22	6.16
	9	38.16	±	0.43	12.61	±	0.10	15.59	±	0.21	8.20
Control before PEF		42.16	±	0.05	17.42	±	0.10	21.30	±	0.12	
PEF	0	41.59	±	0.06	17.07	±	0.05	20.96	±	0.09	0.75
	1	40.57	±	0.34	17.66	±	0.19	21.63	±	0.32	1.64
	2	39.59	±	0.48	16.23	±	0.13	19.03	±	0.23	3.63
	3	41.57	±	0.24	17.14	±	0.07	20.76	±	0.14	0.85
	4	41.32	±	0.34	17.13	±	0.23	20.72	±	0.19	1.07
	5	39.59	±	0.17	14.30	±	0.11	17.36	±	0.11	5.65
	6	40.91	±	0.14	16.59	±	0.07	19.96	±	0.16	2.01
	7	40.86	±	0.10	16.52	±	0.05	19.88	±	0.09	2.12
	8	39.38	±	0.51	15.98	±	0.20	18.81	±	0.48	4.00
	9	39.78	±	0.42	16.15	±	0.17	19.07	±	0.36	3.50

During storage of the PEF treated juice an increase in color change was detected. At week 2 and 5 high values for color difference were detected, which can be explained by statistical difference. The color measurement was performed 5 times with 1 sample. For further investigations more samples should be analyzed. However, up to week 4 a slight color change, which is visible for a trained eye was observed with a ΔE of max. 1.64 neglecting the outliners. In week 6 and 7, the color change increased up to 2.12 followed by a further increase up to 3.50 at week 9. The color difference at the end of the storage period was significant, but not strong. Compared to the color change after thermal treatment, the color differences observed after PEF treatment were smaller than after thermal treatment. This can be demonstrated by calculating the color difference between PEF and thermal treated juice at the same stage of storage. Directly after treatment and at the beginning of the storage, the color difference was less than 1, which means that the color difference was not visible. With increasing storage, the color difference increased up to a maximum of 5.23 after 9 weeks indicating a high color difference between PEF and thermal treated carrot juice. Therefore, less color change can be achieved by using PEF for preservation of carrot juice.

Carrots are important for human health due to their nutrients. They contain antioxidants, which are able to neutralize free radicals causing oxidative damage to lipids, proteins and nucleic acid (Wang et al. 1996). The antioxidant potential may be from compounds, such as vitamin C, vitamin E, β-carotene or also phenolic compounds, like flavonoids (Cao et al. 1996). The method for determination of antioxidant capacity (section 3.5.2) is based on measuring ascorbic acid equivalents, due to the use of ascorbic acid as standard. The antioxidant capacity was analyzed over 9

weeks of storage at ambient temperature (Figure 4-50). The initial AAE before PEF treatment was 201.1 ± 16.8 mg/l. After PEF treatment, AAE increased up to 259.2 mg/l followed by a decrease to 227.9 mg/l after 1 week of storage. At week 2, the AAE increased up to 280 mg/l. This antioxidant capacity remained constant for the rest of the storage time. Only after 3 weeks of storage, a slight decrease to 259 mg/l was observed, but it must be noted that the standard deviation was high.
The initial AAE before thermal treatment was 237.7 ± 3.4 mg/l. Due to the thermal treatment, the AAE decreased directly after the treatment to 177 mg/l. The decrease in free radical scavenging activity after thermal treatment was also reported by Dede et al. (2007). They analyzed antioxidant capacity of carrot juice after thermal treatment at 80 °C for 1 min and observed a decrease. With increasing storage, the AAE increased up to a maximum of 288 mg/l after 4 weeks of storage. After week 4, a slight reduction in AAE was observed. These results demonstrate that the most changes occurred in the first three weeks of storage. The results of PEF treated carrot juice showed directly after PEF treatment no significant change in AAE, but a decrease after 1 week. After week 4, the antioxidant capacity of PEF was slightly higher than the thermal treated juice.
Comparing the different initial AAE, a higher AAE was observed for the thermal treated control. The difference between the control sample for PEF and thermal treatment was the freezing/thawing process. Ancos et al. (2000) reported a decrease in antiradical efficiency of raspberries after freezing by liquid nitrogen at -80 °C from 26 to 12 %. The obtained differences in the study of Ancos et al. (2000) and the performed analysis in this study are not comparable, as the methods of analysis are different and the product differs totally. However, the lower AAE of the sample before PEF treatment can be explained by the freezing process.

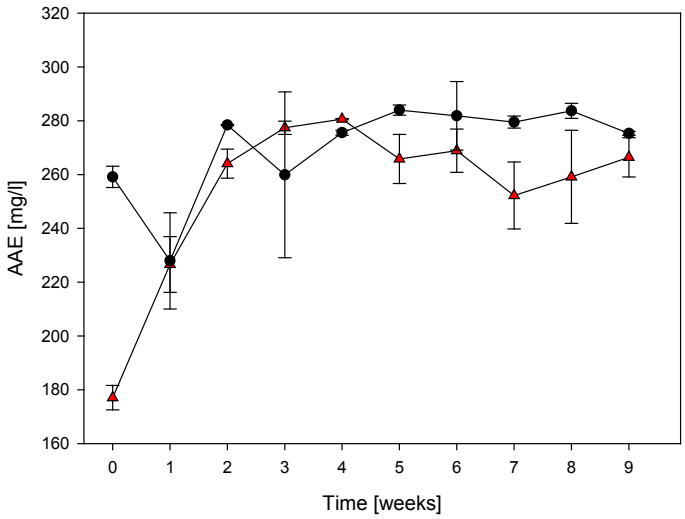

Figure 4-50: Antioxidant capacity (ascorbic acid equivalent (AAE)) of thermal (▲, 121 °C, 45 s) and PEF (●, 198 kJ/kg, 9 kV/cm) treated carrot juice over storage time of 9 weeks at ambient temperature.

Phenols are involved in the antioxidant activity (Wang et al. 1996). The total phenol content was measured over storage of 9 weeks at ambient temperature after thermal and PEF processing. The unit of total phenol content is GAE (gallic acid equivalent), based on the use of gallic acid as standard (section 3.5.2).

The initial GAE before thermal treatment was 305.68 ± 4.2 mg/l. Directly after the thermal treatment, an increase in GAE up to 324.02 mg/l followed by a decrease to 283.2 mg/l was observed. Patras et al. (2009) studied the phenol content in carrot juice after thermal sterilization. The GAE decreased from 173.3 to 99.23 mg/l after treatment at 121 °C for 3 min. Compared to the results obtained in this study, a lower initial GAE was detected and the decrease was higher. Between week 1 and 8, the GAE was more or less constant around 300 mg/ml. After week 8, a decrease in GAE to 271.5 mg/l in the thermal treated juice was obtained. Starting at week 7, the total phenol content of the PEF treated juice decreased from 357.1 to 262.1 mg/l. Directly after the PEF treatment, the GAE decreased from 208.18 to 165.4 mg/l. After 1 week, the GAE increased up to 319.1 mg/l. With increasing storage, the GAE was almost constant with statistical variations. Caminiti et al. (2012) studied the total phenol content of orange-carrot blend, which was filtered orange and carrot juice in a ratio of 1:1 (v:v). After PEF treatment, using an electric field strength of 24 kV/cm and 89 µs treatment time resulting in an outlet temperature of 49 °C, the GAE was 516 compared to 505 for the control sample. The observed decrease in this study can be explained by the higher outlet temperature and the therefore more severe treatment conditions. Moreover, the product in the study of Caminiti et al. (2012) contained orange juice, which had an additional impact on the total phenol content.

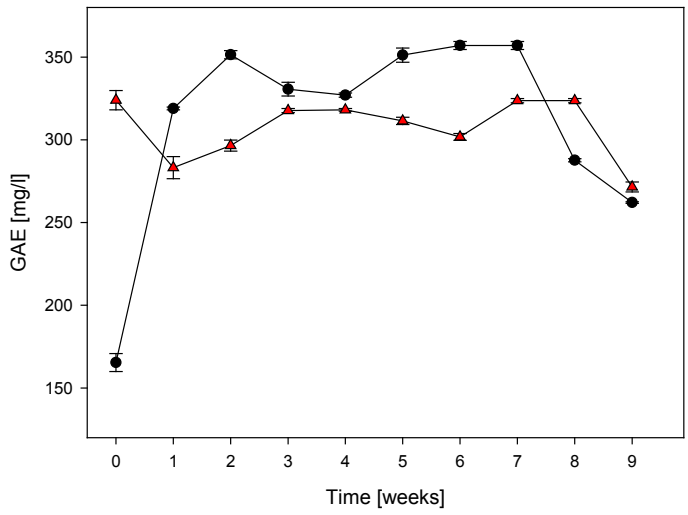

Figure 4-51: Total phenol content (gallic acid equivalent (GAE)) of thermal (▲, 121 °C, 45 s) and PEF (●, 198 kJ/kg, 9 kV/cm) treated carrot juice over storage time of 9 weeks at ambient temperature.

Comparing the initial GAE values for thermal and PEF treatment, a difference of 97.5 mg/l was observed, which can be explained by the freez/thaw process of the PEF treated sample. It is stated in literature, that for example the freezing of Rhododendron leaves causes a decrease in phenols (Chalker-Scott et al. 1989). Other studies analyzing the phenol content after short and long term (12 months) freezing of raspberries reported no change in GAE due to freezing process (Ancos et al. 2000).

Directly after the PEF treatment the GAE was lower compared to thermal treatment. After 1 week storage, the GAE of the PEF treated juice increased up to a higher value than thermal treated juice. Due to the freez/thaw step and the PEF treatment, the total phenol content was reduced, but recovered after 1 week of storage. The inactivation of vegetative microorganisms by PEF in juices, showed no change in total phenol content (Elez-Martínez et al. 2007; Odriozola-Serrano et al. 2008). Odriozola-Serrano et al. (2008) studied the phenol content in tomato juice after PEF treatment at an electric field strength of 35 kV/cm and 8269 kJ/l. The temperature was kept below 40 °C. After storage of 56 days at 4 °C, no change in total polyphenol content was detected. Compared to the analysis performed in this study, the polyphenol content was constant until week 7. The difference in results of both studies can be explained by the product, the storage conditions and mainly the treatment conditions. The aim of the presented study is inactivation of spores, which requires a more severe treatment than inactivating vegetative microorganisms by PEF.

In summary, during storage the PEF treated juice showed a higher GAE than the thermal treated juice. In both cases, a decrease in total phenol content was observed at the end of the analyzed storage time.

Within this section, the quality of the PEF and thermal treated carrot juice was compared over shelf life of 9 weeks. As a result, no changes in pH and soluble solid content were detected over the time. The °Brix of 10.2 and pH of 4.9 was not significantly changed by the thermal and PEF process. Regarding the color, no massive differences were observed after storage of 1 week. During the shelf life, the color changed to browner. The thermal treated juice showed a higher color difference, which means the color was better retained after PEF treatment. The brown color is partly based on oxidation reaction, where enzymes are involved. These oxidases are also involved in inactivation of polyphenols (Oriozola et al. 2008). Consequently, based on the results of color measurement and the higher color retention of the PEF treated juice, less enzyme reaction is expected for PEF treated juice compared to thermal treatment. Therefore, a higher polyphenol content can be expected, which was experimentally shown, after PEF treatment. At this stage the Maillard type reactions need to be mentioned, which might influence the color of the juice. The

darker color of the thermally treated juices might be due to the Maillard reaction. However, polyphenols are compounds involved in antiradical scavenging activity. Thus, a higher polyphenol content should result in a higher antioxidant capacity, which was observed after week 5. In summary, the PEF treated carrot juice had a higher quality in terms of color retention, polyphenol content and antioxidant capacity.

Moreover, the results demonstrated the influence of freezing on the polyphenol and antioxidant capacity. For further trials, the freezing step should be avoided in order to allow a facilitated comparison to thermal treatment as a reference.

4.7 Industrial application of PEF process regarding scale up, safety guidelines and costs

The demonstrated results indicate a possible application of PEF process for inactivation of bacterial endospores. The analysis of the influence of product parameters (section 4.2) showed an application of the developed process to a wide range of products. In this chapter, the scale up, required safety guidelines for the implementation of a HACCP plan and operation costs will be discussed.

The trials in this study were performed in pilot scale with a flow rate of 30 l/h. The equipment used had a maximum capacity of 200 l/h. For higher capacities, different equipment with a higher power have to be used. An overview of available PEF systems and their capacities is presented in Table 4-25.

Table 4-25: Overview existing PEF systems for pilot and industrial scale (na - no information available).

Manufacturer	Average power [kW]	Max. Voltage [kV]	Max. Current [A]	Max. Flow [l/h]	Reference
Divtec	1 - 125	20 - 400	100 - 500	10.000	http://www.divtecs.com/
Elea	30 - 80	25 - 30	200 - 3.000	200 - 5.000	http://www.elea-technology.eu/
KEA TEC	21	50	na	1.000	http://www.kea-tec.de/

Maxwell	150 - 2.000 GW	na	na	na	
Nutri-pulse	na	na	na	na	http://www.innovation-xl.com/en/home.html
PurePulse	16 - 50	40		1.800	http://www.purepulse.eu/
Scandinova	0.4 - 90	450	1.000	na	http://www.sc-nova.com/
Steribeam	3	20 - 30	na	1 l batch	http://www.steribeam.com/

In order to analyze the scalability of the developed process, trials with a flow rate of 50 and 100 l/h were performed (Figure 4-52). By increasing the flow rate, the residence time decreases, which can be seen in Figure 4-53. To apply the same energy input at a high flow rate, the frequency has to be increased. At a flow rate of 30, 50 and 100 l/h the residence time is 0.188, 0.113 and 0.057 s. The frequency to achieve an energy input of 197 kJ/kg varies from 78 Hz at 30 l/h to 306 Hz at 100 l/h. In theory, the same inactivation should be achieved at constant energy levels independent from the flow rate. The results in Figure 4-52 indicate a lower inactivation at higher flow rates. Applying an energy of 197 kJ/kg, an inactivation of 4.4, 0.7 and 0.2 log at 30, 50 and 100 l/h was achieved. The reason for this might be due to energy loss by the switches. At high frequencies, the switches open and close quite fast, which results in a loss of energy. Therefore, it can be assumed, that the energy delivered at high flow rates is lower than indicated by the system. Moreover, due to the lower residence at higher flow rates, the temperature holding time was reduced. As a result of the separation of total inactivation into thermal and PEF induced inactivation (section 4.3.3.1), a thermal inactivation rate for *B. subtilis* spores was observed. Although, the thermal inactivation was lower compared to the PEF induced inactivation, it has to be considered and might influence the inactivation at higher flow rates. At lower temperature holding times, less thermal inactivation is induced and therefore a lower total inactivation at higher flow rates can be assumed. In conclusion, the lower inactivation at higher flow rates is based on the lower delivered energy at high frequencies and the lower temperature holding time during the process.

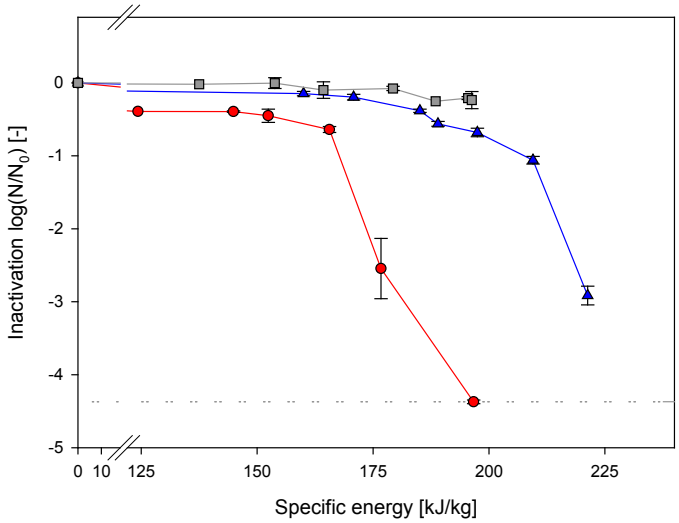

Figure 4-52: Inactivation of *B. subtilis* spores in Ringer´s solution (4 mS/cm, pH 7) by PEF treatment with an electric field strength of 9 kV/cm and an inlet temperature of 80 °C at a flow rate of 30 (●), 50 (▲) and 100 (■) l/h; detection limit (—·—).

However, the obtained results demonstrate that an inactivation of spores can be achieved at higher flow rates, but the parameters, such as frequency, have to be considered. Moreover, a safety validation should be performed in order to ensure the correct treatment. The studies performed in literature were mostly performed with a batch system (Yonemoto et al. 1993; Marquez et al. 1997; Uemura et al. 2003) or on lower lab scale (Bermúdez-Aguirre et al. 2012). Different parameters, such as gap distance and frequency, have to be considered and adapted to the scaled up system. Based on the availability of scale-up PEF system, a spore inactivation by PEF in combination with thermal energy can be achieved.

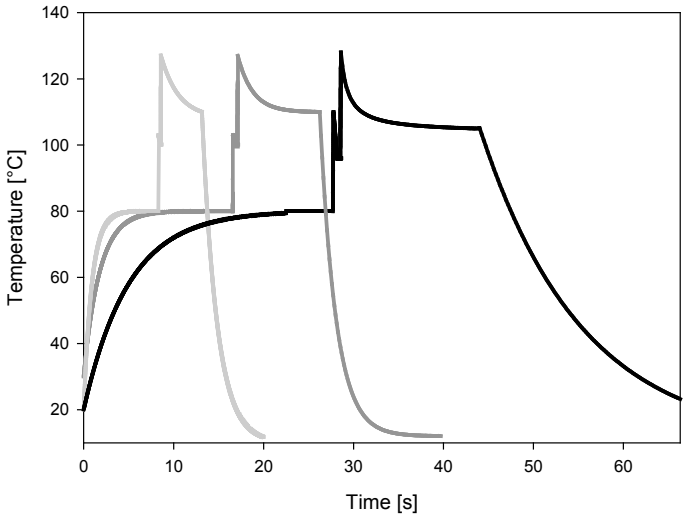

Figure 4-53: Temperature time profile of PEF treatment at 30 (—), 50 (—) and 100 l/h (—) applying an energy input of 197 kJ/kg and an electric field strength of 9 kV/cm.

Nowadays, quality assurance (QA) plays an important role in food production. The aim is to produce products with a constant high quality and therefore prevention of deviation from given quality specifications. The main principle of QA is process control, where a Hazard Analysis Critical Control Point (HACCP) concept can be considered, which is currently the most effective tool for QA (Bockelmann et al. 1998). The requirement for a quality system is outlined in ISO9000. It offers the possibility of certification of a documented quality system of total verification of processes, procedures, quality specifications and suitable organization. Tools for the quality system are Good Manufacturing Practices (GMP), Quality Assurance Control Point (QACP) and HACCP. At the moment HACCP is the most powerful tool for QA. The US Food and Drug Administration (US FDA) published the implementation of HACCP for fish industry as a mandatory action as well as in juices. As HACCP concept is a tool to control production processes and to guarantee publics health's, more countries will render HACCP. The HACCP concept was developed in the 1960s by Pillsbury Company to ensure the food safety and quality (Ropkins et al. 2000). Hulebak et al. (2002) stated the central goal of HACCP as *"establishing targets or standards, innovation and changes will be stimulated to reduce the risk from all sources of foodborne hazards—biological, chemical, and physical—while simultaneously providing a tool for holding establishments accountable for achieving acceptable levels of food-safety performance".*

The implementation of PEF system requires integration into the existing HACCP concept. Therefore, the hazards, which can be compounds or substances leading to harm, and Critical Control Points (CCPs) have to be identified. To simplify the hazards, they can be grouped and separated into physical, chemical and biological hazards (Bockelmann et al. 1998). Foreign material in the product is an example of a physical hazard. A chemical hazard could be contamination of the product by disinfection reagents or pesticides. A biological hazard is the contamination with spoilage or even pathogenic microorganisms. To eliminate or reduce a hazard, CCPs, which can be stations, operations or processes, have to be identified. To control the CCPs, they have to be monitored in order to allow a stop of production, when the critical limit is exceeded. For the PEF process, three main CCPs were suggested by Toepfl et al. (2014) (Figure 4-54). A biological hazard is the contamination of the product with spoilage microorganisms. To reduce the level of contamination, a constant PEF treatment is required. Therefore, the process parameters outlet temperature, specific energy, electrical resistance and flow rate should be controlled. The outlet temperature is a function of specific energy, specific heat capacity and inlet temperature (Equation 13). As the outlet temperatures are higher than 100 °C, pressure is required. A variation in inlet temperature or specific energy would cause a change in outlet temperature and may result in insufficient treatment. The flow rate should be controlled as well, because a change in flow rate causes changes in

residence time and pulse number. To check if the product is pumped through the PEF equipment, the resistance, which is the reciprocal of conductivity, should be measured. After production, a Clean in Place (CIP) procedure should be performed to clean the system properly.

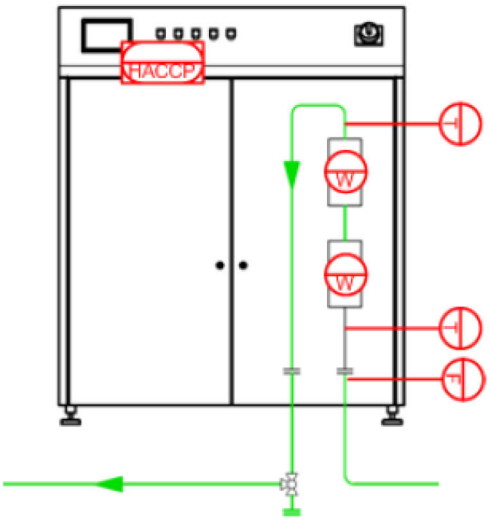

Figure 4-54: Critical Control Points (CCP) for operating PEF system (F- flow rate, T – temperature, W – specific energy input).

Based on previous trials a critical limit can be given, which allows variations of the stated parameter in a given range. The outlet temperature, for example, should be set as a critical control point to monitor, if the specific energy applied, is lower than the given limit, the product is unacceptable, as the product has not been processed correctly. An outlet temperature above the critical limit is unacceptable, because of unsatisfactory product quality. The corrective action in this case would be to reprocess the product at the correct settings. The monitoring and verification of these process parameters are critical in order to guarantee production of safe and high quality products.

So far, the studied PEF process can be implemented in industry, as larger systems are available. The operation costs for the process were calculated for the conditions used in this study and a higher capacity of 1000 l/h (Table 4-26). Working with a flow rate of 30 l/h and a daily production of 20 h, 132 t (metric tons) of product within one year with 220 working days can be produced. This specific PEF system works with a power of 1.8 kW, which results in energy cost of 0.7 ct/l assuming 0.12 €/kWh. An increase in capacity causes a reduction in energy costs to 0.3 ct/l.
For cost calculation of the complete process, the operation costs for pre heating and cooling have to be included. For heating up, various pieces of equipment can be used, which were mainly classified as direct and indirect heating (Fellows 2009). The direct systems are steam infusion and

steam injection, where steam is used to heat up the product. Due to limitations in particle size, low heat recovery and high operation costs, direct systems are unsuitable for combination with PEF processes. With indirect heating systems, the product has no contact with the heating medium, which allows a higher heat recovery. The main equipment configurations for indirect heating systems are plate and tube-in-tube heat exchangers. The advantage of a plate heat exchanger is the high efficiency in energy with a heat recovery of approx. 90 % and high flexibility in terms of production rate. There are limitations if the product contains particulates and/or has a high viscosity. For products with particles, the tube-in-tube exchanger can be used, which is less energy efficient when compared to the plate heat exchanger (Bockelmann et al. 1998).

The power required to heat up the product from 4 to 80 °C with capacity of 30 l/h is 2.53 kW. Including a heat recovery of 80 % and the cooling, the total energy usage is 1.06 kW, which results in energy costs of 0.43 ct/l. Adding the energy costs for pre heating and cooling to the energy costs for the PEF system, the total energy costs for the complete PEF process are 1.15 ct/l.

For a thermal treatment, the product is heated from 4 to 130 °C. The process conditions in terms of capacity and heat recovery were the same as for the PEF process in order to allow a comparison. The resultant energy costs are 0.71 ct/l.

Table 4-26: Operation costs for PEF process and thermal treatment with a capacity of 30 and 1000 l/h assuming 0.12 €/kWh.

	PEF treatment		Thermal treatment	
	5 kW (30l/h)	30 kW (1000 l/h)	30 l/h	1000 l/h
Power [kW]	1.8	25	3.53	117.6
Conductivity [mS/cm]	4	4	4	4
Inlet temperature [°C]	80	80	4	4
Outlet temperature [°C]	-	-	130	130
Heat recovery [%]	-	-	80	80
Specific heat capacity [kJ/kg K]	4	4	4	4
Daily production [h]	20	20	20	20
Operation per year [h]	4400	4400	4400	4400
Flow rate [l/h]	30	1000	30	1000
Production per year [t]	132	4400	132	4400
Energy costs per year [€]	950.4	13200	1862.8	62092.8
Energy costs PEF[€/kg]	0.007	0.003	-	-
Energy costs pre heating cooling [€/kg]	0.004	0.004	-	-
Total energy costs [ct/kg]	1.15	0.73	0.71	0.71

Comparing the energy costs of both processes, the costs for thermal process are lower at a capacity of 30 l/h. Although the energy costs for a thermal process are lower, the product is exposed to a higher temperature, which causes a higher loss of nutrients and therefore loss of quality. By applying PEF, a lower temperature is achieved while ensuring the microbial safety. A way to reduce the energy costs is the increase of inlet temperature, which results in a reduction of power for the PEF treatment. In this case the costs for electrical energy, which were higher than the thermal energy, are reduced and the costs for thermal energy increased. An increase in pre heating up to 90 °C, results in an increase in thermal energy costs up to 0.48 ct/l. This study (section 4.1) and results in literature (Toepfl et al. 2007) demonstrated a reduction in energy at higher inlet temperatures. A decrease to 1.2 kW leads to reduction in energy costs to 0.48 ct/l resulting in a total energy cost of 0.96 ct/l compared to 1.15 ct/l at an inlet temperature of 80 °C and a power of 1.8 kW. The costs for PEF treatment are widely discussed in literature. Heinz et al. (2003) studied the reduction in energy and therefore energy costs by making use of the synergism between PEF and inlet temperature. A reduction in energy and therefore operation costs can be achieved by using the heat of the treated product to pre heat the untreated product prior to PEF treatment. The power consumption for pasteurization applying an energy of 50 kJ/kg is 13 kWh per ton of product (Toepfl et al. 2006). For sterilization a higher energy input is required and therefore a power consumption of 40 kWh per ton product results. However, at higher capacities, the costs for PEF process are comparable to the energy costs for the thermal treatment.

5. Conclusion and outlook

Pulsed electric field technology is widely studied in the area of cell disintegration of solid material, such as grapes or sugar beets (Siemer et al. 2012), and inactivation of vegetative cells in products, such as juices or smoothies (Toepfl et al. 2005). For extension of shelf life of fresh juice, the process is already used in industry (Irving 2012). For inactivation of bacterial endospores by PEF, only limited information is available in literature. Therefore, the focus of the presented research was the performance of a basic study for inactivation of bacterial endospores by PEF in combination with thermal energy.

The influence of process and product parameters on the inactivation of *B. subtilis*, *A. acidoterrestris* and *G. stearothermophilus* spores was analyzed. To facilitate the comparison between the spore types and the influence of the parameters, the experimental data were modeled. Due to occurrence of a shoulder, which is termed as initiation and a linear part of the inactivation data, the model developed by Geereard et al. (2000) was used. The resultant models indicated a good precision. Further work could be done by applying and validating the developed models. Food products with similar product characteristics as analyzed should be tested and compared to the model.

However, the analysis of the impact of process parameters indicated the inlet temperature as the main influencing factor followed by the electric field strength. By increasing the inlet temperature, the energy required for a specific inactivation can be reduced (Siemer et al. 2014a). For the electric field strength, only limited effects could be shown. A broader range, analyzing electric field strengths of more than 12.5 kV/cm should be tested in order to confirm the obtained results. Besides the influence of processing parameters, the influence of product parameters (pH, sugar, fat and salt concentration) on the inactivation of bacterial spores was analyzed.

The salt concentration was modified by using different concentrations of Ringer´s solution varying from 1 to 15 mS/cm. High conductivities are expected in the area of soups and lower in juices. As an example, the conductivity of Gazpacho soup is 8.82 mS/cm (Elbers et al. 2012) and orange juice a conductivity of 4 mS/cm (Elez-Martínez et al. 2005). However, the conductivity has an impact on the applied voltage and on the sensitivity of spores to the PEF treatment. Due to the link of the conductivity to the impedance of the treatment chamber and the resulting impact on the matching of the pulse generator and treatment chamber, it was not possible to reach high electric field strength (> 6 kV/cm) using Ringer´s solution with a conductivity of 15 mS/cm. The resulted low electrical resistance of circuit ranged a higher peak current. Depending on the generator setup a mismatch may occur or the pulse voltage has to be adapted. Due to limitation on peak current, the voltage has to be reduced when treating media with high conductivity. Therefore, salt may

have an influence on the treatment intensity, which has an effect on the inactivation of spores. In this study, no inactivation of *G. stearothermophilus* spores could be achieved in Ringer´s solution with a conductivity of 15 mS/cm and an electric field strength of 4 kV/cm. An increase in electric field strength enabled an inactivation, but required a higher applied pulse voltage and higher peak current. Therefore, a system with a higher maximum peak current should be used.

The salt can also affect the spores in order to increase or decrease the sensitivity to the PEF treatment. The results demonstrated a higher resistance of the spores when suspended in media with high salt content. The presence of salt might have an effect on the stability of the spore envelope, which causes a reduction in water activity and an increase in spore resistance.

The product parameter pH may have an influence on the inactivation of spores by PEF. The obtained results showed only a slight effect of the pH, which means less energy for an inactivation at pH 4 compared to pH 7. For further analysis at pH lower than 4 should be tested in order to check if the effect is increasing with decreasing pH. Moreover, it should be clarified in what way the pH is affecting the spores.

An important ingredient in most soups is fat. Because soups are potential products for the process of spore decontamination, the effect of fat on the inactivation of spores by PEF was analyzed. Within the analyzed fat levels of 5 and 10 % fat, no significant effects on the energy required for a specific inactivation at higher fat levels was observed. An effect would probably occur when analyzing higher fat contents. As an example, the thermal resistance is increasing when spores are suspended in pure fat (Molin et al. 1967; Senhajii et al. 1977; Ababouch et al. 1995). Nevertheless, the level of fat in soups or even juices does not exceed 10 % (anonymus 2014). Therefore, the fat did not affect the intensity of the PEF treatment for inactivating bacterial spores in the range of liquid food that this investigation was focused.

Furthermore, only a slight effect of sugar on the inactivation of spores by PEF was detected in the study. The sugar level in the model solution was increased up to soluble solids content of 10 °Bx. Within this range, the differences in energy for specific inactivation of spores are low. Therefore, products with a sugar level up to 10 % can be treated by the developed process without any significant changes in energy level.

By using a model solution, it was possible to analyze the effect of single product parameters on the inactivation of spores by PEF. At this stage, limited effects of increasing or decreasing the resistance to PEF were observed. The combination of the parameters, which means model solutions containing fat and sugar, might give different results. It is generally accepted, the more complex the media, the higher the resistance of spore to an inactivation process (López et al. 1996; Jagannath et al. 2003; Chmal Fudali et al. 2011). Therefore, the inactivation of spores in food with for

example the same fat content as analyzed in this study, might require a higher energy input than the one observed in this study. Moreover, the spores were prepared under lab conditions and added under controlled conditions to the product. The resistance of spores is partly given by the sporulation temperature. For thermal resistance, a higher resistance was observed when spores sporulated at higher temperatures (Condon et al. 1992; Sala et al. 1995; Palop et al. 1996). The results of this study also indicate an effect of sporulation temperature on the PEF resistance. A higher energy input is required when spores are sporulated at higher temperature. Spores formed in natural conditions might have a different resistance (Abel-Santos 2012) compared to spores sporulated under controlled conditions in a lab. Therefore, it is necessary to validate the obtained results with a naturally spore contaminated food product. A comparison of energy requirement for inactivation of artificial or natural spore contamination will indicate the differences in resistance of spores to the developed PEF process. Consequently, the performed analysis of effect of product parameters on the PEF treatment, demonstrated the required change in process intensity for the inactivation of spores, but a trial with a food product and natural spore contamination is essential to validate the obtained results.

The developed process for spore inactivation by combined PEF and thermal treatment, which means a higher inlet temperature compared to the PEF treatment for inactivating vegetative microorganisms was used. Moreover, the temperature was increasing during the treatment based on Joule heating. As the temperature is increasing up to more than 100 °C depending on the specific energy, the effect of temperature on the inactivation has to be considered. Therefore, a model combining the thermal inactivation kinetics of each spore and the temperature time profile of the PEF process was developed to quantify the thermal effect during PEF process (Siemer et al. 2014b). As a result, the inactivation of *B. subtilis* and *A. acidoterrestris* spores indicate a thermal inactivation part, which is lower than PEF induced inactivation. The inactivation of *G. stearothermophilus* is mostly based on PEF effect, but it has to be stated that the principle of PEF inactivation of bacterial spores is unclear. This model allows a separation of inactivation rate in PEF and thermal induced inactivation. How the temperature is involved in the PEF induced part and how PEF is affecting the spores is unclear. The presented SEM pictures demonstrated a different appearance of the PEF treated spores compared to thermally treated spores, which indicates that PEF acts differently than thermal treatment. The PEF spores appeared shrunken like the thermally treated ones, but additionally a layer was visible on the spores, which might be proteins. For the CLSM analysis to mark the protein, the PEF treated spores appeared red, whereas in the thermally treated sample only a few appeared red. The principle of thermal inactivation of spores is based on protein denaturation and enzyme degradation (Coleman et al. 2007). Based on the obtained

results in this study, the mechanism of spore inactivation by PEF might be different. It might be that enzyme or protein denaturation are involved somehow in the whole mechanism. Moreover, the rapid temperature increase by Joule heating is important. Trials performed in a batch system with mostly isothermal conditions indicated no inactivation of *B. subtilis* spores applying an energy input of 300 kJ/kg at an electric field strength of 25 kV/cm and a temperature of 90 °C. Results using continuous PEF system showed an inactivation of 4.4 log applying an energy of 9 kV/cm and an energy of 196 kJ/kg using an inlet temperature of 80 °C. This result demonstrated the importance of the fast temperature increase. It might be that the fast temperature activates the spore somehow in order to make the spore more sensitive to the PEF treatment. This activation might be a change in spore structure or affecting early stages of germination. The germination of spores is induced by nutrient or non-nutrient pathways. It might be that the high temperature or even the electric field triggers the germination, which makes them more sensitive to the treatment. Nevertheless, it has to be noted that PEF is not causing germination.

However, a possible way to analyze the effect of PEF on spores is the analysis of mutants with lack of important spore components by PEF. The effect of the coat, which is a layer composed mainly of proteins and small amounts of carbohydrates and lipids, was analyzed within this study. As a result less energy was required for an inactivation of the decoated spore compared to the wild type *B. subtilis* spore. Therefore, the coat can be regarded as a protection of the spore against the PEF treatment. Another component responsible for high resistance of spores is dipicolinic acid (DPA). 5 to 15 % of the dry weight of spores is DPA, which accumulates in the spore core with calcium and causes low core water content (Pedraza-Reges et al. 2012). By analyzing the effect of PEF on a mutant with a lack of DPA, the influence of low core water content can be evaluated (Paidhungat et al. 2000). Due to the fact, that the low core water content is the main reason for the high resistance of spores to wet heat (Pedraza-Reges et al. 2012), the effect of temperature during the PEF process has to be considered and evaluated. Moreover, mutants with a lack in α/β type small acid-soluble proteins (SASPs) can be analyzed to determine the effect of the developed PEF process on DNA. In the spore core, the DNA is saturated with α/β type SASP, which represents a protection of the DNA. Due to this protection, the spore has a higher resistance to radiation, chemicals and dry heat (Setlow et al. 1995). It is unlikely, that PEF directly acts on the DNA. In the area of inactivation of vegetative microorganisms, PEF is affecting the membrane and not the DNA (Hamilton et al. 1967; Weaver 2000). Therefore, it might be that PEF is affecting the structure of the spore, so that the induced high temperature can damage the DNA. It is reported, that spores have a special DNA repair system (recA) to repair damages on the DNA during outgrowth. Beside analysis with α/β type SASP mutant, trials with α/β type SASP/recA mutant should be performed,

in order to exclude possible repair of damaged DNA by recA. In summary, the use of different mutants with lack of important components can give an indication for principle of spore inactivation by PEF.

To validate the results obtained from the basic study on inactivation of spores by PEF, case studies were performed. The first study was performed on yeast extract. To enable an inactivation of *B. subtilis* spores, a higher dilution of the product as instructed had to be done based on the high conductivity. A 3.8 log inactivation of the target spore (*B. subtilis*) was achieved at 257 kJ/kg and 4.5 kV/cm resulting in shelf life of more than 68 days at ambient temperature. The shelf life is comparable to the one obtained for a thermally treated product (121 °C for 20 min). The thermal and PEF treated product indicated no changes in pH during storage and slightly better color retention of the PEF treated sample. Due to the high energy input, a high outlet temperature (144 °C) is induced. A reduction in temperature can be achieved by reducing the energy and increasing the electric field strength. A system with higher peak current or a higher dilution of the yeast extract has to be used in order to achieve this temperature decrease. If the product is used in a lower dilution, the application and/or the process after PEF process should be considered. A higher dilution, results in higher water consumption and higher energy demand, if the product is dried afterwards.

The second case study performed was the PEF treatment of carrot juice. The correct treatment intensity was adjusted by previous safety validation, which analyzed the inactivation of *B. subtilis* and *A. acidoterrestris* spores. When compared to thermally treated juice, the shelf life was more than 9 weeks at ambient temperature. The quality, espeacially the analysis of total polyphenols and antioxidant capacity indicated differences compared to the control and the thermally treated juice. A slightly higher total polyphenol content and antioxidant capacity over the storage was obtained. Moreover, after PEF treatment the color was better maintained compared to thermal treatment.

The case studies were performed in pilot scale and demonstrated the treatment of food product by the developed PEF process. Trials scaled up to 100 l/h indicated a higher energy demand for inactivation based on the required higher frequency in order to achieve the same energy. The reason for the higher energy was the loss in energy caused by the switches when operating at high frequencies. The energy loss by the switches could be reduced by using a system with a higher power. Moreover, as for *B. subtilis* and *A. acidoterrestris*, a thermal inactivation was mathematically obtained, the residence time has an influence of the energy demand at higher flow rates. However, PEF systems on a larger scale with higher intensity are available to allow operation at

higher energy levels. Nowadays, process control is an important tool for quality assurance in production. The key parameters for PEF processes are temperature (in- and outlet), flow rate, electrical resistance and energy control. To ensure a constant high quality product, these parameters have to monitored and controlled.

The focus of the study was inactivation of bacterial endospores by PEF in combination with thermal energy. The results demonstrated an inactivation of *B. subtilis*, *A. acidoterrestris* and *G. stearothermophilus* spores by the developed process depending on the applied conditions. Considering the product parameters, an inactivation of spores can be achieved by PEF at a lower heat load compared to thermal processes. Therefore, the process offers the possibility of producing products with higher quality while ensuring the microbial safety.

6. Appendix

Table 6-1: Comparison of yeast concentration in untreated, PEF and thermal treated sample over shelf life of 68 days, n.a. – not analyzed; RT – room temperature.

	Yeast count [cfu/ml] Storage at 4 °C				Yeast count [cfu/ml] Storage at RT		
	Day 0	Day 20	Day 50	Day 68	Day 20	Day 50	Day 68
Control	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	n. a.	n. a.
PEF 1 (244.1 kJ/kg)	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$4.7 \cdot 10^7$ ± $4.0 \cdot 10^6$
PEF 2 (257.0 kJ/kg)	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0
Thermal treatment (121 °C, 20 min)	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0

Table 6-2: Comparison of mold concentration in untreated, PEF and thermal treated sample over shelf life of 68 days, n.a. – not analyzed; RT – room temperature.

	Mold count [cfu/ml] Storage at 4 °C				Mold count [cfu/ml] Storage at RT		
	Day 0	Day 20	Day 50	Day 68	Day 20	Day 50	Day 68
Control	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	n. a.	n. a.
PEF 1 (244.1 kJ/kg)	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0
PEF 2 (257.0 kJ/kg)	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0
Thermal treatment (121 °C, 20 min)	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0	$9.0 \cdot 10^0$ ± 0

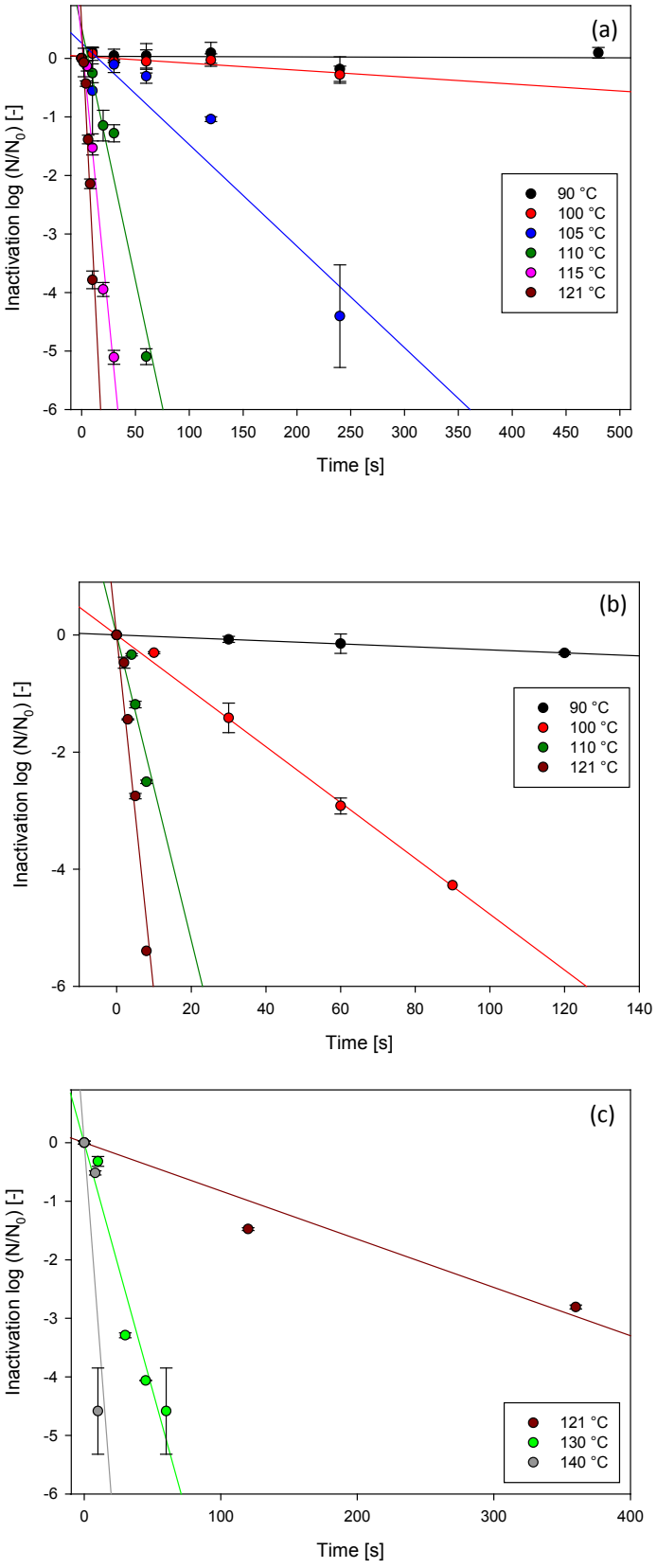

Figure 6-1: D value determination pH 4, (a) *B. sutilis*, (b) *A. acidoterrestris* and (c) *G. stearothermophilus.*

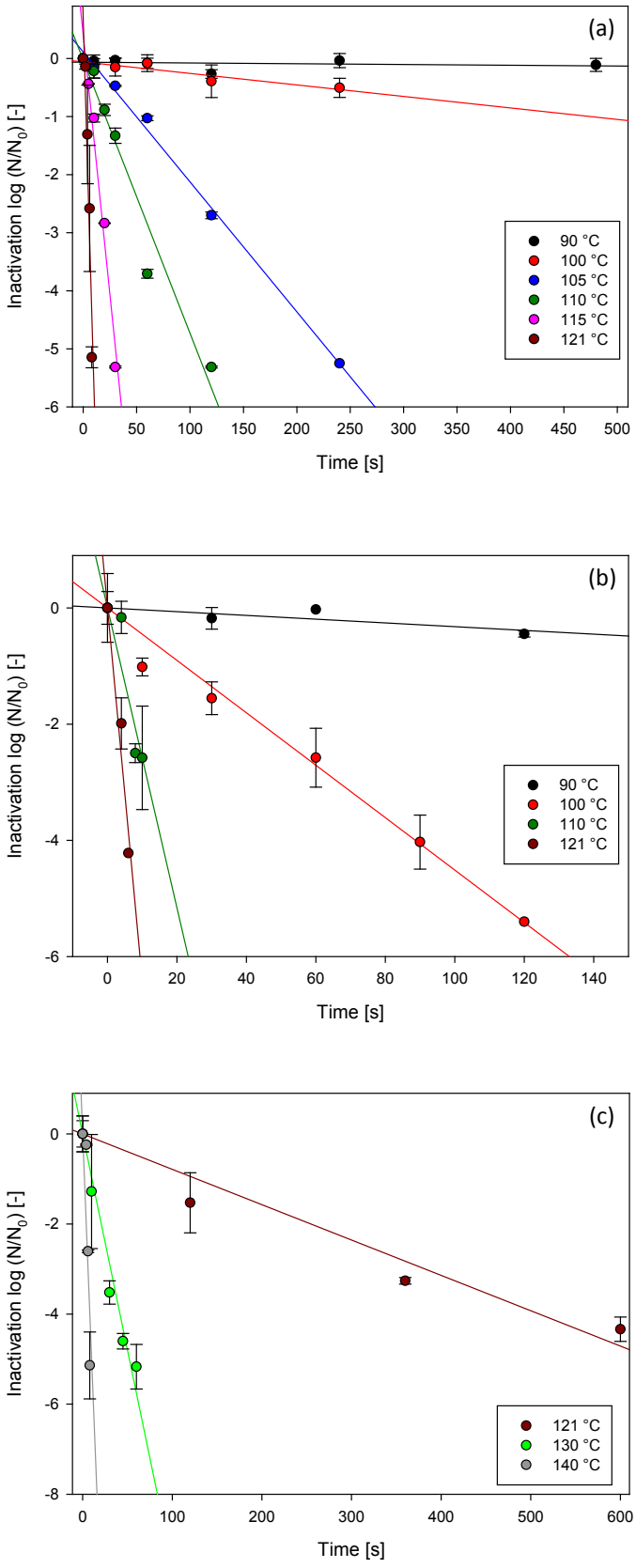

Figure 6-2: D value determination pH 7, (a) *B. sutilis*, (b) *A. acidoterrestris* and (c) *G. stearothermophilus*

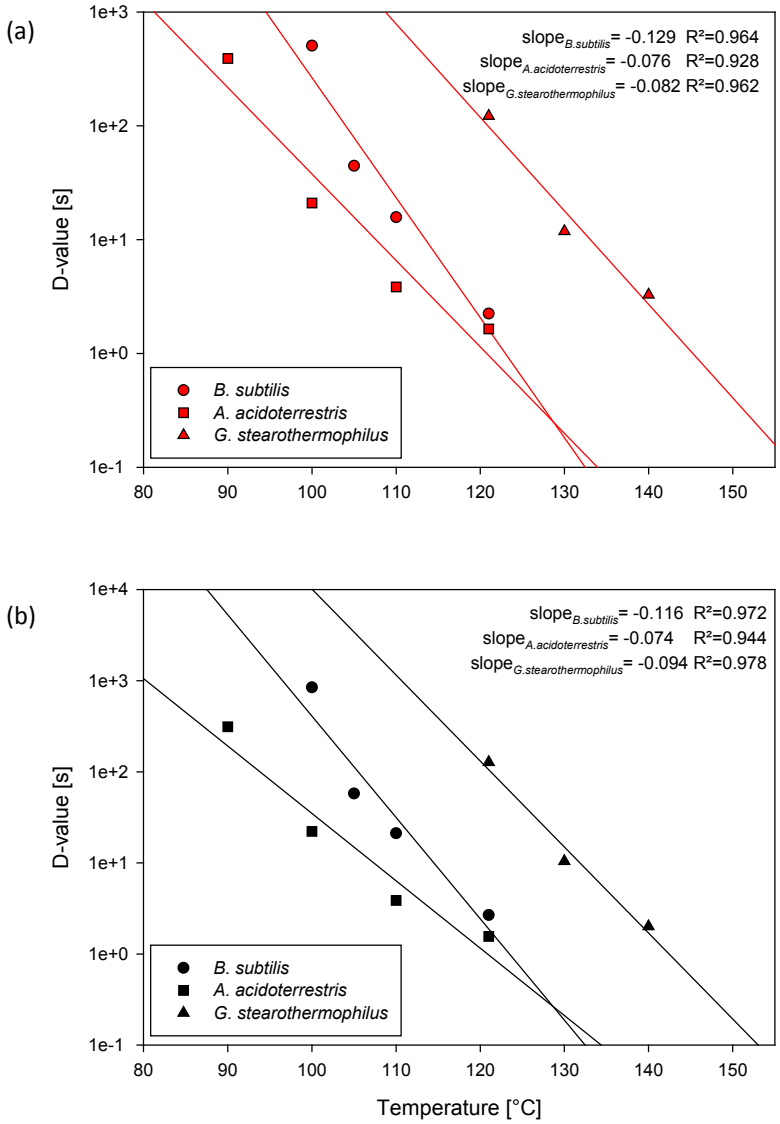

Figure 6-3: z-value determination (a) pH 4 and (b) pH 7.

Table 6-3: Coefficient for Equation 20 to calculate the temperature time profile for cooling phase of PEF process depending in specific energy and spore type.

				Coefficient equation 20			
	pH	Specific energy input [kJ/kg]	Inactivation $\log(N/N_0)$ [-]	a	b	c	r^2
B. subtilis	4	175	-1.3 ± 0.02	10.00	4662.41	5.80	0.999
		180	-2.5 ± 0.12	9.99	4776.38	5.80	0.999
		185	-2.9 ± 0.00	9.99	4760.58	5.80	0.999
	7	164	-0.6 ± 0.04	10.00	180755.48	5.80	0.999
		177	-2.8 ± 0.01	10.00	4646.70	5.80	0.999
		195	-4.4 ± 0.02	9.99	4690.00	5.80	0.999
A. acidoterrestris	4	116	-2.44 ± 0.07	9.99	4300.34	5.87	0.999
		123	-3.56 ± 0.60	10.00	4341.68	5.87	0.999
		137	-3.90 ± 0.00	9.99	163743.47	5.87	0.999
	7	123	-2.6 ± 0.15	10.00	4341.68	5.87	0.999
		137	-3.3 ± 0.00	9.99	163743.47	5.87	0.999
G. stearothermophilus	4	226	-2.01 ± 0.88	9.99	168768.62	5.87	0.999
		242	-2.78 ± 0.12	9.99	170578.52	5.87	0.999
		252	-4.35 ± 0.00	9.99	170578.47	5.87	0.999
	7	242	-1.15 ± 0.17	9.99	170578.52	5.87	0.999
		252	-2.24 ± 0.13	9.99	170578.47	5.87	0.999
		265	-3.38 ± 0.60	9.99	170578.47	5.87	0.999

Curriculum vitae and list of publications

Claudia Siemer, M.Sc.

RESEARCH AREA

- Operation and maintenance of pilot scale food processing equipment, such as High pressure as well as PEF equipments
- Involvement in different projects on European level, e.g. Hightech Europe (FP7-KBBE-2007-2A) and HST Food train (FP7-PEOPLE-2010-ITN)
- Organizing and managing parts of various national and international research projects as well as industrially funded projects resulting in better understanding of concepts, food products and processes
- Responsibility for training graduate, undergraduate students and summer interns
- Organization of workshops for research institutes and industrial companies
- Field of interest: pulsed electric fields processing, spore inactivation, non-thermal processing of food, knowledge transfer to industry

EDUCATION

08/2008 – 10/2010	Master of Science (M.Sc.), Biotechnology, TU Braunschweig Master thesis in cooperation with German Institute of Food Technology (DIL e.V.) and Nestlé Research Center Topic: Pulsed electric field pasteurization of bioactive milk based compositions Masterdegree: 1.3
10/2005 – 08/2008	Bachelor of Science (B.Sc.), Biotechnology, TU Braunschweig Bachelore thesis in cooperation with German Institute of Food Technology (DIL e.V.) Topic: Anwendung von gepulsten elektrischen Feldern zur Konservierung von Lebensmitteln Bachelordegree: 2.4

RESEARCH PROJECTS

Inaktivierung bakterieller Endosporen durch kombinierte Anwendung gepulster elektrischer Felder und thermischer Energie (16798 N). Project coordination. *German Federation of Industrial Research Associations*

Hightech Europe. WP 2 Coordination and writing technology data sheets. *EU FP7*

Science based precision processing for future healthy, structured and tasteful fruit and vegetable based foods: an integrated research and training program (HST Food Train). Coordination and assistance supervision of trainees. *EU FP7*

Phytosana. Decontamination of plant extract (Melisse) by nonthermal technologies. Coordination. *EU Interreg IVa*

Einsatz von Infrarot- und gepulsten Lichtsystemen zur Oberflächendekontamination von Schweineschlachttierkörpern (AiF 17877 BG). *German Federation of Industrial Research Associations*

ORGANIZATION OF TECHNOLOGY WORKSHOPS

Technology workshop for M&S including pocesses, such as PEF, HP and HPH (2012, 2013, 2014), Project leader

PEF demonstration workshops for students, such as University Geisenheim or Konrad Adenauer Stiftung

Summer lab “Special Food Technologies” (2011, 2012, 2013, 2014) in cooperation with University of Applied Science Osnabrueck, supervision of students for the “thermal processing project” and involvement in seminars

Training courses in PEF operation for companies

AWARDS

2nd Poster Prize iFood conference: Siemer, C., K. Elbers, S. Toepfl and V. Heinz. (Nov. 2011) Influence of pH value and conductivity on the inactivation of *B. subtilis* spores by PEF in combination with high temperature.

Received Scholarship from Technische Universitaet Carolo-Wilhelmina zu Braunschweig (June 2010) for outstanding performances

CO-SUPERVISION OF BACHELOR –AND MASTER THESIS

Inaktivierung von *Bacillus subtilis* Sporen durch gepulste elektrische Felder, Master thesis, University Osnabrueck

Inaktivierung bakterieller Endosporen durch die Anwendung von gepulsten elektrischen Feldern und thermischer Energie am Beispiel von Karottensaft – Analyse von Sicherheits- und Qualitätsparametern, Master thesis, University of Applied Science, Osnabrueck

Influence of PEF treatment on the quality of potatoes for the potato mash production, Research and development project, University of Applied Science, Osnabrueck

Application of Pulsed Electric Fields (PEF) in sweet potato juice processing, Master thesis, University of Applied Science, Osnabrueck

Inaktivierung ausgewählter Mikroorganismen in Orangensaft durch den Einsatz von gepulsten elektrischen Feldern und Citral, Bachelor thesis, University of Applied Science, Osnabrueck

Einsatz von PEF als alternatives Schälverfahren von Pfirsichen, Bachelor thesis, University of Applied Science, Osnabrueck

Gelatine removal in dairy desserts – Panna Cotta, Project report, University of Applied Science, Osnabrueck

LANGUAGES

German – native speaker

English – fluent

SCHOOL EDUCATION

07/1996 – 08/2005	Gymnasium Boizenburg Highschool with degree 1.7
07/1992 – 07/1996	Elementary School

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PUBLICATIONS

Presentation

Siemer, C. (2011) Pulsed electric field pasteurization of bioactive milk based compositions, Seminar: Food process design by innovative techniques, NRC, Lausanne

Siemer, C. (2011) Verlängerung der Haltbarkeit frisch gepresster Fruchtsäfte durch gepulste elektrische Felder – technische, kommerzielle und rechtliche Rahmenbedingungen, 2. Symposium DLG Frische Convenience/Fresh cut Produkte, Potsdam

Siemer, C. (2011) Produktionstechnologien zur Verlängerung der Haltbarkeit, Future of Food, Bernburg

Siemer, C. (2012) Einsatz von gepulsten elektrischen Feldern zur schonenden Haltbarmachung flüssiger Lebensmittel, Technologieworkshop FOOD FUTURE, Düsseldorf

Siemer, C. (2012) Pulsed Electric Field Application in Food Processing, Seminar, CNTA, San Adrian, Spanien

Siemer, C. (2012) Industrielle Anwendung von gepulsten elektrischen Feldern, 10. Fresenius-Fachtagung für Technik und QS "Abfüllung sensibler Getränke", Mainz

Siemer, C. (2012) Electroporation in Food, Electroporation based technologies and treatments, Ljubljana, Slowenia

Siemer, C., S. Toepfl, V. Heinz (2013) Inaktivierung *B. subtilis* Sporen durch die kombinierte Anwendung von gepulsten elektrischen Feldern und thermischer Energie, Jahrestreffen der Fachgruppe Lebensmittelverfahrenstechnik, ProcessNet, Quakenbrück

Siemer, C. (2013) Electroporation in Food and Biotechnology, iFood conference, Hannover

Toepfl, S., C. Siemer, K. Aganovic (2014) Höchstdruckhomogenisation als Möglichkeit zur Modifizierung der Funktionalität von Eiprodukten, DLG Symposium „Eier und Eitechnologie“, Celle

Siemer, C. (2014) How new preservation methods open up new markets. Flanders´ Food “Workshop innovatie in pasteurisatie van vloeistoffen - ism Food Pilot”, Melle, Belgium

Siemer, C., S. Toepfl (2014) Pulsed electric field application in food industry - Equipment design and commercial experience, COST TD 1104 Session, 37th International Convention on Information and Communication Technology, Electronics and Microelectronics, Opatija, Croatia

Poster

Siemer, C., M. Schlotmann, S. Toepfl, C. Hertel, V. Heinz (2011) Combination of PEF and citral as antimicrobial substance to inactivate *E. coli* and *L. plantarum* in orange juice, iFood Konferenz, Osnabrück

Siemer, C., K. Elbers, S. Toepfl, V. Heinz (2011) Influence of pH value and conductivity on the inactivation of *B. subtilis* spores by PEF in combination with high temperature, iFood Konferenz, Osnabrück

Siemer, C., J. Schmidgall, S. Toepfl, V. Heinz (2012) Chemical and functional properties of carrot mark and –rind after HP and PEF treatment in comparison to a thermal treatment, 6th International Food Factory Conference, Laval, France

Aganovic, K., C. Siemer, S. Toepfl, V. Heinz (2013) Microscopic analysis of *Saccharomyces cerevisiae* and *Listeria innocua* after PEF, HPP and temperature treatment in Ringer's solution, GDL Kongress Lebensmitteltechnologie, Dresden, Germany

Siemer, C., K. Aganovic, S. Toepfl, V. Heinz (2013) Quality improvement of pea mint soup by Pulsed Electric Field (PEF) processing, iFood Conference, Hannover

Aganovic, K., G. Saldaña, C. Siemer, S. Toepfl, V. Heinz (2013) Production of low fat emulsion using ultra high pressure homogenisation (UHPH) process, iFood Conference, Hannover

Book chapter

Siemer, C., S. Toepfl, V. Heinz (2012). Mass Transport Improvement by PEF - Applications in the Area of Extraction and Distillation. *Distillation - Advances from Modeling to Applications*. S. Zereshki, InTech**.**

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Siemer, C., M. Kiessling, C. Hertel and S. Toepfl (2011). Inaktivierung bakterieller Endosporen. Rundschau fuer Fleischhygiene und Lebensmittelueberwachung 6: 198-200.

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Patent

Mathys, A., S. Toepfl, C. Siemer, L. Favre, J. Benyacoub and C. E. Hansen (2013). Pulsed electric field treatment process and dairy product comprising bioactive molecules obtainable by the process. EP 2 543 254 A1.

Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die Dissertation selbständig verfasst habe. Alle benutzten Hilfsmittel und Quellen sind aufgeführt.
Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.
Veröffentlichungen von irgendwelchen Teilen der vorliegenden Dissertation sind von mir, wie in der vorstehenden Publikationsliste aufgeführt, vorgenommen worden.

Bersenbrück, 29.10.2014

Claudia Siemer

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