MODERATE AND HIGH INTENSITY PULSED ELECTRIC FIELDS

Effect on microbial inactivation, shelf life and quality of fruit juices

Rian Timmermans

Propositions

1. Electric field strength, temperature and pulse width are, in that order, the most important Pulsed Electric Field processing factors for microbial inactivation. (this thesis)

2. Moderate intensity PEF conditions constitute a better alternative pasteurisation process than high intensity PEF.
(this thesis)

3. The observed correlation between children living near fast food outlets and likeness to gain weight does not necessarily imply causation (Pearce et al. "Weight gain in mid-childhood and its relationship with the fast food environment" Journal of Public Health, 2017).

4. "When a measure becomes a target, it is no longer a measure" (Goodhart's law) is a valid argument in preferring Bayesian statistics above *p*-values to judge differences.

5. The energy in- and output during a PhD process could be characterised as a combination of different bipolar pulses.

6. Advertising for exclusive, secret destinations by tourist operators is a *contradictio in terminis*.

Propositions belonging to the thesis, entitled:

Moderate and High intensity Pulsed Electric Fields.
Effect on microbial inactivation, shelf life and quality of fruit juices.

Rian A.H. Timmermans
Wageningen, 19 January 2018

Moderate and High Intensity Pulsed Electric Fields

Effect on microbial inactivation, shelf life and quality of fruit juices

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Moderate and High Intensity Pulsed Electric Fields

Effect on microbial inactivation, shelf life and quality of fruit juices

Rian Adriana Hendrika Timmermans

Thesis

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List of symbols and abbreviations

a	initial fraction
a_w	water activity
A_F	distance from the centre in direction of the external field (m)
A_S	asymptotic value, $\log_{10}(N_{max}/N_0)$
c_p	specific heat capacity (J/kg·K)
cfu	colony forming units
d	diameter (m)
D_T	decimal reduction value (s)
E	electric field strength (V/m) (kV/cm)
e	Euler number, 2.71828
E_c	critical electric field strength (V/m) (kV/cm)
f	frequency (Hz)
$f(A)$	shape factor
$f(\lambda)$	factor for specific conductivity of suspending medium, cytoplasm, cell membrane, ratio of membrane thickness and cell radius
h	Planck's constant, $6.63 \cdot 10^{-34}$ Js
I	current (A)
k	reaction constant
k_b	Boltzmann constant, $1.38 \cdot 10^{-23}$ J/K
K	equilibrium constant of chemical reaction
l	length (m)
m	order of pH equilibrium
n	number of pulses or number of data points
N	number of cells or molecules
N_0	initial number of cells or molecules
N_0^*	initial number of cells after PEF treatment
N_{max}	maximum number of cells
o/n	overnight cultivation
$P_{electric}$	electric power (W)
p	number of parameters
R	gas constant, 8.3145 J/mol/K
R^2	determination coefficient
r	radius (m)
SSr	residual sums of squares
SS_{total}	total sum of squares
t	time (s) (min) (h)
T	temperature (°C) (K)
T_d	denaturation temperature (°C) (K)
T_0	temperature at standard conditions (K)
T_{in}	inlet temperature (°C)
T_{max}	maximum temperature (°C)
T_{out}	outlet temperature (°C)

Tc	characteristic temperature to inactivate 50% of population (°C)
Tr	reference temperature (°C) (K)
u	energy density per pulse (J/m^3)
U	voltage (Volt)
U_{max}	maximum voltage applied (Volt)
V	volume in treatment chamber (m^3) (L)
V_m	transmembrane voltage (V)
w	specific energy, electric energy (J/kg)
$\tilde{y}$	mean
Z	temperature increase needed to reduce the decimal value D_T or Tc by a factor 10

$\Delta^{\ddagger}G^{\Theta}$	change in activation free energy (J/mol)
ΔG_c^{Θ}	change in standard free energy at equilibrium (J/mol)
$\Delta^{\ddagger}G_{pH}^{\Theta}$	change in activation free energy to change pH (J/mol)
$\Delta^{\ddagger}H^{\Theta}$	standard activation enthalpy (J/mol)
ΔH^{Θ}	standard enthalpy (J/mol)
$\Delta^{\ddagger}S^{\Theta}$	standard activation entropy (J/K mol)
ΔS^{Θ}	standard entropy (J/K mol)

α	scale parameter
β	shape parameter
Δ	change in temperature, pH, energy, BIC etc.
φ	flow rate (m^3/s) (L/s) (L/h)
λ	lag time (h)
μ	mean
$\Delta\varphi_m$	transmembrane potential (V)
Θ	angle of the radial direction to the electric field (°)
ρ	density (kg/m^3)
σ	electric conductivity (S/m) or uncertainty in temperature distribution (°C)
σ_y	standard deviation
τ	pulse width (s)
τ_r	reference time (s) (min)
T_c	characteristic time for inactivation (s) (min)
μ_{max}	growth rate (1/h)

AA	Ascorbic acid
AIC	Akaike Information Criterion
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BIC	Bayesian Information Criterion
CBS	Centraal Bureau voor Schimmelcultures, Fungal Biodiversity Centre
CDF	Cumulative Distribution Function
DHAA	dehydroascorbic acid
FCJ	juice from concentrate
GPY	glucose-peptone yeast
HTI	High Temperature Inactivation
HTST	High Temperature Short Time
ISEBTT	International Society for Electroporation-Based Technologies and Treatments
LE	Late Exponential
LTI	Low Temperature Inactivation
MLE	Maximum Likelihood Estimation
MRS	De Man, Rogosa and Sharpe
NFC	juice not from concentrate
PEF	pulsed electric fields
PEu	pectinesterase units expressing activity
PME	pectinmethylesterase
POD	peroxidase
PPO	polyphenol oxidase
PSDF	peptone physiological salt diluent
SP	Stationary Phase
SPME	Solid Phase Micro Extraction
TSB	Tryptic Soy Broth
UHT	Ultra-High Temperature
WT	Wild Type

Erf(x)	cumulative normal (Gaussian) distribution
Erfc(x)	complementary, cumulative normal (Gaussian) distribution
Wo(x)	Lambert W function on the positive branch

Chapter 1

General introduction and outline of the thesis

1.1 General introduction and outline of the thesis

This thesis describes the effect of moderate and high intensity pulsed electric fields as an alternative process to thermal pasteurisation, aiming to inactivate vegetative micro-organisms and enzymes in fruit juices using a lower heat load, intending to improve the shelf life without losing freshness and quality. This introduction chapter provides background information on the preservation of fruit juices, pulsed electric field processing and predictive modelling, and ends with the aim and outline of the thesis.

1.2 Preservation of fruit juice

1.2.1 Definition of fruit juice

Juice is by far the most important product obtained in the citrus fruit industry, which is a multiproduct sector that utilizes the entire fruit (Berk, 2016a). Among citrus fruits, oranges are the variety mostly utilised for production of fruit juices (FAO, 2016). There are various processing techniques and conditions developed to extract the juice from the oranges, to preserve and to store the juice, resulting in orange juices varying in quality, safety and shelf life.

Codex Alimentarius defines fruit juice as *"unfermented but fermentable juice, intended for direct consumption, obtained by the mechanical process from sound, ripe fruits, preserved exclusively by physical means. The juice may be turbid or clear. The juice may be at its original concentration ("not from concentrate" (NFC) juice), or it may have been concentrated and later reconstituted with water ("from concentrate" juice (FCJ)), in a proportion suitable for the purpose of maintaining the essential composition and quality factors of the juice. Recovered aromatic substances and volatile flavour compounds of the same kind of fruit, as well as pulp and cells of the same kind of fruit, may be restored. Single juice is obtained from one kind of fruit, and mixed juice is obtained by blending two or more different kinds of fruits. The addition of sugars or acids can be permitted in nectars, but must be endorsed in the individual standard."* (Codex Stan 247-2005).

"Single-strength juice" is known as 100% juice, and can be either NFC juice, or juice that is reconstituted from a concentrate by dilution with water to its natural single-strength Brix (Berk, 2016a).

1.2.2 Fresh, untreated juice

Freshly squeezed, unpasteurized juice comes very close to consuming the orange itself with respect to aroma and nutrients. Oranges are washed, juice is extracted and immediately bottled, without addition of any additives or application of a pasteurisation treatment. The shelf life of fresh, untreated juice is limited (< 9 days when stored at 4 °C) (Timmermans et al., 2011; Toepfl, 2011), due to outgrowth of spoilage micro-organisms. To minimise product spoilage and presence of pathogens,

the farming and manufacturing operations from picking, fruit washing to packaging must be exceptionally clean. When no pasteurisation is carried out, activity of the enzyme pectin methylesterase (PME) leads to loss of cloudiness through serum/pulp separation, which is considered as a defect in pasteurised juices. However, marketers describe this to consumers as a proof that the product is pure without heat treatment that causes loss of important nutrients (Braddock, 1999a). Although initial fresh juice flavour is usually rated 'good', both enzymes and microbial growth can cause rapid deterioration, even under refrigeration.

In addition to spoilage micro-organisms, also pathogenic micro-organisms can end up in the juice. Some outbreaks of *Escherichia coli* O157:H7 and *Salmonella* spp. have been linked to the consumption of unprocessed apple and orange juice, and an overview is given for the period of 1922 – 2010 by Danyluk et al., 2012. Due to an *E. coli* outbreak in a batch unpasteurised apple juice in 1996, the US Food and Drug Administration introduces a 'Juice HACCP' in 2001, requiring fruit juice producers to achieve a 5 $\log_{10}$ reduction in critical pathogen levels (USFDA, 2001; USFDA, 2004).

1.2.3 Pasteurisation

Pasteurisation aims to stabilise the orange juice by inactivation of micro-organisms and PME enzymes (Berk, 2016a). The common method for industrial pasteurisation is by applying heat exchangers. Typical process conditions used for the pasteurisation of NFC orange juice are temperatures in the range of 90 – 99 °C at 15 – 60 s holding time (Berk, 2016a; Braddock, 1999a). The goal of this pasteurisation step is to inactivate PME, as this enzyme is more heat resistant than the common microbial population present in juices (Berk, 2016a; Braddock, 1999a). Reduction of the enzyme activity of PME is essential for the stability of the cloud and for preventing gelation in concentrates. However, reduction of micro-organisms and enzyme activity is not the only result of thermal pasteurisation. Other effects of the heat treatment include induction of a "cooked taste", reduction of vitamin content and change in colour (Berk, 2016a; Braddock, 1999a). After cooling, juice can be packed (aseptically) and distributed, or stored in a refrigerated, aseptic tank farm, to blend juices to consumers on a year-round basis (Braddock, 1999a).

Alternatively, the NFC juice is pasteurised by thermal treatment at 72 °C for 10 – 30 s (Valero et al., 2010), aiming primarily at microbial inactivation, and allowing some remaining PME activity. As a result of the lower temperature used, a better product quality is obtained when compared to thermally treated NFC juice processed at temperatures > 90 °C.

Juice from-concentrate (FCJ) is fresh juice that is thermally treated in an evaporator to inactivate most enzymes and micro-organisms and to concentrate the product to 65 °Brix (Braddock, 1999a). Due to the lower water activity, typically $a_w < 0.90$, it is microbiologically more stable than single-strength juice (ICMSF, 1998). The treated product is pumped to a (non-aseptic) refrigerated tank farm and stored or transported to the packaging location. Next, concentrate is pumped to a blend room, where water, essence, flavour compounds, pulp, etc. are added to reconstitute the juice to the preferred (single-strength) juice product (Braddock, 1999a). This reconstituted

juice is pasteurised at 80 °C for 15 – 30 s for microbial inactivation, followed by packaging (Braddock, 1999a). The packed juices are stored at ambient temperature with a typical shelf life of 12 months (Ashurst, 2016). Because of the two thermal treatments and evaporation process, the flavour of the product is not comparable to NFC or fresh, although addition of aromas and essences improves the flavour (Berk, 2016b; Braddock, 1999a). Because of economics of storing large bulk quantities of FCJ juice and the consumer preference for a ready-to-serve product, a significant amount of orange juice is packed from reconstituted concentrate and sold as chilled juice (Braddock, 1999a).

Inactivation of the micro-organisms is of paramount importance for food safety and for the prevention of spoilage. Due to the low pH of the juice (< pH 4.6), the germination of microbial spores is inhibited when juice is stored refrigerated (da Silva & Gibbs, 2009). However, at ambient temperatures, various incidents involving the spore-forming spoilage bacterium *Alicyclobacillus* have been registered in high-acid foods (Walker & Phillips, 2008).

1.2.4 Fruit juice consumption in Europe

In Fig. 1.1, the consumption of numbers of single strength fruit juices in Europe is given for 2006 and 2016, excluding numbers of freshly squeezed juice (AIJN, 2010; AIJN, 2017). A drop in volume of total consumption of fruit juice in Europe of 7605 million litres (2006) to 6008 million litres (2016) is shown. The exact reason for this reduction is unknown, but this drop might be related to the debates on high sugar content present in the juice (Boseley, 2013; Rampersaud, 2015). Contradicting results in studies assessing a relationship between consumption of 100% fruit juice and obesity or diabetes have been reported, as shown by reviews of O'Neil & Nicklas (2008) and Xi et al. (2014). WHO advices (2015) 'to limit the total energy intake of free sugars to less than 10% of the total energy intake, which involve sugars added to food and beverages or sugars naturally present in honey, syrups or fruit juices'.

Despite the drop in volume of the total consumption of fruit juice, a growth of NFC juice over FCJ juice was observed in the period 2006 to 2016. Chilled juice shows a similar trend as the NFC segment at the expense of ambient stored juice. With broader availability in retail channels and price discounting activities, consumers are increasingly turning to chilled juice. The exact cause for this shift is not fully understood, but probably relates to a number of factors such as negative framing (Hamilton, 2009), taste and preference for a product to taste 'as close to nature as possible' (AIJN, 2016). This has led to an interest in new processes that focus on microbial inactivation and preservation with a reduced 'thermal load', to improve quality and flavour. These alternative pasteurisation methods for fruit juices include technologies focusing on rapidly heating the product to avoid long heating times and over-processing, such as ohmic heating and microwave heating, but technologies could also aim for pasteurisation at milder temperatures, such as high hydrostatic pressure and pulsed electric fields. In this thesis we focus on pulsed electric fields.

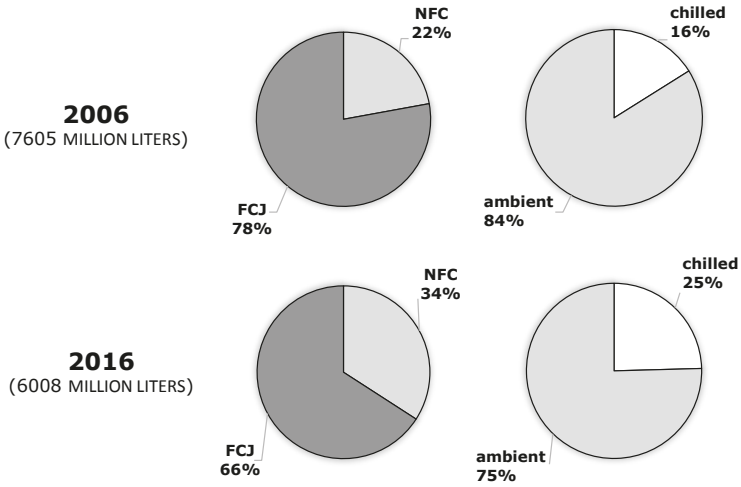

Figure 1.1. Total fruit juice consumption (single strength juice) in Europe (AIJN, 2010; AIJN, 2017).

1.3 Pulsed Electric Field (PEF) processing

1.3.1 Introduction

Although often regarded as a novel technique, the concept of applying electrical current to a food product dates back to the end of the 19th century, shortly after electricity became commercially available (Toepfl, Heinz & Knorr, 2007a). Various forms of application of electricity to reach microbial inactivation have been studied, where some are more controlled than others, including ohmic heating, microwave heating, high voltage arc discharges, low voltage alternating currents, and high-intensity pulsed electric fields (Barbosa-Cánovas et al., 1999; Palaniappan, Sastry & Richter, 1990).

Since the 1960s, applications of pulsed electric fields (PEFs) have been reported. Pioneering work was carried out by Doevenspeck 1960, followed by the research work of Sale and Hamilton that was published as a short series of papers (Hamilton & Sale, 1967; Sale & Hamilton, 1967; Sale & Hamilton, 1968). Then, research of PEF technology diverged into two directions: 1) reversible electroporation promoting electroporation and cell survival; and 2) irreversible electroporation used for microbial inactivation and food preservation. PEF technology is applied in different fields, including medical/anti-cancer therapies, food industry, and biotechnology. Dependent on the purpose and application of the electroporation, different treatment conditions and media are used (Dunn, 2001). Current applications of PEF in the food industry include PEF-assisted processes to enhance cutting, extraction, osmotic treatment, pressing extraction, drying and freezing, and cold pasteurisation focusing on microbial inactivation with improved food quality (Barba et al., 2015). Furthermore, innovations in PEF-assisted processing as a new cooking technology is getting to the market (Goettsch, 2014).

In 2005, the first commercial PEF unit for preservation of juices was installed in the USA, but stopped in 2007 due to technical and commercial limitations (Kempkes, 2010).

In Europe, the first commercial application of PEF aiming at microbial inactivation and shelf life extension of fruit juices was implemented in 2009, with a capacity of 1500 L/h (Toepfl et al., 2014). One year later in 2010, the first application of PEF treatment on solid material immersed in a liquid was installed with a capacity of 50 t/h.

PEF is a technology that causes electroporation of the cell membrane by application of intermittent electric fields of high intensity for a short period of time (Raso, 2016), ranging from microseconds to milliseconds. In liquid foods, electric pulses are delivered into the fluid using two electrodes where the liquid is in electrical contact with both electrodes or alternatively for solid foods coupled to them via an electrically conductive medium. The applied electrical current is a function of the electric field strength, the electrode configuration and its dimensions and depends on the electric conductivity of the product. Depending on the PEF conditions applied, PEF can induce temporarily or permanently changes in permeability of the membrane of a cell, known as 'electroporation'. Optimal PEF process conditions for pasteurisation aim at irreversible damage to microbial cell membranes leading to cell death, with a maximum retention of the quality attributes of the food product, because a lower heat load is required to achieve microbial inactivation.

In the next sections, processing aspects in relation to microbial and enzyme inactivation, quality aspects and shelf life will be further discussed.

1.3.2 Definition of process parameters

Various methods of electric treatment to inactivate micro-organisms were studied. These methods vary primarily in the process control and topology of the electric circuit and the construction of the treatment chamber. The electric current that is generated by the electrical circuit can be either alternating current or direct current, continuously applied, modulated or pulsed. When connected to a treatment device the current through the liquid results in a (peak) value for voltage over the fluid column and to a dissipated power generated inside the fluid.

PEF processing is different from what is known as 'Ohmic heating' by the feature that the electrical peak power exceeds the continuous power taken from the grid. This means that a PEF system is characterised by a storage capacitor and a switch. The capacitor is used to collect and store electrical energy that is released in the treatment chamber by connecting the electrodes of the treatment to the circuit by the switch. This leads to the onset of the pulse where energy stored by the capacitor is transferred through the capacitor. The pulse is turned 'off' when the switch is opened. So in a pulsed system a capacitor is continuously charged from the grid/utility connection and (partly) discharged at the moment the switch is closed. A characteristic of a pulsed system is that the peak power delivered to the load during the pulse is higher than the average continuous power delivery by the utility connection.

Typical PEF pulse durations reported are in the range of nanoseconds to several milliseconds with either a low electric field strength from 100 to 300 V/cm or high field strength ranging from 20 to 80 kV/cm (Barba et al., 2015). Specific PEF pulse characteristics and process parameters will now be discussed.

Various pulse shapes are applied, but the main ones used for PEF processing are exponential decay and square wave pulses. The pulse characteristics of an exponential decay pulse and square wave pulse are shown in Fig. 1.2. Exponential decay pulses are generated by high-power switches with only turn-on capacity, and therefore, discharge the total energy stored in the capacitor bank. These pulses have a sudden onset and therefore a rapid rising rate, but a very slow decaying rate (Fig. 1.2A), causing a long tail section that is ineffective in microbial inactivation and yields extra heat (Raso, Condón & Álvarez, 2014). Square wave pulses can be produced by an incomplete discharge of a capacitor by a switch with on/off capacity or by using a pulse forming network. These pulses are more suitable for microbial inactivation using PEF, because a stationary peak voltage during the entire pulse duration is obtained (Fig. 1.2B) (Raso, Condón & Álvarez, 2014). Both exponential and square wave pulses can be monopolar or bipolar, and an example of bipolar square wave pulse is shown in Fig. 1.2C.

The pulse width (τ) of square wave pulses is usually defined as the time (duration) representing a voltage that is more than 95% of the maximum voltage value (U_{max}). The pulse width of an exponential decay curve is defined by the time required to decay 37 % (U_{max}/e = 37%) of the maximum voltage (Raso et al., 2016), as shown in Fig. 1.2A. Pulse duration is the interval between the 'start' and 'end' capability of a pulse (Raso, Condón & Álvarez, 2014). The time between two pulses is indicated as idle period or 'dead time'.

The electric field strength E (kV/cm) is defined by the voltage divided by the distance between the electrodes of the treatment chamber. Although the range of applied electric field strengths that have been reported in literature is highly variable, no specific definitions are used in the electroporation community to classify a specific range of levels. In this thesis, electric field strength is defined as moderate for $E < 5$ kV/cm and as high for $E > 5$ kV/cm.

The frequency (f, Hz) or repetition rate indicates the number of pulses applied per unit of time (s) and is calculated as the reciprocal of the cycle time, the time between two successive (positive) pulses (Fig. 1.2).

The number of pulses (n) applied per volume element is well defined for batch treatment chambers. For continuous flow chambers an average number n per volume element passing through the treatment chambers can be assigned, according to Eq. 1.1., where V represents volume of treatment chamber (m^3) and φ flowrate (m^3/s).

$$n = \frac{V \cdot f}{\phi} \qquad \text{(Eq. 1.1)}$$

Treatment time is defined as the accumulated time under high field conditions and is calculated as the pulse width of a single pulse multiplied by the number of pulses applied.

Residence time is the time (s) that a specific fluid element is the treatment area between the electrodes of the treatment chamber(s).

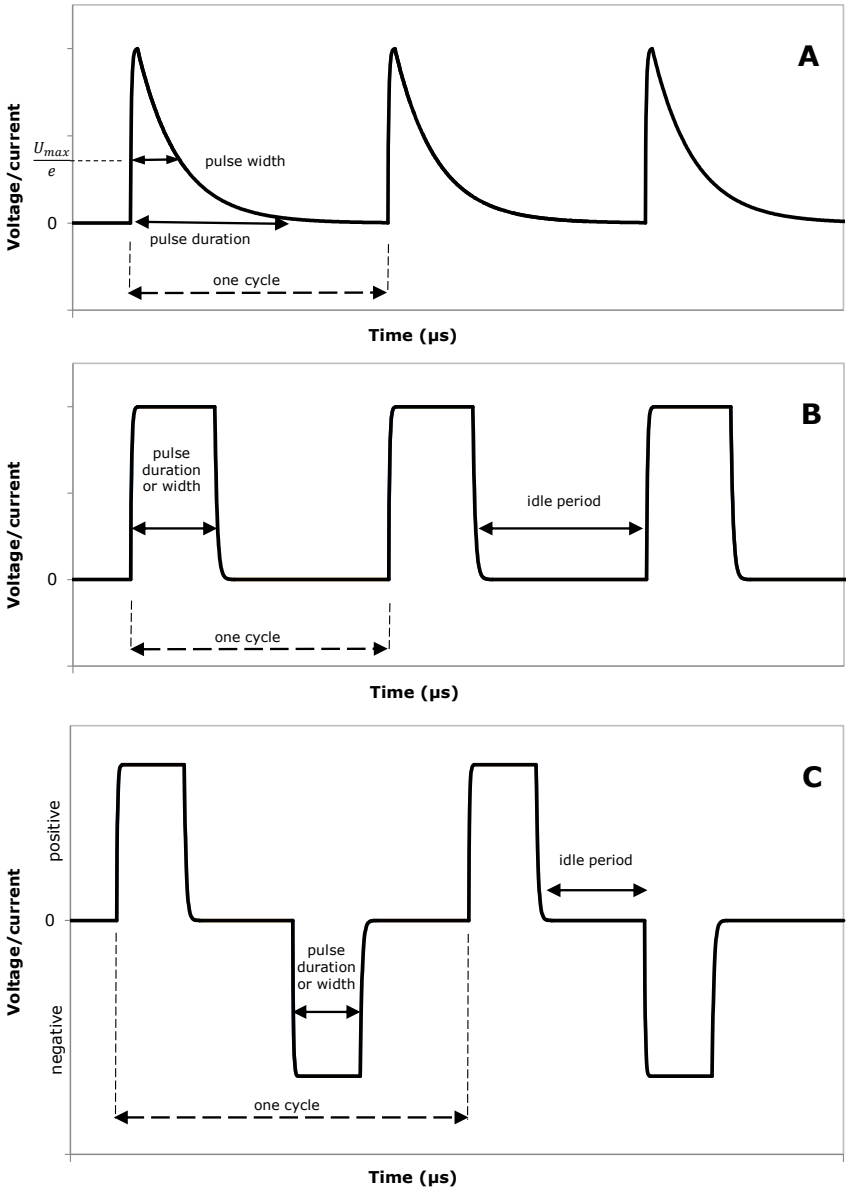

Figure 1.2. Typical pulse shapes used in PEF processing. A) Exponential decay pulse. B) (Positive) Monopolar square wave pulses. C) Bipolar square wave pulses, with alternating positive and negative pulses.

The specific energy (*w,* kJ/kg) of the treatment is the energy density per unit mass. For conductive media the energy is calculated by the electrical energy input according to Eq. 1.2 (energy input) and is balanced by the calorimetric heat as energy output by Eq. 1.3. Here it is assumed that a change in enthalpy does not occur (Mastwijk, 2006). Specific energy for a PEF treatment using continuous flow is calculated as the product of frequency and integral of the voltage (*U*, V) and current (*I*, A) per pulse, divided by

the unit of mass passing through the treatment chamber per unit of time, calculated from the flowrate (φ, m³/s) and product density (ρ, kg/m³), as given by Eq. 1.2a.

$$w = \frac{f \cdot \int U(t) I(t) \mathrm{d}t}{\phi \cdot \rho} \qquad \text{(Eq. 1.2a)}$$

The energy density per pulse is variable and dependent on chosen pulse conditions intensity or electric field strength (E) and pulse duration (τ, s) and electrical conductivity of the product (σ, S/m). Specific energy applied can also be calculated based on these parameters, as given in Eq. 1.2b.

$$w = \frac{f \cdot V}{\phi \cdot \rho} \int \kappa \cdot E^2 \mathrm{d}t \qquad \text{(Eq. 1.2b)}$$

The total amount of electric energy that is converted to heat is balanced by the calorimetric heat leaving the system. The specific energy is related to the temperature difference between the temperature measured just before the first treatment chamber (T_{in}) and the temperature measured just after the last treatment chamber before the cooling section (T_{out}) multiplied by the specific heat capacity of the product c_p (J/kg·K), as given in Eq. 1.3.

$$w = \left(T_{out} - T_{in}\right) \cdot c_p \qquad \text{(Eq. 1.3)}$$

In some process layouts intermediate cooling sections between the treatment chambers are realised (Min et al., 2003; Sharma et al., 2014), to reduce over-heating of the product. As a result, also the heat generated by the PEF treatment is partly lost, so a proper temperature measurement before and after each treatment chamber section should be made to calculate the total amount of energy delivered by the PEF process and the inlet and outlet temperatures.

1.3.3 Effect of the choice of pulse parameters with respect to heat

Although PEF treatment aims for minimal thermal effects, the temperature of the treated medium increases as the result of Ohmic heating, also known as Joule heating. The amount of Joule heating depends on the choice of the pulse parameters: more electrical energy per pulse results in more heating of the product. The pulse parameters electric field strength and pulse width required for a PEF treatment can be chosen arbitrarily. In Fig. 1.3 an example of the temperature rise as a function of total treatment time and electric field strength is shown, based on calculations according to Eq. 1.2b and 1.3 for a medium with a σ = 0.6 S/m, c_p = 3.82 kJ/kg·K and ρ = 1020 kg/m³ (typical numbers for orange juice at 40 °C). Application of short pulses with high intensity, for example τ = 2 µs and E = 23 kV/cm, typically results in a temperature increase of 1.8 °C. However, in practice, more than one pulse will be given, resulting for example in a total pulse time of 10 µs, for 5 pulses of 2 µs, and temperature increase of 9.0 °C. Note that the temperature increase is actually higher than the 9.0 °C calculated as the conductivity also increases as a function of temperature, and this is not incorporated in

the calculations in the plot in Fig. 1.3. The temperature increases rapidly with the pulse duration and/or electric field strength resulting in a process where thermal effects on micro-organisms, enzymes and quality determining compounds present in the product interfere with the electroporation effects. To control the product temperature, intermediate cooling sections are implemented in some continuous-flow PEF equipment or a pausing time between pulses is incorporated in batch processing (El Zakhem et al., 2006a), but this is not incorporated in the contours as shown in Fig. 1.3. Typical process conditions for high intensity PEF to inactivate micro-organisms yield E = 15 – 40 kV/cm, with a total pulse time of 20 µs up to 2000 µs, indicated in Fig. 1.3 in a blue circle (Álvarez et al., 2003a; Toepfl, Heinz & Knorr, 2007b).

A reduction of the electric field strength, in combination with an increase of the pulse duration can result in a similar or even lower temperature increase per pulse when compared to high intensity short pulses. In Fig. 1.3, this domain of moderate electric field strength with E = 0.1 – 5 kV/cm with total pulse time of 100 µs up to 10,000 µs is indicated in a green circle, and is the typical domain where electroporation of plant cells and non-thermal extraction from solid foods occurs (Vorobiev & Lebovka, 2008; Vorobiev & Lebovka, 2010).

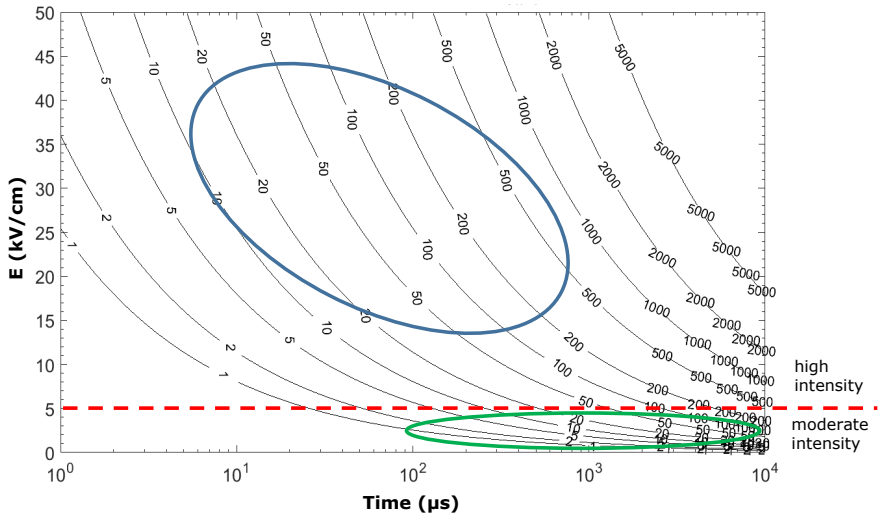

Figure 1.3. Schematic representation of calculated temperature rise (as contours, °C) as a function of total treatment time and electric field strength for a product with conductivity of σ = 0.6 S/m, if no additional cooling section or pause time was used. Typical domain of research at high intensity PEF conditions to electroporate micro-organisms is indicated with a blue circle (Álvarez et al., 2003a; Toepfl, Heinz & Knorr, 2007b) and at moderate intensity PEF conditions to electroporate plant cells or to extract compounds from solids with a green circle (Vorobiev & Lebovka, 2008; 2010). Note that additional cooling sections or pause time could avoid part of the temperature increase as indicated by the contour lines in the indicated blue and green domains. Red dotted line separates the domain of high intensity and moderate intensity PEF as used in this thesis.

The essential difference between ohmic heating, also named moderate electric field treatment (Toepfl, Heinz & Knorr, 2007a) and high intensity pulsed electric field is the differentiation between thermal and non-thermal (electric) effects. High intensity PEF

aims to generate electric effects with minimal heating of the food, while ohmic heating aims at generating thermal effects (Barbosa-Cánovas et al., 1999; Jaeger et al., 2016). However, in practice the differences between ohmic heating and high intensity PEF are not that strict, as high intensity PEF processing is increasingly combined with heat to improve its efficacy (Buckow, Ng & Toepfl, 2013) and moderate intensity PEF processing is sometimes associated with an additional electroporation effect (Lebovka et al., 2005; Loghavi & Sastry, 2009).

A systematic comparison of the effect of high intensity PEF and moderate intensity PEF using similar energy per pulse (at equal temperatures) but different combinations of electric field strength and pulse duration has not been reported in literature, and will therefore be addressed in this thesis.

1.3.4 Mechanism of electroporation

The first important steps in application of high intensity PEF for the treatment of biological matrices were made about 50 years ago. Different research groups observed that PEF treatment induced electrical breakdown of cell membranes, known as electroporation (Neumann & Rosenheck, 1972; Zimmerman, Pilwat & Riemann, 1974). The capacity of PEF to induce cell electroporation to inactivate micro-organisms was demonstrated first by Sale and Hamilton (Hamilton & Sale, 1967; Sale & Hamilton, 1967). Later, the membrane electroporation was investigated and reported to effect the lipid domain (Teissié, Golzio & Rols, 2005; Weaver & Chizmadzhev, 1996), protein channels, transport systems or transport pumps (Tsong, 1990) or cell wall (Pillet et al., 2016).

The most widely accepted explanation of electroporation involves the induction of an (external) electric field on the lipid domain of a cell membrane, that can lead to local instabilities, resulting in formation of pores in the membrane (electroporation). Due to the electroporation, an enhanced permeability across the membrane occurs (electropermeabilization) that, depending on the electric field strength, leads to cell death (irreversible, complete inactivation) or resealing of the cell to its initial viable state (reversible, sub lethal or partial inactivation) (Teissié, Golzio & Rols, 2005; Weaver & Chizmadzhev, 1996). Fig. 1.4 visualises different scenarios of electroporation in the lipid domain of a microbial cell by high intensity PEF.

The transmembrane potential, $\Delta\varphi_m$, generated on a cell membrane by an external applied electric field depends on the intensity of the electric field strength applied (E) and the size and shape of the cell. The external electric field strength required to reach the transmembrane voltage threshold is known as critical electric field strength (E_c), and when $E > E_c$, electroporation is induced (Álvarez, Condón & Raso, 2006). For microbial cells (1 – 10 µm) an electric field strength > 10 kV/cm is required to induce electroporation, while for eukaryotic plant cells (40 – 200 µm) an electric field strength < 5 kV/cm is sufficient (Raso, 2016). When the electric field strength is much higher than the critical electric field strength, or applied for a sufficient long treatment time, electroporation is irreversible (Fig. 1.4).

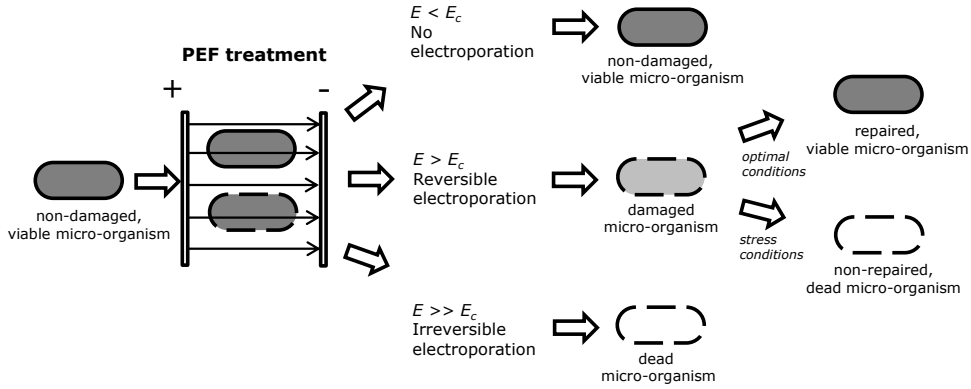

Figure 1.4. Different scenarios of the membrane permeabilization induced by an external electric field for high intensity PEF. Increasing the electric field strength (E) will lead to formation of large reversible or irreversible membrane pores depending on how much the critical electric field strength (E_c) is exceeded (Based on: Raso, Condón & Álvarez, 2014; Sharma, Oey & Everett, 2014).

1.3.5 Effect of PEF on microbial inactivation

The microbial resistance towards a given high intensity PEF process depends on different factors (Álvarez, Condón & Raso, 2006; Wouters, Álvarez & Raso, 2001). Understanding of these factors help to establish process conditions that ensure microbiological inactivation. These factors have been classified into three groups: processing parameters, microbial and culture characteristics and treatment medium or product characteristics (Wouters, Álvarez & Raso, 2001) and are listed in Table 1.1. The most relevant parameters to food processing will be discussed below.

Table 1.1. Factors affecting the efficacy of microbial inactivation using pulsed electric fields. Based on/adopted from Wouters, Álvarez & Raso (2001), and Raso, Condón & Álvarez (2014).

Processing parameters	Microbial and culture characteristics	Product characteristics
Electric Field Strength	Species and strain characteristics	Conductivity
Pulse duration/width	Morphology	pH
Number of pulses	Gram-positive/Gram-negative	a_w
Total treatment time	Size	Composition (lipids, proteins)
Specific energy	Growth condition	Presence of anti-microbials
Preheating temperature	Growth temperature	
Maximum temperature	Incubation time	
Pulse shape	Growth phase	
Frequency	Presence specific compounds	
Configuration of treatment chamber	Recovery conditions	
Residence time	Medium composition	
	Temperature	
	Recovery time	
	Oxygen concentration	

Processing parameters

The efficacy of a high intensity PEF treatment strongly increases with increasing electric field strength (E) (Álvarez et al., 2003a; Heinz et al., 2002; Hülsheger, Potel & Niemann, 1981; Hülsheger, Potel & Niemann, 1983; Sale & Hamilton, 1967). It is suggested that this increased efficacy only occurs, when the external applied electric field strength E exceeds the critical electric field strength (E_c) (Álvarez, Condón & Raso, 2006). The E_c varies for different micro-organisms, as the size and shape of the micro-organisms can be different. Heinz et al. (2002) demonstrated that also the orientation of the electric field on the cell has an impact on the electric field strength required to induce electroporation. Although numerous laboratory studies showed effective microbial inactivation at an electric field strength above 30 kV/cm (Heinz et al., 1999; McDonald et al., 2000; Rodrigo et al., 2001; Walkling-Ribeiro et al., 2008a), this field strength has drawbacks for industrial scale applications in food, due to the risk of arcing inside the treatment chamber (Heinz et al., 2002; Toepfl et al., 2014). Arcing should be avoided at all times, and the field distribution in the treatment chamber should be optimized to avoid excessive high field strengths leading to hot spots. Research relevant for food applications should therefore be aimed at electric field strengths below 30 kV/cm.

Increase of the pulse width was found to increase the PEF lethality to micro-organisms (Aronsson et al., 2001; Wouters et al., 1999). However, in these studies, an increase in pulse width also led to an increased total treatment time. Consequently, when the effect of pulse width is studied as a function of (total) treatment time, no net increased efficiency was observed when the pulse width was increased from 2 µs up to 20 µs (Raso et al., 2000; Sale & Hamilton, 1967; Wouters et al., 1999). Thus application of ten pulses of 2 µs gave a similar inactivation as application of one pulse of 20 µs. An increase of the treatment time leads to an increase in the applied specific energy and hence temperature, if other conditions are kept constant.

Increase of the applied specific energy results in an increased microbial inactivation (Álvarez et al., 2003a; Heinz et al., 2002; Toepfl, Heinz & Knorr, 2007b). The measurement of specific energy has been proposed as a control parameter of PEF processes instead of treatment time (Heinz et al., 2002). Different arguments justify the use of this parameter, one of them is that the variations in actual applied pulse energy are incorporated in this parameter, which is especially relevant when exponential decay pulses are applied, as the treatment time is not controlled for these pulses. Since the electrical specific energy input is calculated as an integrated parameter as shown in Eq. 1.2b that depends on electrical conductivity of the product and process conditions applied, the specific energy is well-defined. Although the microbial inactivation increases with the energy applied, a higher electric field strength applied at the same specific energy was shown to be more effective (Álvarez et al., 2003a). Therefore, it is necessary that both specific energy and electric field strength are reported (Raso, Condón & Álvarez, 2014).

An increase of the specific energy will also result in a higher outlet temperature, as shown in Eq. 1.3. That is, at constant flow and if no additional intermediate cooling sections are used. Both an increase of the inlet temperature as well as an increase in the maximum temperature was found to affect the efficacy for inactivation (Heinz,

Toepfl & Knorr, 2003; Toepfl, Heinz & Knorr, 2007b; Wouters et al., 1999). This was even so at inlet temperatures that are not lethal for microbial inactivation. This effect might be explained by the temperature dependent characteristics of the membrane of the micro-organism. A temperature increase changes the phospholipid bilayer structure of the cell membrane from a gel-like consistency to a liquid crystalline state, affecting the stability and sensitivity of the cell membrane (Stanley & Parkin, 1991). As a result, the E_c will decrease (Coster & Zimmerman, 1975) and consequently electroporation occurs at a lower applied electric field strength, or more inactivation can occur at a constant field strength.

Different treatment chamber configurations such as parallel plates, coaxial cylinders and co- and cross-field geometries have been used, as discussed by Zhang, Barbosa-Cánovas & Swanson (1995). Most of the earlier studies with PEF treatment were using small static treatment chambers held between parallel plates (Grahl & Märkl, 1996; Hülsheger & Niemann, 1980; Sale & Hamilton, 1967). Qin et al. (1995a) showed a greater treatment uniformity in continuous flow systems than in static systems, which was confirmed in a study by Martín et al. (1997) who found more inactivation within a continuous flow system with parallel plate chamber than in a static parallel system. Thereafter, especially in translation of the process to pilot and commercial scales, the preference has been to conduct kinetic studies in continuous flow mode (Sastry, 2016). The uniformity of the electric field strength distribution does not only depends on the fact that a system is static or continuous, but also on the temperature profile and flow dynamics in the treatment chamber (Van den Bosch, 1997). The velocity profile in the treatment chamber (laminar, transitional or turbulent) determines the residence time distribution in the treatment chamber. Longer treatment times will occur near the wall of the treatment chamber due to lower flow velocity, and shorter treatment times occur in the centre of the treatment chamber where a higher flow velocity exists (Jaeger, Meneses & Knorr, 2009), resulting in a larger homogeneity for turbulent flow than for laminar flow conditions.

During the PEF treatment the liquid food is heated as a result of the dissipation of electrical energy and, as a consequence, temperature dependent food properties such as electrical conductivity, density, viscosity and thermal conductivity are modified (Fiala et al., 2001). Changes in electrical conductivity may influence the electric field distribution and changes in viscosity, density and thermal conductivity may affect product flow. Measurements in the small treatment chamber volume are not possible without interference of the measuring device with the product flow and electric field (Jaeger, Meneses & Knorr, 2009), and it is therefore a difficult task to achieve experimental information (Saldaña et al. 2010a). For experimental performance of PEF treatments, the determination of the average outlet temperature is still the most suitable method for an in-line process characterization due to the lack of alternative measuring possibilities (Jaeger, Meneses & Knorr, 2009). However, numerical simulations of the fluid dynamics coupled with the distribution of electric field strength and temperature inside the treatment chamber can provide detailed knowledge on the spatial and temporal distribution of both parameters for various PEF treatment conditions. Numerical simulations have been used for optimization of treatment geometry to avoid over- or underprocessing (Gerlach et al., 2008). Increase in gap/diameter ratio increases homogeneity (Van den

Bosch, 2007) and rounded edges on insulator geometry improve electric field strength distribution (Meneses et al., 2011; Schroeder, Buckow & Knoerzer, 2009). Numerical simulations showed that increase of the flow velocity increases turbulence and provides a better mixing of the fluid, avoiding high (peak) temperatures generated in the zone near the insulator wall, leading to a more homogeneous temperature distribution in the treatment chamber (Jaeger, Meneses & Knorr, 2009; Meneses et al., 2011). To conclude, flow conditions are mostly laminar at laboratory and pilot scale equipment, while they are mostly turbulent at industrial scale equipment. This difference might result in a different distribution of surviving micro-organisms (or retention of heat-sensitive quality compounds) and could lead to differences in the inactivation kinetics between systems and therefore to over- or underestimation of the effectivity of the PEF process.

Microbial and culture characteristics

High intensity PEFs can inactivate vegetative bacterial cells, yeasts and moulds, although differences in sensitivity towards the PEF treatment are observed between micro-organisms (Hülsheger, Potel & Niemann, 1983; Raso et al., 1998; Sale & Hamilton, 1967; Toepfl, Heinz & Knorr, 2007b). It has been suggested that the morphology of the micro-organism plays a role in its sensitivity to high intensity PEF treatment (Heinz et al., 2002). As discussed before, the influence of the cell size and the shape of the micro-organism both influence the transmembrane potential difference created by the external electric field strength. The expression of the transmembrane potential difference, $\Delta\varphi_m$, has been derived from the Maxwell equations (1904) for spherical cells (Neumann, 1989) and for ellipsoidal coordinates (Zimmerman, Pilwat & Riemann, 1974) and is a complex function. Several simplifying restrictions have been proposed, including a factor $f(\lambda)$ which can be approximated by 1, instead of determining the specific conductivities of the suspending medium, the cytoplasm, the cell membrane and the ratio of the membrane thickness and cell radius.

For spherical cells, $\Delta\varphi_m$ can be calculated according to Eq. 1.4, with $f(\lambda)$ as function of the electric conductivities (S/m), r as the radius (m) and Θ as the angle of the radial direction to the electric field (°).

$$\Delta\varphi_m = -1.5 \cdot E \cdot f(\lambda) \cdot r \cdot \cos(\Theta) \qquad \text{(Eq. 1.4)}$$

For cells with non-spherical shape an estimate for $\Delta\varphi_m$ can be obtained by solving Eq. 1.5a and 1.5b, with A_F at distance from the centre in direction of the external field. The shape factor $f(A)$ is a function of the three semi-axes (A_1, A_2, A_3) of elliptical cells.

$$\Delta\varphi_m = -f(A) \cdot A_F \cdot E \qquad \text{(Eq. 1.5a)}$$

$$f(A) = \frac{2}{2 - A_1 A_2 A_3 \int_0^\infty 1 / \left(\left(s + A_F^2 \right) \left(\sum_{n=1}^{3} \sqrt{s + A_n^2} \right) \right)_{ds}} \qquad \text{(Eq. 1.5b)}$$

According to calculations either for spherical coordinates or ellipsoidal coordinates, the smaller the cell size, the lower the value of the induced membrane potential, so the higher the microbial resistance to the treatment (Heinz et al., 2002; Hülsheger, Potel & Niemann, 1983; Toepfl, Heinz & Knorr, 2007b; Zimmerman, Pilwat & Riemann, 1974). A theoretical prediction of the electric field strength required to induce a transmembrane potential of 1 V to three different ellipsoidal micro-organisms varying in shape and size was made by Toepfl, Heinz & Knorr (2007b), and illustrated in Fig. 1.5. It is evident that larger sized cells are more susceptible to electric fields than smaller sized cells.

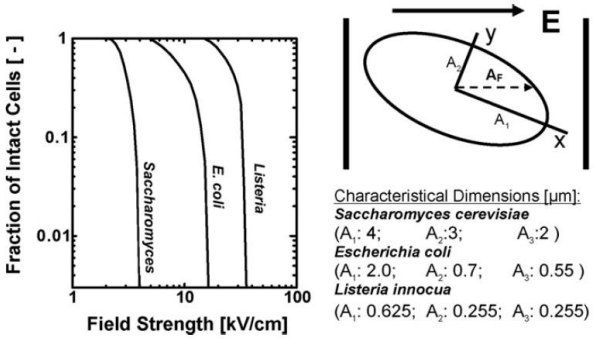

Figure 1.5. Theoretical prediction of impact of cell size and shape of three ellipsoidal micro-organisms relative to electric field strength. Specific threshold required to induce electroporation for the different geometries of the cells are calculated according to Eq. 1.5, and shown as a function of electric field strength (Toepfl, Heinz & Knorr, 2007b).

In general, it has been reported that yeasts are more sensitive to a high intensity PEF treatment than bacteria (Hülsheger, Potel & Niemann, 1983; Sale & Hamilton, 1967; Wouters et al., 1999), that a coccus is more resistant than a rod shaped bacteria (Heinz et al., 2002; Hülsheger, Potel & Niemann, 1983; Qin et al., 1998), and Gram-positive bacteria are more resistant than Gram-negative bacteria (Aronsson et al., 2001; Hülsheger, Potel & Niemann, 1983). The high sensitivity of yeasts to high intensity PEF treatments can be largely explained by its larger cell size (Pagán & Mañas, 2006).

It has been suggested that the thickness of the peptidoglycan layer between the membrane structures of Gram-positive and Gram-negative bacteria may explain the increased resistance in Gram-positive bacteria over Gram-negative bacteria (Hülsheger, Potel & Niemann, 1983) (visualised in Fig. 1.6).

Although high intensity PEF is effective in inactivation of vegetative cells, high intensity PEF is not effective in the inactivation of bacterial spores (Grahl & Märkl, 1996; Hamilton & Sale, 1967), though in combination with preheating at 60 – 95 °C and high intensity PEF heating up to maximum temperatures of 100 – 160 °C and holding times of 14 – 38.5 s inactivation of heat resistant spores has been claimed (Reineke et al., 2015; Siemer, Toepfl & Heinz, 2014). In Fig. 1.6, a schematic representation of the different layers of a bacterial spore is presented. The germ cell wall has a structure similar to that of the peptidoglycan in a vegetative cell wall, and the much thicker cortex has several structural modifications relative to the vegetative peptidoglycan (Popham, 2002). The bacterial spore coats, and mainly the cortex and germ cell wall that enclose

the inner membrane of the spore, probably prevent the permeabilization effect of PEF (Álvarez, Condón & Raso, 2006). Recent work of Pillet et al. (2016) showed changes in coat, cortex and core structure after PEF treatment when using electric field strength of 2.0 – 7.5 kV/cm.

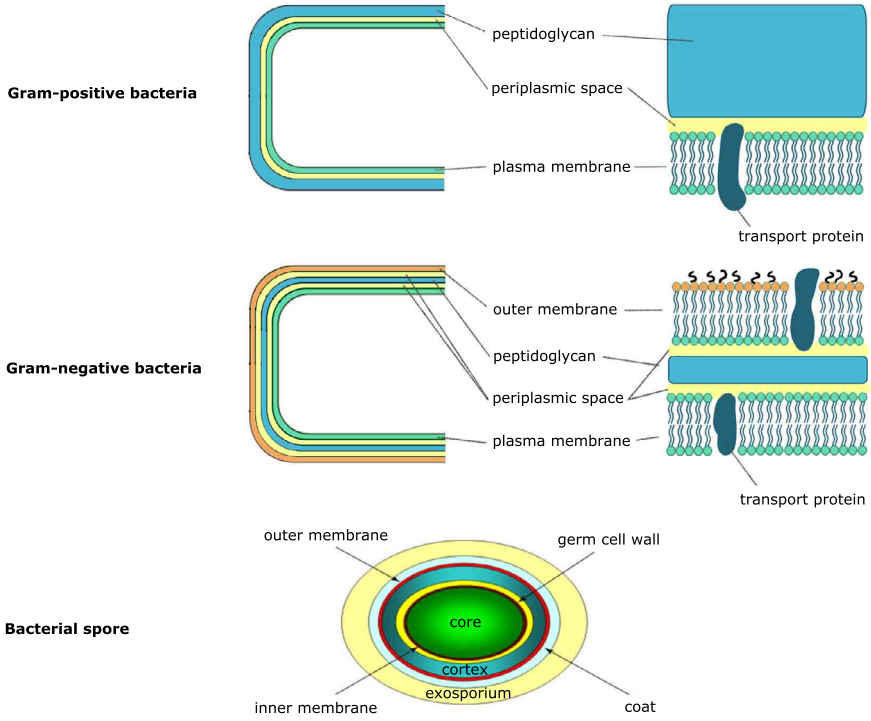

Figure 1.6. Schematic representation of the cell envelope of Gram-Positive and Gram-Negative bacteria and the structure of a bacterial spore. The thickness of the layers can vary between species, and there may some sublayers in the coat and exosporium of the spore. The exosporium layer is only present in some species, e.g. *B. cereus, B. anthracis, Clostridium* spp. (Based on Paredes-Sabja, Setlow & Sarker, 2011).

Apart from the structural characteristics, intrinsic microbial resistance showed to play an important role in PEF resistance, as illustrated by the large diversity in PEF resistance between different strains within the same species (Cebrián et al., 2009; Gurtler et al., 2010; Lado & Yousef, 2003). Culture history plays an important role, as growth phase, growth temperature, and growth medium including the presence of specific compounds can influence the intrinsic properties of a cell subjected to PEF treatment. Several studies have demonstrated that the resistance to high intensity PEF increased with growth phase, meaning that cells in the exponential phase or logarithmic phase are more sensitive towards high intensity PEF than cells in the stationary phase (Álvarez et al., 2002; Hülsheger, Potel & Niemann, 1983; Jacob, Förster & Berg, 1981; Pothakamury et al., 1996). When the growth temperature is sub-optimal, micro-organisms become more sensitive to the high intensity PEF treatment (Álvarez et al., 2002; Cebrián et al. 2008; Cebrián, Condón & Mañas, 2016; Ohshima, Okuyama & Sato, 2002), although differences were never larger than 1 $\log_{10}$ cycle when compared to optimal growth conditions. It has

been proposed that variations in lipid composition of the cell membrane might be induced by growth temperature modifications, resulting in a varying PEF sensitivity (Álvarez et al., 2006; Cebrián et al., 2008; Russell, 2002).

Very little is known about the impact of cell cultivation and historical stress, such as variation in medium pH, salt concentration or presence of specific (antimicrobial) compounds, on PEF resistance. Only the study of Cebrián et al. (2012) measured the effect of acidic, oxidative, hyperosmotic or cold shock stresses given 2 h before high intensity PEF treatment of *S. aureus* and found that conditions had no effect on the PEF treatment. By contrast, heat and alkaline stress increased the resistance towards high intensity PEF. The effect of PEF on *E. coli* and *L. monocytogenes* mutants impaired in specific stress responses invoked by deleted sigma factors (referred to as σ^S and σ^B factors depending on the repertoire of stress genes that is regulated), known to regulate stress response in micro-organisms, was measured. Mutants in the σ^S factor of *E. coli* showed to be more sensitive than their parental strain (Gomes Neto et al., 2015; Somolinos et al., 2008), while contradicting results on σ^B factor in *L. monocytogenes* were observed; the study of Gomes Neto et al. (2015) showed a higher sensitivity in the mutant compared to the parental strain, while a study by Somolinos et al. (2010) did not observe any difference.

In earlier studies, no damaged cells were found for high intensity PEF treatment, as cells were considered either alive or death. Later studies showed that under suitable outgrowth conditions, partly (sub lethal) damaged cells might recover and grow out (García et al., 2005a). The capability of a micro-organism to recover from the damage caused by a high intensity PEF treatment has been associated with the composition of the growth medium. Addition of 0.1% sodium pyruvate (Gurtler et al., 2011) or 1 – 6% NaCl to outgrowth media, dependent on type of micro-organism (García et al., 2005a), can promote the recovery after sub lethal damage. Also longer incubation times (up to several days longer) may contribute to high recovery of the sub lethal damaged cells (Álvarez, Condón & Raso, 2006). This illustrates that it is important to use procedures that recover the largest possible number of cells, to avoid overestimation of the effectivity of the PEF process (Álvarez, Condón & Raso, 2006).

Product characteristics

Physical and chemical parameters describing product properties play an important role on the effectivity of the PEF process, either directly or indirectly.

One of the key parameters influencing PEF processing is the electrical conductivity of the medium, which is an intrinsic property of the treated food product and mostly fixed. The electrical conductivity of a food product depends on the ion activity and temperature, and can vary enormously between food products, as shown in Fig. 1.7.

Due to the ionic conduction of charges, the conductivity increases with temperature and can be approximated by linear functions (Heinz et al., 2002). Electrical conductivity is the capacity of a medium to conduct an electric current, and is the inverse of resistivity.

The product conductivity is an important parameter in the design of the treatment device and the pulsed power circuitry, since it determines the effective electric resistance of the treatment device (Heinz et al., 2002; Toepfl et al., 2014).

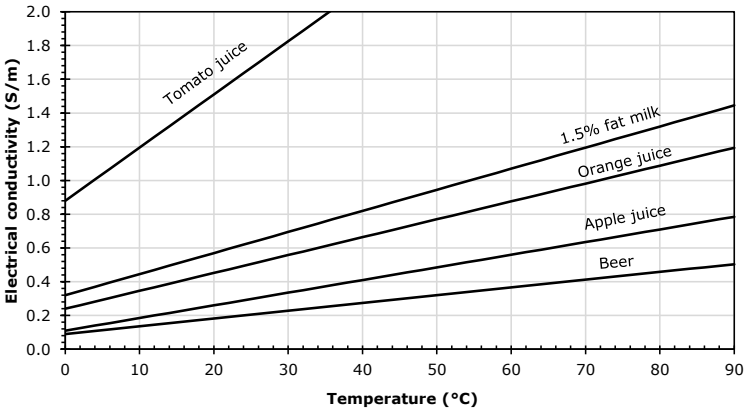

Figure 1.7. Electric conductivity of several food products (Adopted from Heinz et al., 2002).

Many studies conclude that conductivity affects the cell inactivation by high intensity PEF (Jayaram, Castle & Margaritis, 1993; Sensoy, Zhang & Castry, 1997; Vega-Mercado et al., 1996; Wouters et al., 1999), but not all confirm this (Álvarez et al., 2000; Álvarez et al., 2003a). This may (in part) be explained by the fact that when the conductivity has changed other process parameters may change, such as electric field strength or applied energy (Eq. 1.2b), dependent on the design of the electric pulse unit and on the particular applied conditions of the experiment, making it difficult to interpret the data.

The pH effect in relation to microbial inactivation is one of the parameters that has generated most controversy in literature. Some researchers have observed that micro-organisms were more sensitive to high intensity PEF at a low pH (Aronsson & Rönner, 2001; Vega-Mercado et al., 1996; Wouters et al., 1999), others found more sensitivity at neutral pH (Álvarez et al., 2000) or alkaline pH (Jeantet et al. 1999), but also no effect of medium pH was observed (Álvarez et al., 2003b; Hülsheger, Potel & Niemann, 1981). In addition, studies have reported a pH-dependent sensitivity for Gram-positive and Gram-negative cells (García et al., 2005a; Geveke & Kozempel, 2003). García and co-workers (2005a) reported that pH sensitivity was dependent on type of species, and in general, at acidic conditions Gram-negative bacteria were more resistant than Gram-positive bacteria, and at neutral pH Gram-positive bacteria were more resistant to high intensity PEF than Gram-negative bacteria. For conditions that evoked maximum resistance to the bacteria, a large population of sub lethally injured cells was detected. A possible mechanism explaining these observations has not been elucidated yet.

The influence of water activity, a_w, on microbial inactivation by high intensity PEF has been investigated. It has been reported that a decrease in a_w has a protective effect to micro-organisms and was observed at high intensity PEF conditions (Álvarez et al., 2003b; Aronsson & Rönner, 2001). It has been suggested that due to the reduced water activity outside the cell, the water content inside the cell reduces, resulting in shrinkage of the cell size. Also a thickening of the cell membrane has been reported, resulting in a lower permeability and fluidity (Neidhardt, Ingram & Schaerchter, 1990).

Air bubbles or encapsulations may be present in the matrix, but have to be removed to avoid electrical discharges when an electric field of high intensity is applied to the product matrix. Products where foam formation cannot be avoided are not suitable to be subjected to high intensity PEF (Toepfl et al., 2014). Furthermore, Góngora-Nieto et al. (2003) have shown that presence of air bubbles in the treatment chamber led to a drop in electric field strength and inhomogeneity in the treatment chamber, resulting in less microbial inactivation. Formation of air bubbles may be suppressed by application of static backpressure (Mastwijk, 2006).

A similar effect of field inhomogeneity as shown for air bubbles has been shown for agglomerations of micro-organisms and/or particles with different dielectric properties, such as fat globules (Toepfl, Heinz & Knorr, 2007). Experimental work with variations in the composition of the matrix showed that an increase of milk proteins (Jaeger et al., 2009) in the matrix composition led to reduced microbial inactivation. Contrary, other studies showed less than 1 $\log_{10}$ difference in microbial inactivation when changing the matrix composition (Dutreux et al., 2000; Mañas, Barsotti & Cheftel, 2002; Picart, Dumay & Cheftel, 2002; Reina et al., 1998); however these studies used 5– 10 times more energy than the study of Jaeger et al. (2009). Although more studies were carried out on the effect of matrix composition, such as the effect of fat (Grahl & Märkl, 1996), they are often lacking essential information to make a comparison to other studies, and cannot contribute in the formation and evaluation of a hypothesis. Recently, guidelines to report the key information of process settings, equipment, microbial culture and recovery conditions and treatment medium properties are presented by the precursor of the International Society for Electroporation-Based Technologies and Treatments (ISEBTT) to enable researchers to repeat, judge and evaluate experiments and the data obtained (Raso et al., 2016). These guidelines help to compare the various factors that determine the efficiency in microbial inactivation using PEF.

1.3.6 Effect of PEF on enzyme activity

The effect of PEF treatment on enzyme activity has been investigated by many research groups. In general, a more severe PEF treatment or combination of PEF with a higher temperature is required for reduction of enzyme activity than for the reduction of microbial activity (Grahl & Märkl, 1996; Ho, Mittal & Cross, 1997), although exceptions are reported (Jaeger et al., 2010). As some enzymes have interesting functionalities for the food industry, a food product could be treated with high intensity PEF to inactivate micro-organisms, while maintaining the activity of useful enzymes (Elez-Martínez et al., 2007).

The effect of PEF on inactivation of enzymes is not fully understood. The available scientific literature suggests that both electrochemical and thermal effects associated with PEF can result individually or in synergy in changes in the secondary and tertiary structure of enzymes, which may lead to an activity loss (Castro et al., 2001a; Terefe, Buckow & Versteeg, 2013; Yeom, Zhang & Dunne, 1999). Enzymes are globular proteins with catalytic activity that depends on the native configuration of their active site (Whitaker, 1996). Due to the alteration in enzyme structure or its functional groups, the substrate cannot fit the active site, preventing the conversion of the substrate into products and resulting in a loss of biological activity (Hendrickx et al., 1998).

Several other effects have been described in literature responsible for enzyme activity loss by PEF. Application of an external electrical pulse that is sufficiently long in duration, could entail: (i) polarization of the protein molecule, (ii) dissociation of non-covalently linked protein sub-units involved in quaternary structures, (iii) changes in the protein conformation so that buried hydrophobic amino acids or sulfhydryl groups are exposed, (iv) attraction of polarized structures by electrostatic forces, and (v) hydrophobic interactions or covalent bond forming aggregates (Castro et al., 2001a; Perez & Pilosof, 2004). In addition, PEF-induced electrolysis and free radical formation may result in localized pH shifts near the electrodes in aqueous systems (Meneses, Jaeger & Knorr, 2011), and oxidation of amino acid residues important for activity and stability of enzymes (Buckow, Ng, & Toepfl, 2013). Finally, the instantaneous temperature increase during pulsing as well as localized hot spots in the PEF treatment chamber may also contribute to PEF-induced denaturation of enzymes (Jaeger, Meneses & Knorr, 2009; Schilling et al., 2008).

Many studies have investigated the effectivity of PEF processing on enzyme activity, and controversial results have been published. Some studies report complete or nearly complete (> 95%) inactivation of enzymes after PEF treatment (Elez-Martínez, Aguiló-Aguayo & Martín-Belloso, 2006; Giner et al., 2001; Giner et al., 2003; Marsellés-Fontanet & Martín-Belloso, 2007), while others observed that PEF did not affect, or only partly affect, enzyme activity (Buckow et al., 2012; Ho, Mittal & Cross, 1997; Van Loey, Verachtert & Hendrickx, 2002; Vervoort et al., 2011). An increased enzyme activity after PEF treatment of particular enzymes has also been reported (Ho, Mittal & Cross, 1997). These controversial results could be mainly attributed to the different PEF equipment and conditions used, the enzyme sources and the media of treatment (Elez-Martínez et al., 2007), similar to the factors described for microbial inactivation, as discussed in the previous section. The most important parameters affecting enzyme inactivation are shown in Table 1.2, and will be discussed below.

Table 1.2. Factors affecting the efficacy of enzyme inactivation using pulsed electric fields.

Processing parameters	Enzyme characteristics	Product characteristics
Electric Field Strength	Type	Conductivity
Pulse duration/width	Complexity	pH
Number of pulses	Size	Composition (lipids, proteins)
Total treatment time	Source	
Specific energy	Isoform	
Temperature		
Pulse shape		
Frequency		
Batch/continuous flow operation		

Processing parameters

Among the processing parameters, an increase of the values of electric field strength (Bendicho, Barbosa-Cánovas & Martín, 2003; Elez-Martínez, Aguiló-Aguayo & Martín-Belloso, 2006; Giner et al., 2000; Ho, Mittal & Cross, 1997), total treatment time (Bendicho, Barbosa-Cánovas & Martín, 2003), number of pulses (Giner et al., 2000) or pulse width (Elez-Martínez, Aguiló-Aguayo & Martín-Belloso, 2006; Elez-Martínez,

Suárez-Recio & Martín-Belloso, 2007) lead to an increased enzyme inactivation. The effect of these parameters can be combined in the electrical energy applied (as shown in Eq. 1.2), and increase of this parameter reduced enzyme activity (Elez-Martínez, Aguiló-Aguayo & Martín-Belloso, 2006; Espachs-Barroso, Barbosa-Cánovas & Martín-Belloso, 2003). It is important to realise that the electrical energy used in these high intensity PEF studies is very large, up to 8 – 50 MJ/kg, therefore it seemed that heat denaturation due to ohmic heating and increased temperature cannot be ignored. Temperature has been reported to play an important role in enzyme inactivation during high intensity PEF processing, and increase of the temperature reduces enzyme activity (Min, Min & Zhang, 2003; Van Loey, Verachtert & Hendrickx, 2002; Yeom, Zhang & Chism, 2002).

PEF processing can be carried out in batch or continuous flow equipment, and some studies indicate that batch-mode is more effective in the inactivation of enzymes than a continuous system (Bendicho et al., 2002; Martín-Belloso & Elez-Martínez, 2005).

Different results have been reported about the effect of pulse polarity. Bipolar pulses led to more inactivation of polyphenol oxidase and pectin methylesterase than monopolar pulses (Elez-Martínez, Suárez-Recio & Martín-Belloso, 2007; Giner et al., 2002), while monopolar pulses were reported to be more effective in inactivation of peroxidase (Elez-Martínez, Aguiló-Aguayo & Martín-Belloso, 2006). Contrary, Giner et al. (2000) did not observe any effect of pulse polarity on PME activity.

Enzyme characteristics

The enzymatic inactivation using high intensity PEF also depends on type of enzyme, isoform and source. The sensitivity of enzymes to PEF treatment varies from enzyme to enzyme (Grahl & Märkl, 1996; Yang, Li & Zhang, 2004). Grahl & Märkl (1996) observed that lipase was most sensitive to PEF, followed by peroxidase (POD) and alkaline phosphatase. Yang and co-workers (2004) measured a difference in sensitivity as well, with most sensitive enzyme towards PEF in the order: polyphenol oxidase (PPO) > pepsin > peroxidase > chymotrypsin > lysozyme.

Yang and co-workers (2004) suggest that the sensitivity of the tested enzymes to high intensity PEF has no relationship with their thermostability and size. Peroxidase is the most thermostable enzyme among the tested enzymes, but it is not the most PEF resistant one. Therefore, Yang and co-workers suggest that the mechanism involved in inactivation of enzymes by PEF may be different from that by heat.

Different sensitivity was also observed for a different enzyme source and isoform. Giner et al. (2001) observed that PPO from pear was more resistant towards high intensity PEF than PPO from apple. Rodrigo et al. (2003a) reported more inactivation of a thermal sensitive isoform of PME than for a thermal resistant isoform, although the researchers ascribe this observation to be concentration dependent.

Product characteristics

Treatment medium characteristics form a third aspect that affects PEF efficiency in the reduction of enzyme activity. Contradicting results have been reported about the

effect of conductivity. Yang and co-workers (2004) reported that a higher electrical conductivity is more effective using high intensity PEF to inactivate pepsin and PPO, while Van Loey and co-workers (2002) reported no influence of medium conductivity on the inactivation of PPO and POD.

The pH of the medium has been reported to affect the inactivation of high intensity PEF. Van Loey and co-workers (2002) observed an increased PEF effectivity to inactivate POD when the pH was decreased to 4, while changes in pH did not influence the stability of PPO to PEF treatment. Yang and co-workers observed that pepsin was most stable at its optimum pH.

The effect of composition of the medium (presence of particles) on the effectiveness of high intensity PEF to inactivate enzymes is unclear. Some researchers report that the fat content of the medium affects the activity of enzymes by PEF (Bendicho, Barbosa-Cánovas & Martin, 2003; Castro et al., 2001b), while others did not observe a significant difference when changing the medium composition (Van Loey, Verachtert & Hendrickx, 2002). Similar controversial results are found about the effect of proteins, where some authors found a protective effect (Vega-Mercado et al., 2001), others found an enhancing effect (Bendicho, Barbosa-Cánovas & Martín, 2002) and a third research group found no effect (Van Loey, Verachtert & Hendrickx, 2002).

For citrus juice, inactivation of pectin methylesterase (PME) is important to improve shelf life. A summary of representative studies on high intensity PEF inactivation of PME in citrus juice was given by Buckow, Ng & Toepfl (2013). Based on this study, it can be concluded that most studies report substantial inactivation (78.0 – 92.7 %) of PME in orange or other citrus juice. Nevertheless, very high specific energy inputs (up to about 8 MJ/kg) were used in many of these studies to achieve a substantial level of PME reduction, which is much more than typically required for PEF inactivation of vegetative micro-organisms, ranging between 40 – 1000 kJ/kg (Toepfl et al., 2006), although exact conditions depend on the physicochemical properties of the food product, target micro-organism as well as process conditions as discussed in the previous section. Studies with a similar energy input as required to have microbial inactivation of target micro-organism in orange juice, showed about 40% inactivation of PME (Vervoort et al., 2011).

Therefore, the goal should be to optimize the PEF process by making use of the synergistic effects of PEF and mild heat on microbial and enzyme inactivation so that the specific energy input is lower than that of conventional thermal pasteurisation to achieve better product quality (20 kJ/kg assuming 95% heat recovery) (Toepfl et al., 2006).

1.3.7 Effect of PEF on quality attributes and shelf life

High intensity PEF processing of citrus juices at moderate temperatures ($T < 60$ °C), showed only a minimal impact on the physicochemical quality properties (pH, soluble solids, electric conductivity, colour, viscosity), nutritional values (vitamin C content, carotenoids) and the sensorial quality when compared to untreated juice (Cserhalmi et al., 2006; Hartyáni et al., 2011; Timmermans et al., 2011; Vervoort et al., 2011).

Studies comparing the effect of high intensity PEF at moderate temperatures (T < 60 °C) towards a thermal pasteurisation process (typically in the order of 95 °C for 30 s) showed less degradation of vitamin C (Elez-Martínez & Martín-Belloso, 2007; Min et al. 2003; Torregrosa et al., 2006), carotenoids (Cortés et al., 2006; Sánchez-Moreno et al., 2005), and volatile aroma compounds (Jia, Zhang & Min, 1999; Min, Min & Zhang, 2003; Yeom et al., 2000) in the high intensity PEF treated orange juices than in the thermal treated ones. However, it is important to note that both the intensity of the PEF treatment and the accompanying temperature involved, as well as the intensity of the thermal treatment (temperature and holding time) applied play an important role in the comparison, as no significant differences in colour, pH, soluble solids, sugar profile, the organic acid profile, bitter compounds, vitamin C, carotenoid profile, furfural and 5-hydroxymethylfurfural were observed between high intensity PEF (T_{max} = 58 °C) and a mild heat treatment (72 °C for 20 s) (Timmermans et al., 2011; Vervoort et al., 2011).

The shelf life of a product is defined as the period in which the product is still acceptable for human consumption (Jaeger, Meneses & Knorr, 2014). The shelf life of a high intensity PEF treated product is mainly determined by outgrowth of micro-organisms and remaining enzyme activity, although other chemical reactions can take place as well during the storage period, that can result into a loss of fresh flavour, degradation of ascorbic acid, cloud degradation and discoloration (Timmermans et al., 2011; Vervoort et al., 2011; Vervoort et al., 2012).

It has been demonstrated that the vitamin C content (Min et al., 2003; Torregrosa et al., 2006; Yeom et al., 2000), carotenoids (Cortés et al., 2006), and volatile content (Min et al., 2003; Yeom et al., 2000) was better retained in high intensity PEF-treated juices than in thermally processed juice (typically 95 °C for 30 s) and that less browning occurred in the high intensity PEF treated juice (Min et al., 2003; Yeom et al., 2000), when stored over a period of 60-196 days at refrigerated storage (4°C). Commercial application of PEF treatment at a preheating temperature of 40 °C, electric field strength of 20 kV/cm, energy input of approximately 120 kJ/kg and maximum temperatures < 60 °C (using intermediate cooling) resulted in a 5 $\log_{10}$ reduction of total bacterial count and *E. coli,* and a shelf life extension from 7 to approximately 21 days, while maintaining fresh-like taste and quality (Toepfl, 2011). To reduce the degree of spoilage and quality degradation due to enzyme reactions, a chilled distribution and storage is required.

1.4 Predictive modelling of inactivation kinetics

Understanding inactivation kinetics will help in the development and validation of optimal PEF process parameters; too mild electric treatment conditions will not lead to microbial and enzymatic inactivation, while too severe conditions will compromise food quality or damage the equipment. To determine these optimal PEF conditions, reliable data and appropriate modelling of inactivation kinetics are required to allow predictions of microbial and enzyme inactivation and establish the conditions for electric treatment.

Predictive models are useful tools that can help to understand a system or to predict future behaviour (Van Boekel & Zwietering, 2007), i.e. mathematical models can predict the responses of micro-organisms and enzymes to process parameters and environmental variables (McDonald & Sun, 1999). This section will mainly focus on predictive modelling of microbial inactivation kinetics, as there are not many studies on modelling the inactivation of enzymes after PEF processing, which is an interesting phenomenon in itself, and a reason to spend attention on this in the present thesis.

The construction of a successful predictive model depends on the availability of sufficient experimentally determined inactivation data and the accuracy of the model. Two widely used methods to collect data are end-point methods and methods based on inactivation curves (kinetics) (Álvarez, Condón & Raso, 2006). Analysis of only one end point (the effect of a single treatment condition) as employed in most published PEF experiments will not provide sufficient information to correctly model the inactivation of a micro-organisms or enzyme during PEF treatment (Álvarez, Condón & Raso, 2006), and is more suitable for validation studies in which individual death data points are compared with predictions by the model. Kinetic series are a preferred method to collect data. Generally, a PEF inactivation curve is a plot where the logarithm of survivors (microbiology) or remaining enzyme activity (enzymes) is plotted against the duration (time) of the inactivation process for a given treatment intensity, and a primary model is used to define a particular parametric line shape that is fitted to experimental inactivation data (Álvarez, Condón & Raso, 2006).

For thermal and non-thermal inactivation of micro-organisms, generally four types of microbial inactivation curves can be observed: linear inactivation curves, curves with a shoulder (having a delay in time or temperature before inactivation starts), curves with a tail (having inactivation up to a point and beyond this point the degree of inactivation reduces), and sigmoidal like curves with both a shoulder and a tail. The presence of a shoulder is assumed to indicate that some threshold must be exceeded before cells are affected by the stress, that is more often observed upon exposure to mild stress, whereas the presence of a tail is assumed to indicate that a minority of the population is more resistant to the stress than the majority of population (Metselaar, 2016). Several empirical models have been proposed to describe microbial inactivation curves using high intensity PEF, such as the Hülsheger-model (Hülsheger, Potel & Niemann, 1981), sigmoidal inactivation curves based on the Fermi equation (Peleg, 1995) or log-logistic model (Raso et al., 2000), but the Weibull model is most frequently used (Cebrián et al., 2008; Gómez et al., 2005a; Huang et al., 2014; Rodrigo et al., 2001), probably due to its simplicity and flexibility. Enzyme inactivation is modelled using the same models as used for microbial inactivation, including first-order inactivation, Hülshegers model, Fermi's model, Weibull model and second order polynomial equations (Giner et al., 2000; Giner et al. 2005; Min, Min & Zhang, 2003; Zhong et al., 2007).

Secondary models describe how the primary model parameters vary when changing the treatment conditions and environmental conditions. These secondary models can increase in complexity when the number of processing variables investigated increases (Saldaña et al., 2013).

According to the principle of parsimony, or Ockham's razor, the number of parameters in a model should be minimised to avoid overfitting. However, modelling is a delicate balance between under- and over-parameterisation and 'should be as simple as possible, but not simpler', according to a famous quote of Einstein. Model discrimination can be a helpful tool to determine the best performing model. A proper procedure will contain a penalty function for an increase in the number of parameters, as can be calculated and optimised using the Akaike Information Criterion (AIC) or Bayesian Information Criterion (BIC) (Van Boekel, 2009a).

When microbial inactivation kinetics of vegetative micro-organisms by high intensity PEFs are investigated, a concave downward survival curve including tailing as function of treatment time is usually observed (Álvarez et al., 2000; Álvarez et al., 2002; Álvarez et al., 2003b; Gómez et al., 2005a; Jayaram, Castle & Margaritis, 1992; Ohshima, Okuyama & Sato, 2002; Saldaña et al. 2010b). Several other studies have indicated first order kinetics for inactivation of vegetative micro-organisms by high intensity PEF and therefore a linear relationship between the logarithm of the number of survivors and the treatment time was established (Pérez et al., 2007; Rodrigo et al., 2003b; Sensoy, Zhang & Sastry, 1997). The microbial inactivation in these studies usually does not exceed more than 3-4 $\log_{10}$ cycles, so these studies might refer to the majority population ignoring more resistant minority populations (tailing). In most of the PEF studies that were performed in batch systems, pulse duration and number of pulses were found to have a similar effect on the inactivation of micro-organisms, and therefore these two are combined in the parameter treatment time, and inactivation kinetics is expressed as a function of this joint parameter (Abram et al., 2003). However, several authors reported that in a continuous PEF system, at constant treatment time, energy input and field strength, longer pulse width resulted in more inactivation than shorter ones (Abram et al., 2003; Aronsson et al. 2001; Wouters et al. 1999). These observations suggest that the treatment time is not an adequate parameter to describe inactivation kinetics in a continuous PEF system.

Besides that the empirical models are not mechanistic and hence have no physical meaning, they are not suitable for extrapolation of the data outside the experimental region (Smelt et al., 2002; Van Boekel & Zwietering, 2007). This calls for an alternative, preferably rational, model that can describe microbial inactivation with a minimal number of parameters, with independent variables that allows comparison to conventional heat treatment.

1.5 Aim and outline of this thesis

The effect of high intensity pulsed electric fields as alternative, mild process to thermal pasteurisation of fruit juices aiming for a better product quality, without compromising safety and shelf life has been intensively studied, as discussed in the previous sections in this chapter. Knowledge gaps were identified and will be discussed below, and research questions following from that are described.

Knowledge gaps

Although the effect of high intensity PEF processing parameters on the quality aspects of several food products has been extensively studied and show a good retention of quality, the influence of high intensity PEF processing parameters on microbial inactivation has mainly been studied in buffer systems and not in real foods. Besides the use of buffer systems, microbial inactivation is often studied using conditions or designs that are not (directly) applicable on an industrial scale: batch wise treatment, use of exponential decay pulses, long total treatment times and electric field strengths over 30 kV/cm. Moreover, the effect of microbial culture conditions and recovery conditions is not always taken into account, what could lead to an overestimation of the efficiency of the PEF process. The overview presented in this chapter points to the relevance and contribution of the influence of process parameters, microbial characteristics and treatment medium properties to microbial inactivation. Therefore, evaluation of process conditions that are technically applicable on an industrial scale using relevant micro-organisms in real food matrices is required to search for optimum treatment conditions, aiming at maximum safety with the best product quality.

The most important parameters in PEF processing are electric field strength, pulse width and temperature. The temperature of the treated product will rise proportionally with the treatment intensity (electric field strength and total treatment time), allowing a further inactivation of micro-organisms through a synergistic effect between pulses and temperature. Increase of the temperature or electric energy by the PEF process will result in more microbial inactivation, however the higher thermal load involved in the PEF treatment will also lead to a reduced product quality, in terms of freshness.

Most publications on PEF processing as alternative preservation technology focus on the effect of high intensity pulsed electric fields (E > 20 kV/cm) for a short period of time, using a pulse width between 2 and 20 µs with a typical total treatment time of several milliseconds, to minimize the heat load.

The use of moderate electric field strengths, such as those between 0.5 – 5.0 kV/cm, has been studied in the field of biology, and results showed electroporation effects in micro-organisms, when exposure time was long enough. However, application of the pulses took up to hours, as a temperature increase of the sample was avoided in these studies (El Zakhem et al., 2006a; El Zakhem et al., 2007). These moderate intensity conditions have been extensively studied as a pre-step in mass transfer processes in food- and biotechnology, aiming to mildly disintegrate plant cells. Although the electroporation effect in plant cells was observed, these process conditions have received no attention for application as an alternative preservation process to inactivate micro-organisms, probably due to small size of the microbial cells, demanding for a higher transmembrane potential difference to induce electroporation than larger sized eukaryotic cells.

Aim and research questions

This thesis aims at providing insight how moderate and high intensity Pulsed Electric Field processing conditions, product matrix and characteristics of target species affect microbial inactivation, shelf life, enzyme activity and quality of fruit juices.

In order to achieve this aim, the following research questions were formulated:

- Can moderate intensity PEF combined with heat act as an alternative process to high intensity PEF to preserve fruit juice?
- What are the critical process parameters required to achieve microbial inactivation for target species in different product matrices?
- Is there an additional, possibly synergetic, non-thermal pulse or electric field effect next to thermal effects responsible for microbial inactivation?
- What is the effect of high intensity PEF parameters and storage temperature on shelf life of a fruit smoothie?
- How would a combined process of moderate electric field strength and heat affect the quality and enzyme activity of a fruit juice?

Outline

This leads to the following outline of the thesis:

Chapter 2 describes the impact of high intensity PEF processing conditions on the inactivation of four spoilage and pathogenic micro-organisms in different fruit juices that vary in pH and conductivity.

In Chapter 3, the effect of high intensity PEF processing conditions and storage temperature on the inactivation and outgrowth of yeasts and moulds naturally present in a fresh fruit smoothie and impact on shelf life was studied.

In Chapter 4 a primary, rational thermodynamic model based on Gaussian distributions and Eyrings rate constant was developed to describe inactivation kinetics of enzymes and micro-organisms exposed to heat and chemical (acid) treatment. This model, named Gauss-Eyring, was evaluated in Chapter 5 to describe non-linear thermal inactivation data for five different micro-organisms in orange juice. Based on the properties of the model, predictions of thermal inactivation as a function of temperature for short holding times were made, to facilitate the comparison to a PEF process.

In Chapter 6, the impact of high intensity PEF conditions and moderate intensity PEF conditions combined with heat was studied on the inactivation of five relevant spoilage and pathogenic micro-organisms in orange juice. Inactivation kinetics were fitted using the Gauss-Eyring model and compared to a thermal reference process with same holding time as the PEF treatment, based on parameters estimated in Chapter 5, to study if the PEF conditions comprise a synergetic non-thermal pulse effect to the thermal effect responsible for the inactivation of the micro-organisms. Based on these results, the most interesting pulse conditions were tested on two micro-organisms in fruit juices varying in pH and conductivity.

In Chapter 7 the effect of the most promising moderate intensity PEF conditions established in chapter 6, was evaluated on quality characteristics of freshly squeezed orange juice, including colour, vitamin C content, pectin methylesterase inactivation and volatile flavour compounds. A kinetic series of PEF treated orange juice with varying maximum temperatures was compared to untreated juice and two conventional thermal treatments, based on industrial conditions used. Based on the results, optimal PEF conditions could be established.

Finally, the results of this work are discussed in Chapter 8 and concluding remarks and recommendations for future research are presented.

Chapter 2

High Intensity Pulsed Electric Field processing of different fruit juices: Impact of pH and temperature on inactivation of spoilage and pathogenic micro-organisms

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Abstract

High intensity pulsed electric field (PEF) technology can be used for the inactivation of micro-organisms and therefore for preservation of food products. It is a mild technology compared to thermal pasteurisation because a lower temperature is used during processing, leading to a better retention of the quality. In this study, pathogenic and spoilage micro-organisms relevant in refrigerated fruit juices were studied to determine the impact of process parameters and juice composition on the effectiveness of the high intensity PEF process to inactivate the micro-organisms. Experiments were performed using a continuous-flow PEF system at an electric field strength of 20 kV/cm and pulse width of 2 μs with variable frequencies to evaluate the inactivation of *Salmonella* Panama, *Escherichia coli*, *Listeria monocytogenes* and *Saccharomyces cerevisiae* in apple, orange and watermelon juices.

Kinetic data showed that under the same conditions, *S. cerevisiae* was the most sensitive micro-organism, followed by *S*. Panama and *E. coli*, which displayed comparable inactivation kinetics. *L. monocytogenes* was the most resistant micro-organism towards the treatment conditions tested. A synergistic effect between temperature and electric pulses was observed at inlet temperatures above 35 °C, hence less energy for inactivation was required at higher temperatures. Different juice matrices resulted in a different degree of inactivation, predominantly determined by pH. The survival curves were nonlinear and could satisfactorily be modelled with the Weibull model.

2.1 Introduction

There is a demand for mild preservation processes that can enhance the shelf life of high quality foods, without affecting the quality and safety of the food products or using chemical preservatives. High intensity pulsed electric field (PEF) processing is one of these mild preservation techniques that has been investigated during the last decades as an alternative for thermal processing without compromising sensorial and nutritional properties of food (Hartyáni et al., 2011; Vervoort et al., 2011). PEF effectiveness in the inactivation of vegetative bacterial cells, yeast and moulds, has been demonstrated, however, microbial spores are resistant to PEF treatment (Grahl & Märkl, 1996; Van Heesch et al., 2000; Wouters et al., 2001). Therefore, PEF should be considered as a pasteurisation method, and fruit juices are a suitable candidate product, since the acid conditions of these products control the germination of microbial spores (Raso & Barbosa-Cánovas, 2003).

The mechanism of PEF processing to inactivate micro-organisms is not fully elucidated, but could be explained as a combination of electroporation and electropermeabilization (Teissié, Golzio & Rols, 2005; Weaver and Chizmadzhev, 1996; Wouters et al., 2001). Induction of an (external) electric field on the membrane of a micro-organism can lead to local instabilities, resulting in pores in the membrane (electroporation). As a result of electroporation, an enhanced permeability across the membrane occurs (electropermeabilization) that, depending on the applied electric field, leads to cell death (irreversible, complete inactivation) or resealing of the cell to its initial viable state (reversible, sub-lethal or partial inactivation). For high intensity PEF processing, strong electric fields (E = 5 – 50 kV/cm) are applied to ensure irreversible effects, leading eventually to cell death.

A considerable number of studies have been conducted to determine the inactivation kinetics of micro-organisms by PEF in liquid food products. Comparison of the published experimental data is not straightforward, since the degree of microbial inactivation is strongly dependent on the type and design of the equipment, pulse shape, and intensity of the pulses in terms of field strength, energy and number of pulses applied (Heinz, Toepfl & Knorr, 2003). Moreover, inactivation is influenced by the properties and type of (food) matrix, and by the potential addition of preservatives (García et al., 2005b; Gurtler et al., 2011; Saldaña et al., 2011).

A large number of studies have been carried out in buffer systems in order to gain insight into the mechanism of inactivation and process conditions required for microbial inactivation using varying conditions, including electrical conductivity, pH, water activity and ionic composition (Álvarez et al., 2003a, 2003b; Aronsson & Rönner, 2001; Aronsson, Rönner & Borch, 2005; Gómez et al., 2005a, 2005b; Toepfl, Heinz & Knorr, 2007b; Wouters et al., 1999). Inactivation studies of micro-organisms in fruit juices using PEF have been described for a number of studies (Elez-Martínez et al., 2004a, 2004b; Gómez et al., 2005a, 2005b; Gurtler et al., 2010; Heinz, Toepfl & Knorr, 2003; Mosqueda-Melgar, Raybaudi-Massilia & Martín-Belloso, 2007; Qin et al., 1995b; Rodrigo et al., 2001, 2003; Saldaña et al., 2011), however only a single micro-organism or one type of juice was selected to evaluate the effect of PEF. Here, we describe a comparative analysis including species representing spoilage and pathogenic micro-organisms in varying juices using

treatment conditions based on an existing industrial scale system for fresh fruit juice processing with a capacity of 1500 L/h and electric field strength of 20 kV/cm.

For industrial implementation, it is of paramount importance to determine and validate effective process conditions and constraints in real food systems with pathogens or realistic surrogates to minimize microbial hazards. Furthermore, it is important to test this on a representative PEF system, with the same configuration as the industrial system, as the configuration of the system has a considerable effect on the effectiveness of inactivation.

The objectives of this study were to investigate inactivation of spoilage and pathogenic micro-organisms in fruit juice, to assess the impact of process parameters and juice composition on the effectiveness of the PEF process, and to find a suitable model to predict the minimum required process conditions.

2.2 Material and Methods

2.2.1 Selection of juice products

Fruit juices used in this study were fresh, non-heated not-from concentrate (NFC) juices delivered by a commercial producer of fruit juices directly after production (orange juice, orange-strawberry juice, tropical juice) or delivered frozen at −18 °C (lime juice). Watermelon juice used to evaluate the survival of relevant micro-organisms was extracted directly from watermelons obtained from a local supermarket, using a table juice extractor type HR 1861 (Philips) to obtain watermelon juice. One single batch of 30 L juice was squeezed, mixed and frozen in batches of 1 L. Prior to an experiment, the watermelon juice was thawed and prepared similar as the other juices. Since the matrix composition of NFC juices is subject to seasonal variation, apple juice (Appelsientje Goudappel) and orange juice (Minute Maid) from concentrate (FCJ) were used for inactivation kinetics studies to ensure reproducibility of experiments over time.

2.2.2 Preparation and analysis of juices

For all experiments, juices were pasteurized by heating them in glass bottles filled with 1000 mL of fruit juice for 45 min in a water bath at 85 °C. For inactivation kinetics, orange juice and watermelon juice were sieved after pasteurisation using a sterile sieve (0.225 mm pore size) to remove pulp, while apple juice did not contain any pulp. Electric conductivity (Hi 933000, Hanna instruments) and pH (Metrohm 744, Metrohm, Herisa, Switzerland) were measured of each juice after inoculation with micro-organisms, and shown in Table 2.1. For some experiments, the pH of watermelon juice was changed from 5.3 to 3.6 by adding HCl.

Table 2.1 pH and conductivity measured at 20 °C of juice matrices after inoculation with micro-organisms.

Matrix	pH	Conductivity (S/m)
Apple juice	3.5	0.26
Orange juice	3.7	0.38
Watermelon juice	5.3	0.30
Watermelon juice	3.6	0.35

2.2.3 Micro-organisms and culture conditions

Pathogenic and spoilage micro-organisms were selected based on their association with and prevalence in fruit juices, their ability to survive and to grow in the juice matrices selected in this study and if known, their resistance towards PEF, as this is inherent to species and strain level (Gurtler et al., 2010). The micro-organisms were tested in fresh NFC juices and FCJ juices with variation in pH, and evaluated after 1, 4 and 7 days of incubation. Comparable results of survival were found between both types of juices. The mentioned criteria lead to the choice for the micro-organisms shown in Table 2.2. No survival (i.e., decline in number of micro-organisms) was observed in high acidic juices (pH 1.8), survival (i.e., a stable number of micro-organisms) was observed in acid juices (pH 3.6) and growth (i.e., an increase in number of micro-organisms) was observed for all micro-organisms in weak acid juices (pH 5.3).

Fresh cultures of the yeast strain *Saccharomyces cerevisiae* (Table 2.2) were prepared by plating from frozen stocks on glucose-peptone yeast (GPY) agar plates, containing 40 g glucose, 5 g peptone, 5 g yeast extract and 15 g agar per 1 L distilled water. Plates were incubated overnight at 25 °C. One single colony isolate was used for inoculation of a 50 mL flask containing 10 mL GPY broth and cells were cultivated for 24 h at 25 °C in a shaking air incubator (Innova, 180 rpm). Of this overnight culture, 0.1 mL was used for inoculation of 9.9 mL fresh GPY medium supplemented with 1% of glucose in a 50 mL flask and incubated for exactly 24 h at 20 °C and 180 rpm. Fresh cultures of *Salmonella enterica* serotype Panama and *Escherichia coli* strains (Table 2.2) were prepared from frozen stock cultures that were plated on TSB (Oxoid) agar plates and incubated overnight at 37 °C. *Listeria monocytogenes* strain (Table 2.2) was cultivated on BHI (Oxoid) agar plates and incubated overnight at 30 °C. A single colony isolate was used to inoculate a 50 mL flask with 10 mL TSB (*S*. Panama, *E. coli*) or BHI broth (*L. monocytogenes*) and cultivated for 24 h at 20 °C in an Innova shaking incubator (180 rpm). Of this culture, 0.1 mL was used to inoculate 9.9 mL fresh TSB or BHI broth supplemented with 1% glucose (50 mL flask) and incubated for 24 h at 20 °C and 180 rpm.

Table 2.2. Bacterial strains and yeast strain used in this study.

Species/strains used	Source of isolation	Reference
Escherichia coli ATCC 35218	PEF resistant surrogate for *E. coli* O157:H7	Gurtler et al. (2010)
Listeria monocytogenes Scott A	Clinical isolate	Fleming et al. (2010)
Saccharomyces cerevisiae CBS 1544	Fermenting fruit juice isolate	Zhang et al. (1994)
Salmonella Panama 10908	Human outbreak isolate	Noël et al. (2010)

ATCC: American Type Culture Collection, USA
CBS: Centraal Bureau voor Schimmelcultures (Fungal Biodiversity Centre, Utrecht, The Netherlands)

2.2.4 Determination of PEF inactivation kinetics

Inactivation kinetics of selected spoilage and pathogenic micro-organisms associated with fruit juice were determined to identify optimal PEF conditions for inactivation of the target organisms in apple juice and orange juice (FCJ) and watermelon juice (NFC). Inactivation kinetics were determined for single strain pathogens (or a pathogen surrogate) or spoilage micro-organisms rather than for a cocktail of strains. Overnight grown cells of *S. cerevisiae* (GPY), *S.* Panama and *E. coli* (TSA) or *L. monocytogenes*

(BHI) were pelleted by centrifugation (4000 rpm, 5 min) at 20 °C, washed with 100 mL PSDF and resuspended in 100 mL juice. Cells were diluted (1/10 ratio) in 900 mL juice in a glass bottle to reach approximately 10^7 cfu/mL (*S. cerevisiae*) or 10^8 cfu/mL (*E. coli, S*. Panama, *L. monocytogenes*).

Two individual one liter bottles were inoculated with the selected microbial strain and used to perform duplicate PEF experiments.

All samples, prior to or after PEF treatment were immediately placed on ice after collection. The number of viable cells was determined by plating 100 μL of serially diluted PEF-treated juice in sterile peptone physiological salt diluent (PSDF) in duplicate on suitable agar plates supplemented with 0.1% sodium pyruvate to enhance outgrowth of sub-lethally damaged cells (McDonald, Hackney & Ray, 1983; Sharma et al., 2005). Surviving cells were enumerated after 5 – 7 days at 25 °C (*S. cerevisiae*), at 37 °C (*S*. Panama, *E. coli*) and 30 °C (*L. monocytogenes*).

2.2.5 PEF processing

The PEF system used was a continuous-flow system, where the configuration was a downscaled copy from the pilot-scale PEF equipment described by Mastwijk (2006) and Timmermans et al. (2011) and from an industrial scale system of 1500 L/h capacity. Specific attention was paid to design criteria, to guarantee the homogeneity of the electric field when downscaling the treatment device. Higher field strengths than 30 kV/cm will lead to arcing and the formation of toxic substances (Mastwijk, 2006), and are therefore not used in this study.

The inoculated juice was pumped by means of a peristaltic pump (Masterflex L/S pump, Cole-Parmer) through a 6 mm (disposable) silicone hose (Masterflex 6424-16, Cole-Parmer) at a flow rate of 14 – 16 mL/min. Before PEF treatment, the juice was preheated, by heating in continuous flow through a 1 m × 6 mm diameter SS316 heat spiral that was immersed in a water bath at 43 ± 1 °C. Next, the liquid was pumped through a co-linear PEF treatment device, comprising two vertically positioned treatment chambers with each a diameter of 1.0 mm and a gap of 2.0 mm, involving a residence time of 13.5 ms in the treatment chambers at a flow rate of 14 mL/min. The temperature at the outlet was measured using a 0.3 mm thermocouple type K (TM-914C, Lutron, Taiwan), and yielded 36 ± 1 °C when juice was preheated and PEF was turned off. Subsequently, the juice was pumped through a cooling spiral of 2 mm diameter and 500 cm length cooled on melting ice. After cooling, the samples of the liquid were collected at the exit under aseptic conditions. The throughput of the juice in the system was periodically measured using a digital scale analytical balance (Sartorius, Gottingen, Germany) by recording the weight of the collected juice.

Prior to the experiment, all parts that came into contact with the juice were sterilized by moist heat in an autoclave for 20 min at 120 °C. The system was started using 1 L of pasteurized juice to fill the system and to obtain stationary processing conditions of flow in the range of 14 – 16 mL/min. A sample of this start-up liquid was taken at the exit as a negative control. After this, the pasteurized juice at the inlet was replaced by a bottle of inoculated juice. The start over occurred at the most intense

PEF conditions (i.e. highest repetition rate). A stationary state was reached before the inoculated juice reached the PEF treatment chamber. The PEF treated juice was collected every 5 min after the repetition frequency was set to lower levels. The collected samples were treated at the following repetition frequencies and number of pulses are indicated when a flow rate of 14 mL/min was used: 964 Hz (13 pulses), 785 Hz (10.6 pulses), 650 Hz (8.8 pulses), 560 Hz (7.5 pulses), 390 Hz (5.3 pulses), 270 Hz (3.6 pulses), 220 Hz (3.0 pulses), 180 Hz (2.4 pulses), 140 Hz (1.9 pulses), and 120 Hz (1.6 pulses) and finalized by sampling of a positive control (no treatment). Monopolar square wave pulses of τ = 2.0 µs duration at a field strength of 20 kV/cm were used. Pulse waveform, voltage, and intensity in the treatment chambers were recorded with a digital oscilloscope Tektronix TDS3052B (Tektronix Inc., Beaverton, USA). Energy balance was made up and correct for at least 90%, according to Mastwijk et al. (2007). Specific energy used for each condition was calculated according to Eq. 2.1, where w is the specific energy (kJ/kg), T_{out} is the outlet temperature (°C), T_{in} is the inlet temperature (°C) and c_p is the specific heat capacity, which is 3.8 kJ/kg·K for fruit juice of 10 – 12° Brix.

$$w = \left(T_{out} - T_{in}\right) \cdot c_p \qquad \text{(Eq. 2.1)}$$

2.2.6 Experimental design and statistical analysis

Data was collected for each species in two independent PEF experiments, with cleaning and sterilisation between the two experiments. Different inactivation models were evaluated (linearized first order model, exponential decay, sigmoidal and Weibull). Based on the goodness of the fit, evaluated by calculating R^2 and root-mean-square error (RMSE) values, it was found that the empirical model of Weibull was the one that fitted best to all data. A mathematical model based on the Weibull distribution was therefore used to fit the survival data ($\log_{10}$ survival fraction vs. specific energy) of all micro-organisms, as shown in Eq. 2.2 where N is the number of micro-organisms that survived the treatment, N_0 is the initial number of microbial population, w is the specific energy used (kJ/kg) and α and β are the two parameters of the distribution; α is called the scale parameter (a characteristic of the specific energy used) and β is the shape parameter (Van Boekel, 2002).

$$log_{10}\left(\frac{N}{N_0}\right) = -\left(\frac{w}{\alpha}\right)^{\beta} \qquad \text{(Eq. 2.2)}$$

Parameters were estimated via nonlinear regression using least squares. Individual results were modelled using Gnu-plot.

2.3 Results and Discussion

2.3.1 Influence of inlet temperature prior to PEF treatment

Initial inactivation experiments were performed in apple juice at an inlet temperature of 20 °C and an electric field strength of 20 kV/cm. Under the tested conditions, inactivation of *S*. Panama was virtually absent up to an input of specific energy of

60 kJ/kg. Further increase of specific energy with a concomitant temperature rise did inactivate cells. To substantiate whether the inactivation resulted from the higher specific energy input or that cells were more susceptible to PEF at elevated temperature, the experiment was repeated using an inlet temperature of 36 °C. This experiment confirmed that at 36 °C *S*. Panama cells were inactivated at lower specific energy input (Fig. 2.1A). Similar findings were obtained for *S. cerevisiae*, where the inactivation was enhanced by an elevated inlet temperature from 20 °C to 36 °C (Fig. 2.1B). Data showed that the initial temperature had a strong influence on the specific energy required to inactivate the micro-organisms, with less specific energy required to obtain a similar level of inactivation at elevated temperatures. Similar observations have been described for *Listeria innocua* and *E. coli* (Heinz, Toepfl & Knorr, 2003; Toepfl, Heinz & Knorr, 2007b; Wouters et al., 1999).

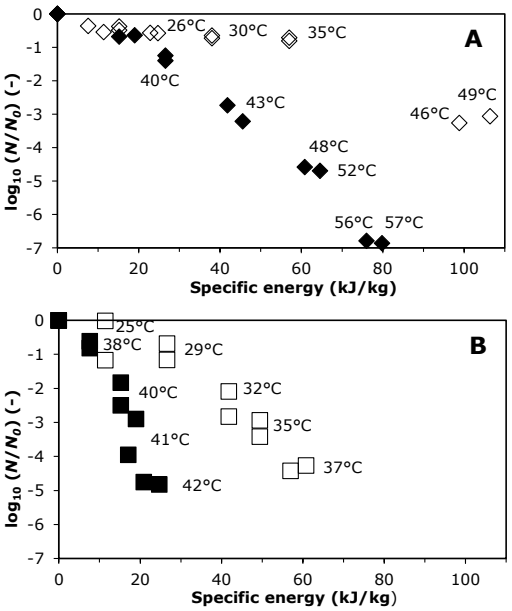

Figure 2.1. Reduction of viable counts of A) *S*. Panama added to apple juice after PEF treatment, preheated at 20 °C (◇) and 36 °C (◆) and B) *S. cerevisiae* precultured in apple juice after PEF treatment, preheated at 20 °C (□) and 36 °C (■).

The effect of the starting temperature on the efficacy of the PEF treatment may be explained by the temperature dependent characteristics of the membrane of the micro-organisms. Phase transitions of the phospholipids from gel to liquid-crystalline phase are temperature related, which affects the stability of the cell membrane at higher temperatures (Stanley & Parkin, 1991). The critical membrane breakdown potential decreases when the temperature of the solution increases (Coster & Zimmermann, 1975), and consequently electroporation occurs at lower external electric fields. This suggests, together with data shown in Fig. 2.1, a synergistic effect between temperature and electric pulses, implying that every additional PEF pulse is more effective than the

previous one: the temperature increases as a result of pulsing, leading to a weaker cell and making it more vulnerable towards the next PEF pulse.

For further experiments, an inlet temperature of 36 °C was chosen. At inlet temperatures exceeding 36 °C, the outlet temperature reaches critical levels where product quality was compromised.

2.3.2 PEF effectiveness towards different microbial species in diverse juice matrices

The inactivation of *S. cerevisiae*, *S*. Panama, *E. coli* and *L. monocytogenes* by PEF treatment was investigated in apple juice (Fig. 2.2A), orange juice (Fig. 2.2B) and watermelon juice (Fig. 2.2C). All species were susceptible to PEF treatment in apple juice, and inactivation curves obtained showed a non-linear response (Fig. 2.2A). With the exception of *L. monocytogenes*, similar findings were obtained for orange juice (Fig. 2.2B), but a reduced susceptibility was pronounced in watermelon juice (Fig. 2.2C). PEF sensitivity followed the order *S. cerevisiae* > *S*. Panama > *E. coli* > *L. monocytogenes*, meaning that the energy expense to inactivate the bacteria is higher than for yeast. Therefore, process validation using yeast species can lead to an overestimation of effectiveness of the process conditions. Validation designs that include (pathogenic) bacteria on identical small scale equipment are therefore preferred.

The reduced sensitivity of *L. monocytogenes* towards electric pulses compared to that of other micro-organisms might be explained by two factors. First, the size and shape of the micro-organisms affect the required electric field to lethally damage the cells, where at smaller cell sizes, a lower membrane potential is induced by an external field, leading to a higher microbial resistance to the treatment (Álvarez, Condón & Raso, 2006; Hülsheger, Potel & Niemann, 1983; Zimmermann, Pilwat & Riemann, 1974). Moreover, the shape of the micro-organism has an influence on the membrane potential, where a rod-shaped cell requires an electric field more than five times stronger than that required by a spherical shaped cell with the same characteristic dimensions (Heinz et al., 2002). As *L. monocytogenes* is much smaller (short rods, 0.4 – 0.5 µm × 0.5 – 2 µm) than *S*. Panama (straight rods, 0.7 – 1.5 µm × 2 – 5 µm) and *E. coli* (straight rods, 1.1 – 1.5 µm × 2.0 – 6.0 µm), it costs more energy to inactivate this micro-organism, hence it requires less energy to inactivate the large *S. cerevisiae* (ellipsoidal shape, 3 – 15 µm × 2 – 8 µm) (characteristic dimensions taken from Bergey (1986)). A second argument is that *L. monocytogenes* is a Gram-positive bacterium with a cell membrane structure different from the other tested bacteria that are Gram-negative. Research of Hülsheger, Potel & Niemann (1983), and Toepfl, Heinz & Knorr (2007b), also described that this could have an influence on PEF sensitivity.

The reduced sensitivity of *L. monocytogenes* towards PEF was also found by Gómez et al. (2005a), and Saldaña et al. (2010c), where at field strengths up to 20 kV/cm inactivation was virtually absent. Increase of the high intensity field strength up to 35 kV/cm significantly improved sensitivity of *L. monocytogenes* towards PEF.

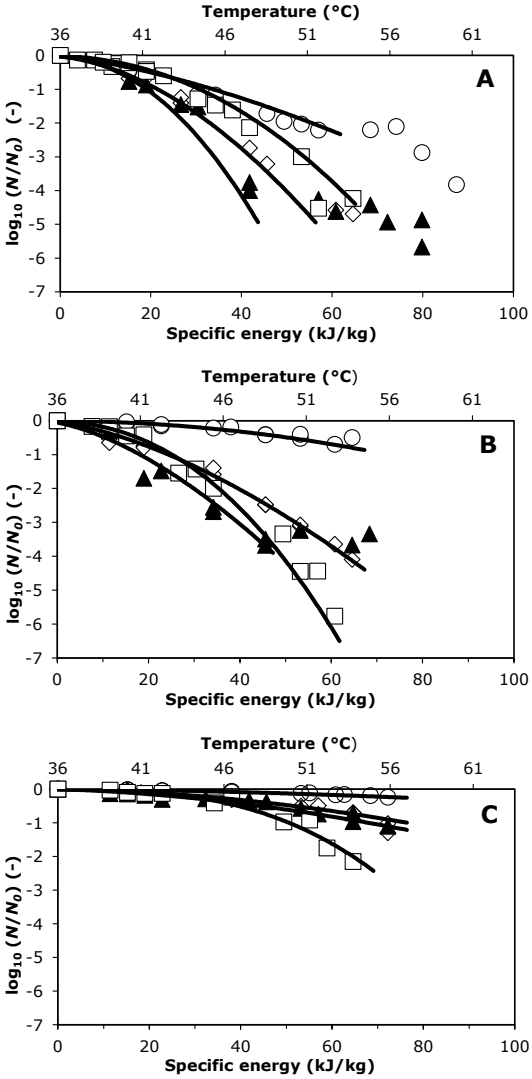

Figure 2.2. Reduction of viable counts of *S.* Panama (◇), *E. coli* (▲), *L. monocytogenes* (○) and *S. cerevisiae* (□) added to apple juice, pH 3.5 (A), orange juice, pH 3.7 (B), and watermelon juice, pH 5.3 (C) after PEF treatment, preheated at 36 °C.

The high resistance of *L. monocytogenes* in orange juice shown in this study is remarkable, showing virtually no inactivation, where for apple juice, 3.5 $\log_{10}$ inactivation at a specific energy input of 87 kJ/kg was reached. There was a slight difference in the pH of apple juice (pH 3.5) and orange juice (pH 3.7) which could have influence on the observed difference in sensitivity. Similar findings were reported previously for heat treated *E. coli* O157:H7 in apple and orange juices with a higher resistance in orange juice (Mazzotta, 2001). The reduced sensitivity of *L. monocytogenes* was pronounced even more in the watermelon matrix (pH 5.3) (Fig. 2.2C), and a remarkably higher

resistance was observed for the other tested species as well. To determine if this higher PEF resistance of the micro-organisms in watermelon juice could (in part) be explained by a pH effect or, alternatively, induced by the presence of other components in watermelon juice, experiments were carried out where the pH of watermelon juice was decreased with HCl from pH 5.3 to 3.6. The results, presented in Fig. 2.3, show an enhanced performance of PEF inactivation of micro-organisms when the pH was reduced. The shape of the inactivation curve of micro-organisms in watermelon juice with an adapted pH is comparable to the curves shown for apple and orange juices, which suggests that the observed differences were caused by pH effects.

It is assumed, that the role of pH of the medium in the inactivation of micro-organisms is related to the fact that most micro-organisms maintain the cytoplasmic pH near neutrality (Corlett & Brown, 1980). The pH of the medium affects the effectivity of weak organic acids, present in fruits, by influencing the ratio of non-dissociated and dissociated organic acids. A low pH favours formation of non-dissociated organic acids, the form that may pass the cell membrane and enter the cytoplasm. Inside the cell it can dissociate in the cytoplasm and lead to acidification. Protons may enter the cell by passive influx or via proton dependent transporters. Reduction of the intracellular pH thereby affects the biochemical processes in the cell including transport processes over the membrane, redox state, and enzyme activities (Cotter & Hill, 2003). Similar observations in pH difference were found for Gram-positive and Gram-negative micro-organisms in buffer solutions (Álvarez et al., 2002; García et al., 2005b; Geveke & Kozempel, 2003; Gómez et al., 2005a, 2005b; Saldaña et al., 2010c; Wouters et al., 1999).

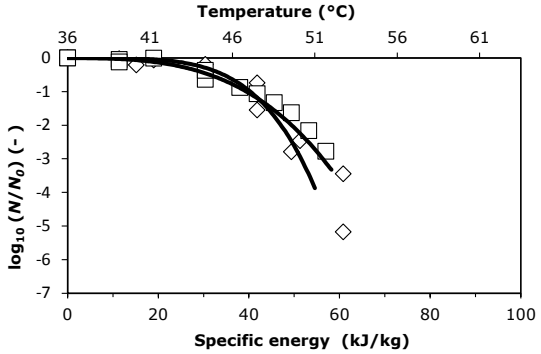

Figure 2.3. Reduction of viable counts of *S.* Panama (◇) and *S. cerevisiae* (□) in watermelon juice after addition of HCl to pH 3.6 and PEF treatment, preheated at 36 °C.

2.3.3 Mathematical modelling

Microbial inactivation data for the different juice matrices were used to construct a predictive model for an optimal PEF process. The empirical model of Weibull (Eq. (2.2)) was fitted to inactivation data obtained from plotting the decimal logarithmic inactivation of the survival fraction *versus* specific energy up to the point that tailing started. The estimated parameters α and β obtained from the different micro-organisms in the tested juices are shown in Table 2.3. The goodness of fit was evaluated by calculating

R^2 and RMSE values. The determination coefficient R^2 for each model describing each micro-organism in varying medium was higher than 0.93, which means that less than 7% of the total response variation remained unexplained by the Weibull equation. The values for parameter RMSE were in the range of 0.01 to 0.56, and can be assumed close to the observed data (Saldaña et al., 2011).

Another test for judging the applicability of the Weibull model is by plotting the observed data to the model calculations, as shown in Fig. 2.4. The difference between a point in the graph and the line of equivalence is a measure of the inaccuracy of the corresponding estimation. Since the data points of all micro-organisms are randomly distributed above and below the equivalence line, no systematic tendency is found, and therefore the Weibull model studied was satisfactory in terms of describing all data being analysed.

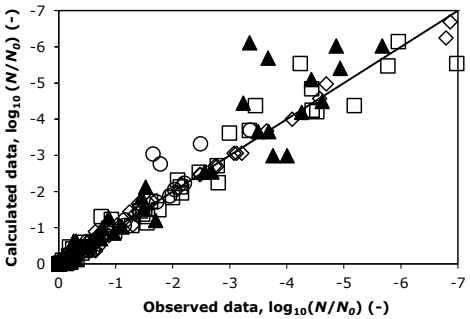

Figure 2.4. Plot of observed values of microbial inactivation of *S.* Panama (◇), *E. coli* (▲), *L. monocytogenes* (○) and *S. cerevisiae* (□) in apple, orange, watermelon and adapted watermelon juice vs. calculated values using the Weibull equation, with the line y = 1 representing a perfect fit.

As an indication for the quality of the estimated model, the statistical correlation between the parameters α and β (expressed in the correlation coefficient) has been calculated. If this correlation coefficient is > 0.99, it signals that the parameter estimates could not be well estimated in the regression procedure (Van Boekel, 2002). The results show that there is no such problem with the current dataset. Modelling of the data where the pH of watermelon juice was decreased showed an increase in the statistical correlation.

The shape factor β in Table 2.3 indicates that the survival curves of *S*. Panama, *E. coli*, *S. cerevisiae* and *L. monocytogenes* fitted with the Weibull model were all concave downward (β > 1), which was also obvious from Figs. 2.1, 2.2 and 2.3. Downward concavity (β > 1) indicates that remaining cells become increasingly damaged, whereas upward concavity (β < 1) indicates that remaining cells have the ability to adapt to the applied stress (Van Boekel, 2002). Therefore, concave downward (β > 1) survival curves of all micro-organisms tested in this research can be interpreted as evidence that the microbial cells show the tendency to become weaker when specific energy and temperature increase, indicating that accumulated damage due to synergy occurs. This synergistic effect between temperature and pulses is in line with the results of the experiments on the influence of inlet temperature on PEF inactivation,

as described in Section 2.3.1. Contradicting, in literature concave upward ($\beta < 1$) survival curves of *E. coli* (Álvarez et al., 2003c; Rodrigo et al., 2003c; Saldaña et al., 2010c), *Lactobacillus plantarum* (Gómez et al., 2005b; Rodrigo et al., 2001), *Yersinia enterocolitica* (Álvarez et al., 2003b), *S. enterica* serovars (Álvarez et al., 2003d; Saldaña et al., 2010c) and *L. monocytogenes* by Álvarez et al. (2003a) and Gómez et al. (2005a) were reported. It is difficult to compare these studies with our results, since different PEF equipment with varying treatment chambers and settings were used. All these studies with an upward concavity had in common that they operated at a maximum temperature of 35 °C and used square-wave pulses. If we consider the data up to 35 °C, we observe the same findings: survival of *S*. Panama up to 35 °C as shown in Fig. 2.1A (open symbols) gave comparable inactivation curves as described by the abovementioned studies with concave upward parameters (α = 124.95, β = 0.34, R^2 = 0.96, RMSE = 0.05), and when more pulses were given, PEF showed to be more effective in inactivation, leading to concave downward parameters when Weibull fit was performed over data up to 49 °C (α = 50.43, β = 1.56, R^2 = 0.92, RMSE = 0.32). This suggests that the synergistic effect between temperature and pulses is apparent at temperatures above 35 °C, and was therefore not observed in the other studies.

Evaluation of the Weibull parameters α and β in the aforementioned studies with respect to the variable electric field strengths, showed no influence on the β parameters when higher or lower electric field strengths were used. Nevertheless, increase of the electric field strength greatly affects the α parameter, showing a more efficient PEF process, what is expressed by a lower energy use or shorter treatment time. When the parameters α and β are compared for all different micro-organisms, it can be seen that the β-value for the *S. cerevisiae* dataset was higher compared to that for other micro-organisms, confirming that *S. cerevisiae* is more susceptible to the PEF treatment than other micro-organisms. The energy needed to inactivate 5 $\log_{10}$ cycles is calculated to facilitate the comparison among micro-organisms and matrices. Based on the estimated α and β parameters, a calculation is made to estimate the amount of specific energy necessary to have a 5 $\log_{10}$ reduction of a certain micro-organism in a specific fruit juice (Table 2.3). Most energy is necessary to inactivate the micro-organism *L. monocytogenes* in watermelon juice. The dependence of the parameters α and β on the pH of the different juices is shown in Fig. 2.5. Although limited experiments with a variable pH were carried out, it can be concluded from Fig. 2.5A that the β parameter does not depend on pH in a systematic way. Contrary, the α parameter, depicted in Fig. 2.5B, seems to be dependent on the pH of the fruit juice for all micro-organisms tested. In low-acid fruit juices, more specific energy is needed to inactivate micro-organisms than in high acidic fruit juices. The effect of acidification of the low-acid watermelon juice of pH 5.3 to pH 3.6 is indicated with the filled symbols in Fig. 2.5, showing no effect on the β parameter, but a reduction of the α parameter. Similar results for the shape independency and scale dependency towards pH were found for PEF treatment of *L. monocytogenes* and *L. plantarum* in media with varying pH (Gómez et al., 2005a, 2005b).

Table 2.3. α and β values estimated from the fitting of the mathematical model based on the Weibull distribution to experimental data for different micro-organisms in varying juices and the calculated specific energy necessary for a 5 $\log_{10}$ reduction.

Micro-organism	Medium	pH	α (kJ/kg)	(CI 95%)[a]	β	(CI 95%)[a]	R^2 [b]	RMSE[c]	Correlation between α and β	Calculated specific energy necessary for 5 $\log_{10}$ reduction [kJ/kg]
Salmonella Panama	Apple juice	3.5	20.52	(18.39-22.65)	1.40	(1.29-1.51)	0.96	0.19	0.98	64.8
	Orange juice	3.7	23.63	(21.79-25.47)	1.38	(1.25-1.51)	0.99	0.20	0.96	75.9
	Watermelon juice	5.3	70.15	(67.36-72.95)	2.60	(1.86-3.34)	0.96	0.10	0.39	130.3
	Watermelon juice	3.6	41.12	(38.76-43.47)	3.05	(2.37-3.73)	0.99	0.19	0.86	69.7
Escherichia coli	Apple juice	3.5	15.15	(8.65-21.65)	1.08	(0.72-1.44)	0.93	0.56	0.97	67.2
	Orange juice	3.7	16.39	(13.16-19.62)	1.27	(0.99-1.55)	0.97	0.26	0.95	58.2
	Watermelon juice	5.3	71.29	(65.89-76.68)	1.58	(1.16-2.01)	0.94	0.09	0.62	197.4
Saccharomyces cerevisiae	Apple juice	3.5	29.56	(24.36-34.75)	2.19	(1.64-2.74)	0.96	0.53	0.96	61.6
	Orange juice	3.7	24.85	(22.63-27.08)	1.90	(1.71-2.09)	0.99	0.24	0.98	58.0
	Watermelon juice	5.3	51.28	(48.52-54.04)	2.92	(2.35-3.50)	0.97	0.16	0.89	89.0
	Watermelon juice	3.6	38.43	(33.78-43.07)	3.22	(2.27-4.17)	0.93	0.44	0.95	63.4
Listeria monocytogenes	Apple juice	3.5	28.88	(27.66-30.09)	1.18	(1.09-1.27)	0.97	0.05	0.89	113.0
	Orange juice	3.7	88.46	(73.05-103.87)	1.51	(1.05-1.97)	0.91	0.07	0.91	256.8
	Watermelon juice	5.3	187.07	(146.81-227.33)	1.61	(1.31-1.92)	0.97	0.01	0.98	508.3

[a] CI 95%: confidence interval

[b] R^2: determination coefficient

[c] RMSE: root mean square error

[d] Calculation based on estimated α and β parameters and the Weibull model (Eq. 2.2).

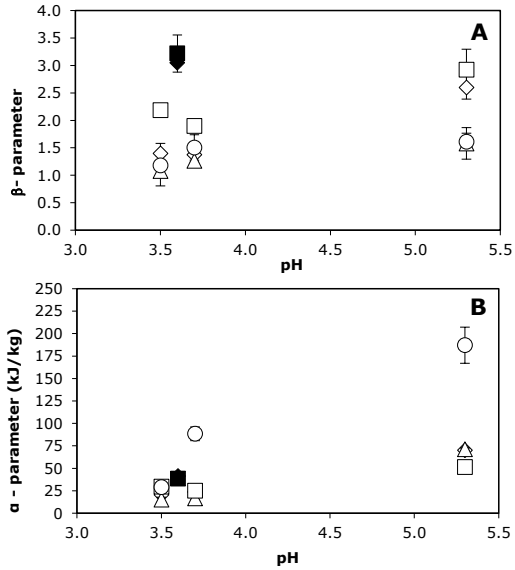

Figure 2.5. Graph of A) shape parameter β and B) scale parameter α, both with 95% confidence interval as function of pH for inactivation experiments of *S*. Panama (◇), *E. coli* (△), *L. monocytogenes* (○) and *S. cerevisiae* (□) in fruit juices, where filled symbols are presenting acidified watermelon juice.

2.4 Conclusion

High intensity PEF processing conditions for fruit juice were assessed and inactivation of micro-organisms was dependent on pH, the type of microbial species and inlet temperature of the matrix.

A synergistic effect between temperature and PEF treatment was demonstrated and suggests that optimization of the PEF conditions to reduce the energy input should aim for processing at higher inlet temperature to allow more effective inactivation per pulse.

The diversity in PEF resistance across the different microbial species shows the importance to validate industrial processes with relevant micro-organisms (spoilage and pathogens) for the food products. Testing of pathogens has to be done in the food matrix desired to be PEF pasteurised, as intrinsic factors such as pH and conductivity, influence the amount of energy required to reach the required reduction of micro-organisms

Chapter 3

Effect of electric field strength applied by high intensity PEF processing and storage temperature on the outgrowth of yeasts and moulds naturally present in a fresh fruit smoothie

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Abstract

High intensity pulsed electric field (PEF) technology offers an alternative to thermal pasteurisation of high-acid fruit juices, by extending the shelf life of food products, while retaining its fresh taste and nutritional value. Substantial research has been performed on the effect of electric field strength on the inactivation kinetics of spoilage and pathogenic micro-organisms and on the outgrowth of spoilage micro-organisms during shelf life. However, studies on the effect of electric field strength on the inactivation and outgrowth of surviving populations during shelf life are missing. In this chapter, we assessed the influence of electric field strength applied by high intensity PEF processing and storage temperature on the outgrowth of surviving yeast and mould populations naturally present in fresh fruit smoothie over time.

Therefore, an apple–strawberry–banana smoothie was treated in a continuous-flow PEF system (130 L/h), using similar inlet and outlet conditions (preheating temperature 41 °C, maximum temperature 58 °C) to assure that the amount of energy across the different conditions was kept constant. Smoothies treated with variable electric field strengths (13.5, 17.0, 20.0 and 24.0 kV/cm) were compared to smoothies without treatment for outgrowth of yeasts and moulds. Outgrowth of yeasts and moulds stored at 4 °C and 7 °C was analysed by plating and visual observation and yeast growth was modelled using the modified logistic growth model (Zwietering model).

Results showed that the intensity of the electric field strength had an influence on the degree of inactivation of yeast cells, resulting in a faster outgrowth over time at lower electric field strength. Outgrowth of moulds over time was not affected by the intensity of the electric field strength used.

Application of PEF introduces a trade-off between type of spoilage: in untreated smoothie yeasts lead to spoilage after 8 days when stored at 4 or 7 °C, whereas in PEF treated smoothie yeasts were (partly) inactivated and provided outgrowth opportunities for moulds, which led to spoilage by moulds after 14 days (7 °C) or 18 days (4 °C).

3.1 Introduction

High intensity pulsed electric field (PEF) technology offers an alternative for thermal pasteurisation to extend the shelf life of high acid-fruit juices. Target spoilage micro-organisms and most pathogens can be inactivated using high intensity PEF (Aronsson & Rönner, 2001; Timmermans et al., 2014). During the last years, transition from lab- and pilot-scale equipment to industrial scale equipment took place (Irving, 2012), and the first PEF processed fruit juices and smoothies are on the market in different countries, including The Netherlands, Germany, the United Kingdom and Austria (Buckow, Ng & Toepfl, 2013). The benefit of PEF over conventional thermal treatment is that a shelf life extension from 7 to 21 days is obtained, while retaining the fresh taste and nutritional value (Hartyáni et al., 2011; Irving, 2012; Vervoort et al., 2011). Many process parameters of PEF technology have been studied to determine the effect on the inactivation kinetics of micro-organisms. Among the process parameters, electric field strength and treatment time are considered as the most important parameters influencing the effectiveness to inactivate micro-organisms (Raso, Condón & Álvarez, 2014).

Microbial inactivation could be enhanced by increasing the electric field strength to a level where external electric field strength (E) exceeds the critical electric field strength (E_c) of the membrane of the micro-organism ($E > E_c$) (Álvarez, Condón & Raso, 2006). The E_c varies for different micro-organisms, but in general microbial inactivation requires electric field strengths above 5 kV/cm (Raso, Condón & Álvarez, 2014). Substantial research has been performed on the effect of electric field strength on the inactivation kinetics of spoilage (Marsellés-Fontanet et al., 2009; Puértolas et al., 2009) and pathogenic micro-organisms (Evrendilek, Zhang & Richter, 1999; Gurtler et al., 2010; Huang et al., 2014) in fruit products or on the outgrowth of spoilage micro-organisms studied for one electric field strength during shelf life (Evrendilek et al., 2000; Qiu et al., 1998; Timmermans et al., 2011; Walkling-Ribeiro et al., 2010). However, studies on the effect of electric field strength on the inactivation and outgrowth of the residual population not targeted by PEF during shelf life are missing.

Microbial spoilage of food products causes large economic losses (Dantigny, Guilmart & Bensoussan, 2005). It has been suggested that fungal spoilage causes more losses within the food supply chain than any other group of micro-organisms (Betts, 2010). Spoilage of fruit juices is dominated by three acid-tolerant groups: aciduric bacteria, moulds and yeasts (Graumlich, Marcy & Adams, 1986). Especially yeasts, but also moulds, form the main type of organisms naturally present on fresh fruits (Roberts et al., 2005), leading to fungal spoilage even in high-acid products like lemons, at pH 2.2 (Castillo et al., 2014; Moss, 2008).

Fungal contamination of fruits can be introduced at various steps in the production chain, including cultivation, harvesting, handling, transport, post-harvest storage (Tournas & Katsoudas, 2005), processing and marketing conditions. Controlling post-harvest spoilage is therefore an important aspect and storage under controlled atmosphere or refrigeration reduces the fungal growth dramatically and prolongs the shelf life of the fruits (Tournas & Katsoudas, 2005). Washing and handling of some fruits may be difficult, e.g. shape of branched table grapes shields other grapes for removal of micro-organisms and red fruit products like strawberries and blackberries are very susceptible to physical damage.

Therefore, these fruits are associated with high microbial loads of yeast and moulds (Pitt & Hocking, 2009).

Yeast can grow at fruit juice conditions unfavourable for other species such as low pH and high sugar concentration. Combined with its capacity to grow at refrigeration temperatures, this makes them potential spoilers of fresh fruit juices (Tribst, Sant'Ana & Massaguer, 2009). Yeasts can produce undesired metabolic products such as carbon dioxide and alcohol during growth in fruit juice, and release of pectin degrading enzymes that enhance turbidity and cause flocculation and phase separation (Jay & Anderson, 2001). Moulds, similar to yeast, tolerate low pH values and high sugar concentrations but are mostly aerobic and cannot grow under low oxygen tension (Tournas, 1994; Tribst, Sant'Ana & Massaguer, 2009). Their growth results in undesired effects such as gas production, change of odour and the formation of a mycelial mat on the surface of the juice (Jay & Anderson, 2001). Moulds may produce highly heat resistant spores that survive the pasteurisation step. Upon germination of the spores, outgrowth to visible hyphae follows and the juice becomes spoiled (Dantigny, Guilmart & Bensoussan, 2005).

Furthermore, moulds have the ability to produce secondary metabolites, known as mycotoxins, which are toxic to humans. Mycotoxins are natural contaminants, which are chemically stable during storage and processing, even when heated at temperatures of 80 – 120 °C, and therefore they may be present in the food even when fungi are no longer present due to the processing (Kabak, 2009).

Our objective was to investigate the effect of electric field strength applied by pulsed electric field processing and storage temperature on the outgrowth of yeasts and moulds naturally present in a fresh fruit smoothie, and to model the outgrowth of surviving yeasts as a function of electric field strength and storage temperature.

3.2 Material and methods

3.2.1 Product

One batch of 1000 L fresh fruit smoothie was supplied by a commercial processer, by extraction of fresh apples (45%), strawberries (40%), and banana (15%). The smoothie was not inoculated with micro-organisms, so only the population naturally present in the fruits was studied in this research. Conductivity of the fruit smoothie was 0.310 ± 0.003 S/m at 20 °C, pH was 3.6 ± 0.1 at 20 °C, viscosity 11.1 ± 0.6 Pa·s at 20 °C, and density 1040 kg/m^3.

3.2.2 Selection of PEF conditions

The objective of this study was to investigate the effect of the process parameter electric field strength. Increase of electric field strength (E, V/m) will lead to a higher energy density per pulse (u, J/m^3) (Eq. 3.1), leading to more heating up of the product (Eq. 3.2), if the other parameters conductivity (σ, S/m), pulse width (τ, s), density (ρ, kg/m3) and specific heat (c_p, kJ/kg·K) are kept constant.

$$u = E^2 \cdot \sigma \cdot \tau \quad \text{(Eq. 3.1)}$$

$$\mathrm{d}T = \frac{u}{\rho \cdot c_p} \quad \text{(Eq. 3.2)}$$

Temperature showed to be an important parameter in the efficacy to inactivate micro-organisms during PEF-processing (Heinz, Toepfl & Knorr, 2003; Timmermans et al., 2014; Toepfl, Heinz & Knorr, 2007b), and we would like to exclude this effect from electric field strength in this study. Therefore, processing conditions were selected to deliver an equal amount of energy for each electric field strength. In practice, this means that the inlet temperature (measured just before entering the PEF treatment chamber) and outlet temperature (measured directly after leaving the PEF treatment chamber) were controlled and set at 41 ± 1 °C and 58 ± 1 °C, respectively. This choice implied that more pulses were delivered at a lower electric field strength than at higher electric field strength to provide the same amount of energy (Table 3.1).

The outlet temperature of 58 °C was selected as it allows a 5 $\log_{10}$ reduction of target pathogen *Escherichia coli* in fruit juice at pH 3.7 at an electric field strength of 20 kV/cm and pulse width of 2 µs (Timmermans et al., 2014). Based on the design of the equipment, and a maximum energy input of the pulser, the inlet temperature was chosen as low as possible to retain optimal product quality. Electric field strengths were chosen in the range 13.5 – 24.0 kV/cm, as this is the electric field strength typically applied at industrial scale.

3.2.3 PEF processing

The PEF equipment used was a continuous-flow system. The technical drawing of the treatment chamber is provided by Mastwijk et al. (2007) and the process setup is discussed in detail by Timmermans et al. (2011). In short, a co-linear treatment chamber with diameter of 10mm and a gap of 20mm was used. Electrodes were made of stainless steel and the insulators were made of polyetherimide (Ultem™ resin). Prior to processing, the entire system was cleaned (CIP) and sterilized (SIP 120 °C for 20 min). Fruit smoothie was prepared 24 h before PEF treatment and stored in a 1000 L bag-in-box at 4 °C overnight. PEF processing was performed at t = 0 h, where after start-up with salt–water (3 g/L NaCl-solution), the chilled fruit smoothie was pumped at 130 L/h through the system and treated with high intensity PEF. First, conditions with the highest electric field strength, E = 24.0 kV/cm, were processed, followed by E = 20.0 kV/cm, E = 17.0 kV/cm and E = 13.5 kV/cm. Fruit smoothie was preheated to 41 ± 1 °C, subsequently PEF treated to reach a maximum temperature of 58 ± 1 °C, and immediately cooled down to a filling temperature of 14 ± 1 °C. Samples were manually bottled, under hygienic conditions in a laminar flow cabinet, using sterile 250 mL PET bottles (γ-irradiated, 15 kGy, Synergy Health, The Netherlands) with an atmospheric headspace.

When all PEF-treatments were carried out, preheating was turned off and untreated smoothie was pumped through the system and filled at 11 ± 1 °C as a control. Monopolar pulses with a duration of 3 µs were used. Pulse waveform, voltage and current in the treatment chamber were recorded with a digital oscilloscope (Rigol

DS1102E). The electric energy was obtained by numerical integration of the voltage and current traces, and equals to the caloric power within the experimental error (10%), according to Mastwijk (2006) and Timmermans et al. (2014). Specific electric energy per treatment is shown in Table 3.1, with calculations based on Eq. 3.3, wherein w is the specific energy (kJ/kg), $P_{electric}$ is the electrical power (W), ϕ is the flow rate (m^3/s), f is the frequency (Hz), U is the voltage signal (V), I is the current signal (A) and ρ is the density of the smoothie (kg/m^3).

$$w = \frac{P_{electric}}{\phi \cdot \rho} = \frac{f \cdot \int U(t) I(t) dt}{\phi \cdot \rho} \qquad \text{(Eq. 3.3)}$$

Table 3.1. Process conditions used for PEF treatment of fruit smoothie.

E **(kV/cm)**	**Actual peak voltage (kV)**	**Actual peak current (A)**	**Frequency (Hz)**	**Number of pulses**	**Flow rate (L/h)**	**T_{in} (°C)**	**T_{out} (°C)**	**ΔT (°C)**	***w*** **(kJ/kg)**
0	0	0	0	0	123	11	11	0	0
13.5	27.5	100	290	24.1	136	41	58	17	54
17.0	34	125	185	15.7	133	40	58	18	53
20.0	40	150	130	10.8	136	41	58	17	52
24.0	48	175	100	8.7	130	40-41	58-59	18	55

3.2.4 Storage conditions and sampling

Immediately after filling, samples were transferred to a refrigerator at 4 °C or 7 °C. Microbial analysis was carried out in triplicate, where samples were taken from three different bottles, at fixed points in time during the shelf life study. Next, visual observation of 20 selected bottles of each treatment condition at both storage conditions was carried out daily, for a maximum period of 27 days at 7 °C and 76 days at 4 °C.

3.2.5 Visual observations of fruit smoothie

Visual spoilage of the fruit smoothie was determined by daily observations of 20 individual bottles that were scored for different categories including; no change, slime formation, development to a visible mould, gas formation (bubbles), expansion of package and final phase (end). Photos supporting the classification of the categories are added in the Appendix. Results of the daily scores were summarized in a grid, where a colour was allocated to each stage of spoilage, to facilitate comparison.

3.2.6 Quantification outgrowth of yeasts and moulds

To quantify the number of yeasts and moulds present in the fruit smoothie during storage, a representative sample (10 – 15 g) was taken from the bottle at indicated time points. Sample was diluted with sterile peptone physiological salt diluent (PSDF) (ratio 1:10) and homogenised for 1 min. Next, samples were serially diluted in PSDF and plated on dichloran-rose bengal chloramphenicol (DRBC) agar. Surviving yeasts and moulds were enumerated after 5 – 7 days incubation at 25 °C according to an

established method, ISO 21527-1 (2008). Discrimination of yeasts and moulds was performed based on morphological differences where yeasts appeared as matte or shiny round colonies, and moulds could be characterised as flat or fluffy propagules/germs or colonies often coloured with fruiting or sporing structures (ISO 21527-1, 2008).

Photos of the agar plates were made to evaluate if trends in number and type of morphological characteristics of yeasts and moulds developed during storage. No trends were observed, and therefore 12 yeasts and moulds dominating the outgrowth were further characterised. Isolates were sent to a commercial lab (BaseClear, The Netherlands) for further characterisation based on a PCR amplification and DNA sequencing of D2-LSU rRNA gene. The amplicons obtained were analysed for identification using capillary electrophoresis and results were analysed against the validated MicroSEQ library of BaseClear B.V (Leiden, The Netherlands).

All samples had a match to the library of at least 99.2%.

3.2.7 Statistical analysis and mathematical modelling

For modelling purposes, the individually measured plate counts were used (measured in triplicate). Models were fit to the results taken from the logarithm of number of yeasts grown out during storage time. A distinction was made between the yeast and mould data set, and between PEF-treatment and no treatment.

3.2.8 Microbial growth of yeasts in untreated smoothie

Growth of yeasts in untreated smoothie was fitted according to a linear model (Eq. 3.4), where log N is the decimal logarithm of number of plate counts (cfu/mL) at storage time t, μ_{max} is the specific growth rate (1/h), t is the storage time (h) and N_0 is the decimal logarithm of the initial number of counts (cfu/mL).

$$\log_{10} N(t) = \mu_{max} \cdot t + N_0 \qquad \text{(Eq. 3.4)}$$

3.2.9 Microbial growth of yeasts in PEF-treated smoothie

Growth of yeast after PEF treatment was fitted according to the modified logistic growth model (Zwietering et al., 1990) (Eq. 3.5), where log N (t) is the decimal logarithm of number of plate counts (cfu/mL) at storage time t, A_s corresponds to a more or less asymptotic value with no dimension, representing the ratio $\log_{10}(N_{max}/N_0)$ with N_{max} as maximum number of counts (cfu/mL) and N_0 the initial number of counts (cfu/mL), μ_{max} is the specific growth rate (1/h), t is the storage time (h), λ is the lag time (h) and N_0* is the decimal logarithm of the initial number of counts after PEF treatment (cfu/mL).

$$\log_{10} N(t) = \frac{A_s}{1 + exp\left[\frac{4 \cdot \mu_{max}}{A_s}(\lambda - t) + 2\right]} + N_0^* \qquad \text{(Eq. 3.5)}$$

This modified Zwietering model introduces four model-parameters: start concentration, lag time, maximum growth rate in exponential phase and maximum concentration reached in the stationary phase

Model building

Since no (primary) models are available describing the relation of electric field strength and storage temperature on outgrowth of yeasts after PEF treatment, their dependency was initially studied using global fitting where storage time *t* is treated as continuous variable and electric field strength *E* and storage temperature *T* are treated as categorical variables. Relations are later expressed into secondary models.

Global fit (for primary model).

Global modelling was carried out to describe the effect of *E* and *T* on the implicit model-parameters; N_0^* (*E*, *T*), λ (*E*, *T*), μ_{max} (*E*, *T*) and A_s (*E*, *T*).

Therefore, the single response of the micro-organism in terms of outgrowth to time, electric field strength and storage temperature was fitted at once (Motulsky & Christopoulos, 2003). To study the dependency of *E* and *T* on the model-parameters, models with variation in the total number of parameters were proposed, having a maximum of 32 parameters (all parameters for outgrowth are dependent on *E* and *T*) and a minimum of 4 parameters (no dependency of neither *E* nor *T* on outgrowth). Evaluation of the model performance was used as a tool to establish if a parameter was dependent on either *E* or *T*. The total number of data points was n = 357 (yeasts) and n = 272 (moulds).

Evaluation of the (primary) models.

Evaluation of the residuals was a first check to evaluate the model performance. Next, the goodness-of-fit of the models with a variable number of parameters (4 – 32) was compared using two criteria: Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). In Eq. 3.6 and Eq. 3.7, definitions for AIC and BIC are given, respectively, wherein p is the number of fitted parameters. A penalty for introducing more parameters in a model is given, where the BIC gives a stronger penalty (p*ln (n)) for introducing more parameters than the AIC (2(p+1)) (van Boekel, 2009a).

$$\mathrm{AIC} = n \cdot \ln\left(\mathrm{SSr}/n\right) + 2\left(p+1\right) \qquad \text{(Eq. 3.6)}$$

$$\mathrm{BIC} = n \cdot \ln\left(\mathrm{SSr}/n\right) + p \cdot \ln\left(n\right) \qquad \text{(Eq. 3.7)}$$

Secondary models

Based on the dependence of the model parameters on *E* and *T*, secondary models were proposed. As only two temperature conditions (4 °C and 7 °C) were measured, no secondary models for temperature were considered. For the growth curve of yeasts, the decimal log of initial number of organisms (N_0*) and the lag time λ were found to depend on the electric field strength. Linear models were proposed and evaluated.

Software package

Software package Athena Visual Studio version 14.2 was used for parameter estimation and model discrimination. Parameters were obtained by fitting growth curves to the data by non-linear least squares using the Levenberg–Marquardt algorithm.

3.3 Results and discussion

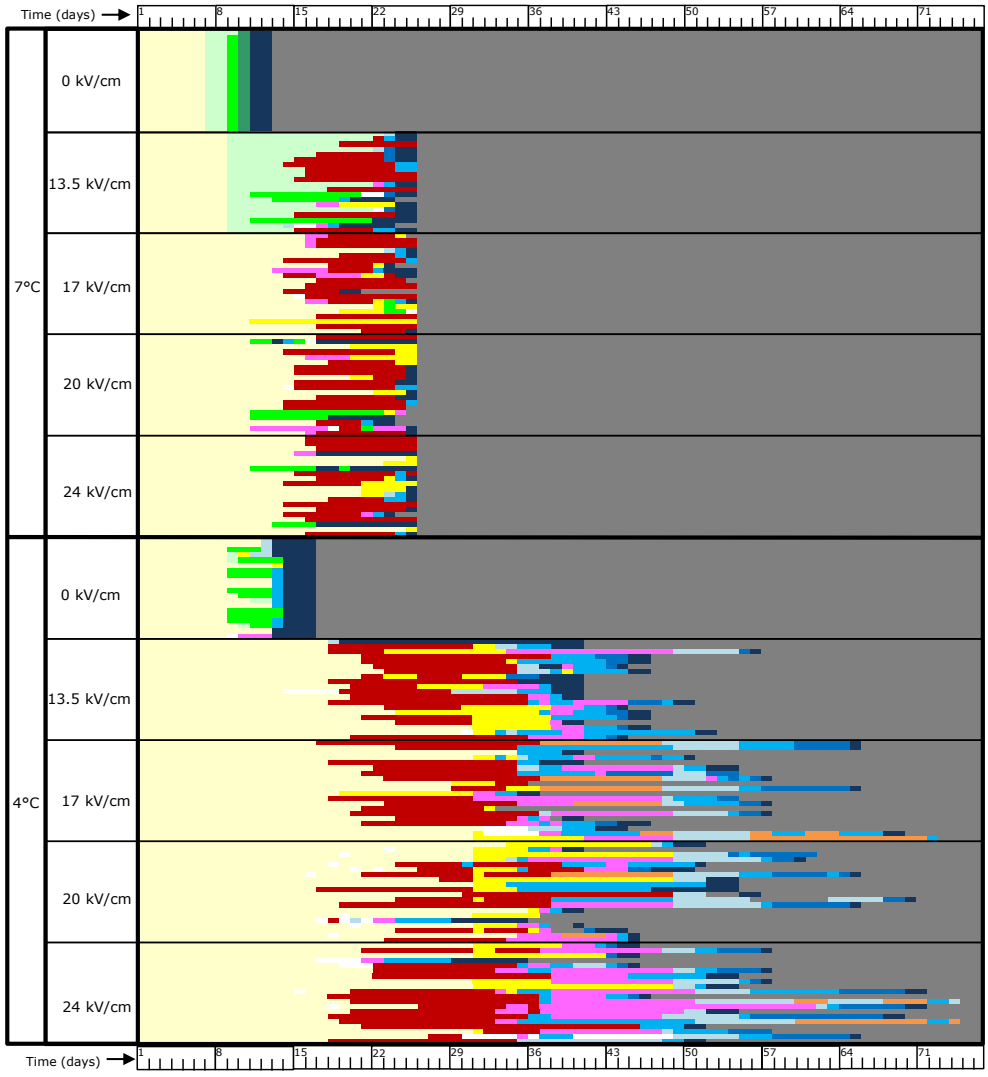
3.3.1 Visual observations of fruit smoothies

First the impact of electric field strength on the quality of the different smoothies was evaluated based on visual inspection of untreated and PEF treated smoothies stored at 4 °C and 7 °C. The individual observations of 20 bottles of each treatment at both storage conditions were scored daily and the results are summarized in Fig. 3.1. Observation of untreated fruit smoothie (E = 0 kV/cm) could be characterised by the formation of slime, followed by gas formation leading to deformation and expansion of the bottle. No mould growth was observed. Comparison of untreated smoothies stored at 7 °C and 4 °C showed that slime formation was visible at day 9 for both storage temperatures, but gas formation eventually leading to swelling of the package developed faster at 7 °C than at 4 °C, with blown packages after 11 (7 °C) or 13 (4 °C) days.

The PEF treated smoothies showed virtually no slime formation, and the first visual change was the formation of mycelial mats (moulds) on the surface of the fruit smoothie or in the headspace of the bottle. Generally, moulds were visible after 14 days at 7 °C and 18 days at 4 °C. Only storage temperature influenced the visual appearance of moulds, but no differences in time to detect mould growth were observed for the varying electric field strengths. After the appearance of moulds, gas formation developed over time in the bottles, eventually leading to expansion of the bottles. The gas, probably carbon dioxide, could have been produced by either yeasts or moulds (Tribst, Sant'Ana & Massaguer, 2009). In some bottles phase separation of the fruit smoothie was visible, possibly caused by the action of microbial enzymes formed by yeasts and/or moulds (Sieiro et al., 2012). These pectolytic enzymes degrade the pectin substances, leading to a clarification and destabilisation of the fruit smoothie.

3.3.2 Characterisation of yeasts and moulds

Yeasts and moulds dominating in the spoiled smoothies were selected based on morphology differences and identified based upon LSU rDNA D2 sequence. Dominating yeast species were *Candida* spp., *Metschnikowia* spp., *Saccharomyces* spp., *Pichia* spp. and *Hanseniaspora valbyensis*. These yeast species are typical spoilers of fruit juices (Fleet, 2003; Tribst, Sant'Ana & Massaguer, 2009). All moulds belong to the *Penicillium* family with high identity to the following species *P. commune, P. clavigerum, P. crustosum, P. expansum,* or *P. griseofulvum*. Furthermore, the entomopathogenic fungus *Lecanicillium longisporum* (previously known as *Verticillium lecanii*) and plant pathogen *Mucor piriformis*, known to cause soft rot of several fruits, were isolated.



No change	Little slime	Moulds	Phase separation	Little pressure	Little expansion package	End phase
	Slime		Gas formation	Pressure	Expansion package	
	Slime, gas and little expansion package		Underpressure			

Figure 3.1. Visual observations of the fruit smoothie during storage, showing time (days) on the x-axis and scores for the 20 individual bottles for each different treatment- and storage condition on the y-axis. Colours for the different stages of spoilage are allocated to the start of a new observation.

3.3.3 Outgrowth of yeasts and moulds in untreated fruit smoothie

Microbial spoilage of untreated fruit smoothie (E = 0 kV/cm) was quantified by plate counting at regular time points during storage. Data showed an increase of yeasts and a decrease of moulds over time (Fig. 3.2). A linear model was fit to the yeast data (Eq. 3.4.). Model performance was evaluated and the residuals were randomly distributed. Results of the growth curves are shown in Table 3.2.

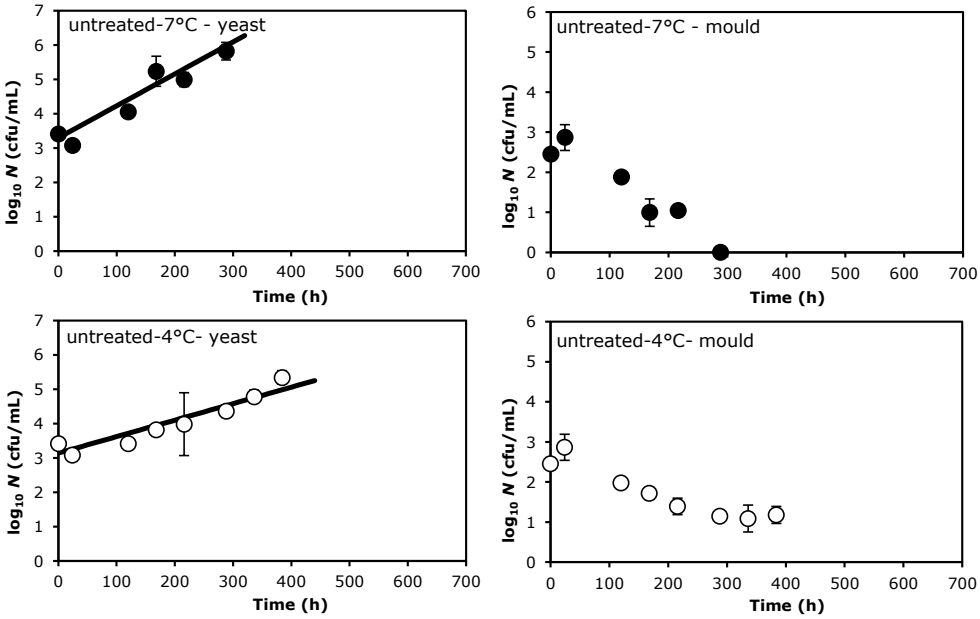


Figure 3.2. Outgrowth of yeasts (left panels) and decrease of moulds (right panels) naturally present in untreated fruit smoothie stored at 7 °C (upper panel: filled symbols) and 4 °C (lower panel: open symbols). Symbols and bars represent the mean and standard deviation of data ($\log_{10}$ cfu/mL) ($\bar{y} \pm \sigma_y$) whereas continuous lines depict the model fit according to Eq. 3.4.

Modelling of the outgrowth of moulds in the smoothie was not possible, as the enumeration of the moulds may be inaccurate, as formed colonies may consist of a mixture of mycelium and spores. Furthermore, fragmentation of the mycelium and breaking of spore clumps during dilution might give non-linearity of the counts by dilution plating (Jarvis et al., 1983). Therefore, counts can only be used to give an indication of the number of moulds present in the fruit smoothie.

Yeast may inhibit growth of bacteria and several mechanisms have been proposed in the literature (Liu et al., 2013). These mechanisms include competition for nutrients and space, parasitism, secretion of antifungal compounds, induction of host resistance, biofilm formation, and involvement of reactive oxygen species (Liu et al., 2013). Limited information is available in the literature on the inhibiting effect of yeast on mould growth.

Research has demonstrated that some yeast species contain antifungal activity and that they can significantly inhibit the growth of moulds (Fleet, 2003). For instance, *Candida* ssp. induce chitinase and β-1,3-glucanase, which controls decay caused by *Botrytis cinerea* in apples (El Ghaouth, Wilson & Wisniewski, 2003) and strawberries (Guinebretiere et al., 2000). This biocontrol activity is comparable with earlier work of Wisnieuwski et al. (1991), who showed secretion of cell wall degrading enzymes and attachment of the yeast *Pichia guilliermondii* to the hyphae of *B. cinerea* and *P. expansum.* Interestingly, some of the isolated yeasts (*Candida* spp. and *Metschnikowia* spp.) are associated with anti-fungal activity (Fleet, 2003; Manso & Nunes, 2011), possibly explaining the competition with moulds, although other competition factors could not be excluded.

Table. 3.2. Results of parameter estimation for the model shown in Eq. 3.4 for outgrowth of yeasts in untreated fruit smoothie.

	T (°C)	μ_{max} (1/h)		N_0 ($\log_{10}$ cfu/mL)		R^2
		mean	**95% CI interval**	**mean**	**95% CI interval**	
yeast	4	0.0048	0.0037 — 0.0059	3.1	2.9 — 3.4	0.78
yeast	7	0.0093	0.0075 — 0.0110	3.3	3.0 — 3.6	0.89

3.3.4 Outgrowth of yeasts in PEF-treated fruit smoothie

The development of number of yeasts during storage after PEF treatment is shown in Fig. 3.3. Yeast growth curves were of sigmoidal nature, starting with a lag-phase where no growth occurred (plateau around 1 $\log_{10}$ cfu/mL). After adaption and recovery of the cells, the exponential phase developed, characterised by a maximum specific growth rate of the yeasts (tangent in the inflection point). This stage is followed by a stationary phase, due to lack of nutrients or formation of secondary products (plateau around 4 – 5 $\log_{10}$ cfu/mL). A relatively large standard deviation of the data was obtained, possibly caused by the set-up of this study, where we studied a heterogenic yeast population present in a complex food matrix under sub-optimal growth conditions.

Yeast growth was fitted using the modified logistic growth model (Eq. 3.5). Performance of this primary model, including 5 variations in the number of independent parameters (between 4 and 32), was evaluated. For all 5 model-fits, residuals were randomly distributed. Table 3.3 shows the results for the goodness-of-fit for the 5 proposed models. Comparison of the models was done based on the Akaike and Bayesian Information Criterion, both introducing penalties for the number of parameters applied in each model. Based on these criteria, model 3 is favoured, as it has the lowest AIC value and differences in BIC value are comparable to model 2, both showing the lowest value.

Model-parameter estimates and their precision, expressed in a 95% confidence interval, of model 3 are shown in Table 3.4. Evaluation of these model-parameters showed that N_0* was dependent on electric field strength, lag time λ was dependent on both electric field strength and storage temperature, growth rate μ_{max} was dependent on storage temperature and A_s was similar in all outgrowth curves.

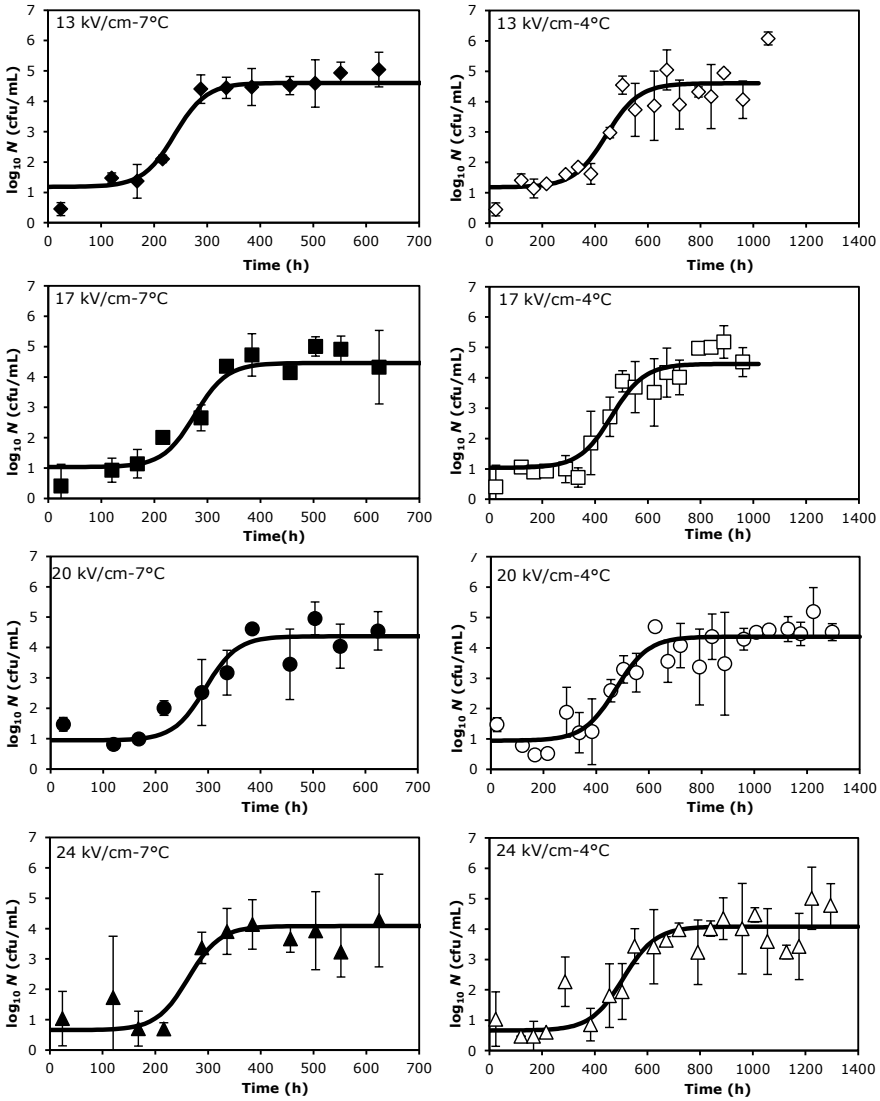


Figure 3.3. Model fit of modified logistic growth model (15 parameters) of natural present yeasts in fruit smoothie treated with PEF at various electric field strength: ◆13.5 kV/cm, ■ 17 kV/cm, ● 20 kV/cm and ▲ 24 kV/cm stored at 7°C, and ◇13.5 kV/cm, □ 17 kV/cm, ○ 20 kV/cm, △ 24 kV/cm stored at 4°C. Symbols and bars represent the mean and standard deviation of data ($\log_{10}$ cfu/mL) ($\tilde{y} \pm \sigma_y$) whereas continuous lines depict the model fit according to Eq. 3.5.

In Fig. 3.4, the electric field strength dependent model-parameter N_0^* and initial concentration of yeast present in the fruit smoothie (N_0) illustrate the reduction of number of yeast cells after PEF treatment, showing more inactivation of yeast cells at a higher electric field strength compared to lower electric field strength. This result is in accordance with other studies on the effect of electric field strength on inactivation kinetics of yeasts described in the literature (Hülsheger, Potel & Niemann, 1983; Marsellés-

Fontanet et al., 2009; Puértolas et al., 2009). A linear regression model was proposed to show the relation between electric field strength and the degree of inactivation, being $N_0^* = -0.05 \cdot E + 1.8$ ($R^2 = 0.96$) (valid for E ranging from 13.5 to 24.0 kV/cm), and this model was fitted to the data via linear regression.

The degree of yeast inactivation at $E = 20.0$ kV/cm was less than expected based on earlier studies (Timmermans et al., 2014), where inactivation of *Saccharomyces cerevisiae* resulted in 7 $\log_{10}$ inactivation in low viscous fruit juice of similar pH and conductivity, whereas in the present study still some colonies could be detected, indicating that less than 3.5 $\log_{10}$ inactivation was obtained. A possible explanation might be that the size of some of the yeast species of the mixed population isolated in this study was smaller than that of *S. cerevisiae*, showing less sensitivity for the PEF treatment, as size of the micro-organism plays a role in the effectiveness of the PEF treatment (Timmermans et al., 2014). Another aspect could be that the viscosity and presence of seeds in the smoothie influenced the homogeneity of the electric field. No scientific studies on the influence of seeds on microbial inactivation by PEF were found and remain to be determined.

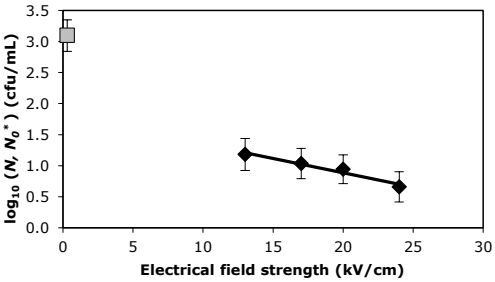


Figure 3.4. Initial concentration of yeast N_0 (□) and estimated concentration of yeast after PEF treatment (N_0^*) (◆) obtained from fitting the modified logistic growth model (15 parameters) for variable electric field strength applied. Symbols and bars represent the mean and standard deviation of data and parameter ($\log_{10}$ cfu/mL) ($\bar{y} \pm \sigma_y$). Continuous line represents the relation between electric field strength and N_0^*, $N_0^* = -0.05 \cdot E + 1.8$, valid for E ranging from 13.5 to 24.0 kV/cm) ($R^2 = 0.97$).

In Fig. 3.5, the relation between model-parameter λ of PEF treated yeasts for variable electric field strength and storage time is shown. A longer lag time was observed when fruit smoothie was treated at a higher electric field strength, and the relation was shown as a linear model: $\lambda = 5.8 \cdot E + 241$ (E ranging from 13.5 to 24.0 kV/cm) ($R^2 = 0.98$) for the dataset of 4 °C, where the fit was obtained by linear regression.

Table. 3.3. Model discrimination test for five models describing outgrowth of yeasts in fruit smoothie after PEF treatment during storage.

Model	*p*	*n*	AIC	BIC
1	4	357	5.9	19.4
2	12	357	-184.3	-139.8
3	15	357	-194.8	-138.7
4	18	357	-180.9	-113.1
5	32	357	-183.5	-65.3

[a] *p*, number of parameters; *n*, number of data points including replicates;
AIC, Akaike Inforamtion Criterion;
BIC, Bayesian Information Criterion

A similar trend at the dataset of 7 °C was found. Based on the linearity of the model, the data point for E = 24 kV/cm may be considered as an outlier. This outlier could be explained from the primary data (Fig. 3.3), where no data points in the exponential phase were measured. The lack of these data points influences the estimation for model parameters. However, the trend is similar to the results of 4 °C, showing a dependence of electric field strength on degree of lag time.

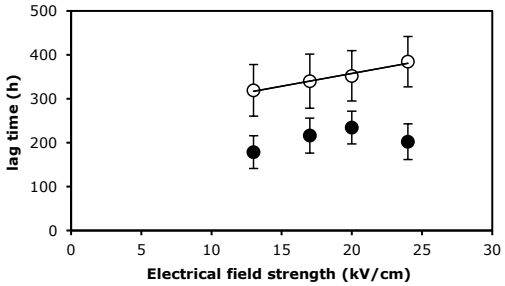


Figure 3.5. Relation between electric field strength and model-parameter lag time (λ) obtained from fitting modified logistic growth model (15 parameters) of natural present yeasts in fruit smoothie after PEF treatment at storage temperature 4 °C (○) and 7 °C (●). Symbols and bars represent the mean and standard deviation of model-parameter ($\tilde{y} \pm \sigma_y$). Continuous line represents the relation between electric field strength and lag time at 4 °C, $\lambda = 5.8 \cdot E + 241$ (E ranging from 13.5 to 24.0 kV/cm) ($R^2 = 0.98$).

This suggests that yeast cells required a longer time to recover from damage caused by more intense electric field strength than at reduced electric field strength. Where the parameter N_0^* indicates the $\log_{10}$ of number of cells damaged, and λ indicates the time to recover from the degree of damage made by PEF.

Temperature influences the metabolic rates of the yeasts. Optimal growth temperature of yeast lies between 20 and 30 °C (Battcock & Azam-Ali, 1998). Therefore, faster recovery during the lag phase and higher growth rate could be found at 7 °C than at 4 °C.

Table. 3.4. Parameter estimation of best model (p = 15) describing outgrowth of yeasts in fruit smoothie after PEF treatment.

parameter	dimension	E (kV/cm)	T (°C)	Mean	95% CI interval
A_s	-	-	-	3.4	3.2 — 3.7
μ_{max}	1/h	-	4	0.014	0.010 — 0.018
μ_{max}	1/h	-	7	0.028	0.018 — 0.039
λ	h	13.5	4	319	260 — 378
λ	h	17	4	340	278 — 402
λ	h	20	4	352	295 — 409
λ	h	24	4	384	327 — 442
λ	h	13.5	7	178	141 — 215
λ	h	17	7	215	176 — 256
λ	h	20	7	234	197 — 272
λ	h	24	7	202	162 — 243
N_0^*	$\log_{10}$ cfu/mL	13.5	-	1.2	0.9 — 1.4
N_0^*	$\log_{10}$ cfu/mL	17	-	1.0	0.8 — 1.3
N_0^*	$\log_{10}$ cfu/mL	20	-	0.9	0.7 — 1.2
N_0^*	$\log_{10}$ cfu/mL	24	-	0.7	0.4 — 0.9

A_s is an asymptotic value that represents the ratio $\log_{10}(N_{max}/N_0)$, μ_{max} is maximum growth rate, λ is lag time, $N_0^*$is the decimal logarithm of the initial number of counts after PEF treatment

3.3.5 Outgrowth of moulds in PEF-treated fruit smoothie

The impact of the PEF treatment on the number of moulds present before and after PEF treatment in fruit smoothie is shown in Fig. 3.6. The number of moulds present in untreated smoothie at t = 0 h and t = 24 h can be compared to PEF treated smoothie (t = 24 h). Analysis of untreated smoothie was carried out at two time points, to be able to compare the effect of PEF, as storage of the smoothie might be affected by other micro-organisms, i.e. yeasts as discussed in Section 3.3. Although the number of moulds in untreated smoothie showed some deviation between t = 0 and t = 24 h, it is evident that the number of moulds was reduced by the PEF treatment. Furthermore, mould reduction increased with intensity of the electric field strength.

The development of number of moulds during storage after PEF treatment is shown in Fig. 3.7. A high variation between the triplicate samples was found. Results of visual observations, described in Section 3.1 and shown in Fig. 3.1 were added to Fig. 3.7, where the arrows indicate when moulds were visible.

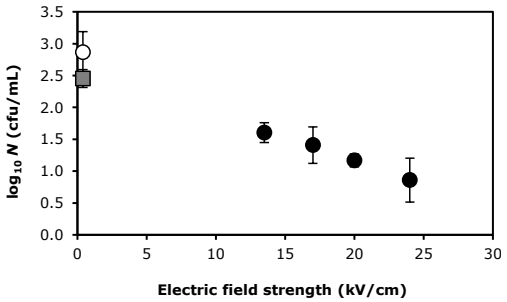


Figure 3.6. Number of moulds at t = 0 h (□) and after 24 h storage at 4 °C having no treatment (○) (E = 0 kV/cm) and measured 24 h after PEF treatment, storage at 4°C for variable electric field strength applied (●). Symbols and bars represent the mean and standard deviation of data ($\log_{10}$ cfu/mL) ($\bar{y} \pm \sigma_y$).

The number of moulds after PEF treatment stored at 7 °C increased after ~350 h of storage. This time point is comparable to the time when the first mould was visible in the bottles. Outgrowth of the number of moulds after PEF treatment stored at 4 °C gives a similar result. The number of moulds increased after ~450 h of storage, comparable to the time when the first moulds were visible.

Comparison of the development of number of moulds over time after PEF treatment for the various electric field strengths applied did not show a noticeable difference in outgrowth.

It is proposed that the outgrowth of moulds during storage could be PEF-resistant mould spores, although this is also related to the chosen temperature conditions. The study of Evrendilek et al. (2008) showed that germination tube elongation and spore germination of *P. expansum* after PEF treatment during the first 24 – 48 h after treatment were greatly inhibited by increased electric field strength and treatment time. Meanwhile, ascospores *Neosartorya fischeri* and *Byssochlamys nivea* showed to be resistant towards PEF (Grahl & Märkl, 1996; Raso et al., 1998).

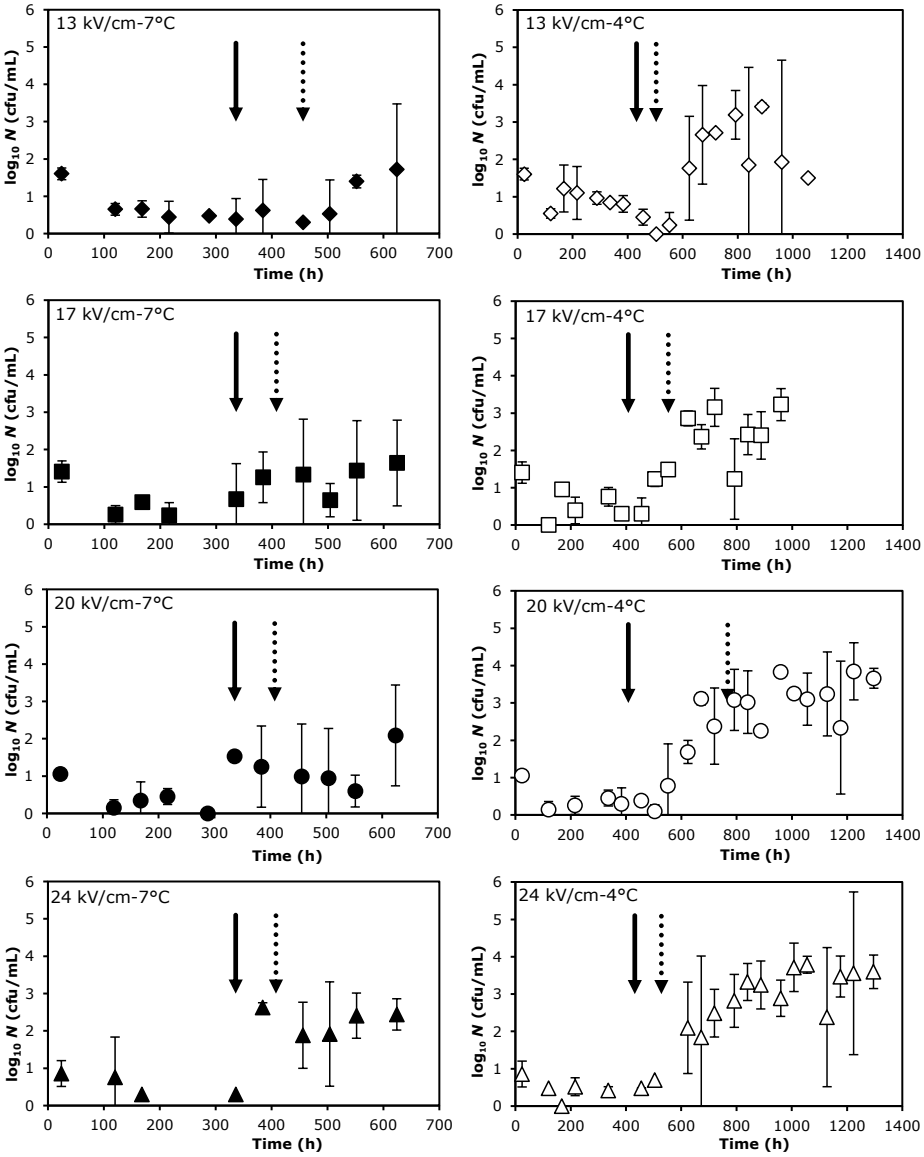


Figure 3.7. Outgrowth of moulds naturally present in fruit smoothie treated with PEF at various electric field strength: ◆13.5 kV/cm, ■ 17 kV/cm, ● 20 kV/cm and ▲ 24 kV/cm stored at 7°C, and ◇13.5 kV/cm, □ 17 kV/cm, ○ 20 kV/cm, △ 24 kV/cm stored at 4°C. Symbols and bars represent the mean and standard deviation of data ($\log_{10}$ cfu/mL) ($\tilde{y} \pm \sigma_y$). Arrows indicate when the first mould is visible in at least 1 bottle (continuous line), and dotted line arrows indicate when at least 50% of the bottles (10 bottles) contain a visible mould (taken from Fig 3.1).

3.4 Conclusion

Spoilage of untreated smoothie is dominated by the outgrowth of yeasts, leading to spoilage after 8 days (4 °C and 7 °C). Application of PEF treatment (partly) inactivates the yeasts, providing outgrowth opportunities for moulds, which lead to spoilage by moulds after 14 days (7 °C) or 18 days (4 °C)

Outgrowth of yeast after PEF treatment was affected by electric field strength and storage temperature. A model was established describing the impact of electric field strength on the degree of inactivation of yeast and lag time during outgrowth, where a higher electric field strength led to more damage and longer lag time.

The number of moulds was reduced by the PEF treatment, where the inactivation increased with an increased electric field strength. During the storage period no noticeable difference in outgrowth for variable electric field strength was observed. It was therefore suggested that electric field strength has influence on vegetative moulds, and not on mould spores.

The data show that for PEF treated smoothie spoilage is dominated by moulds and underpins the importance of sufficient cooling of the production chain and oxygen scavenging to reduce outgrowth of moulds to obtain a longer shelf life of the smoothie.

Future research should focus on understanding how production of primary and secondary metabolites and limited nutrients influence the competition of PEF treated spoilage micro-organisms, and on the effect of electric field strength on vegetative hyphae and mould spores.

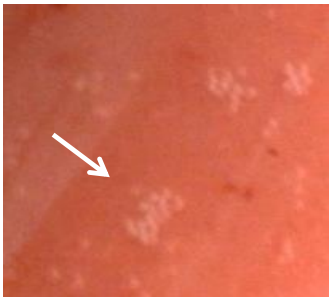
3.5 Appendix

Visual observations of the fruit smoothie during storage were scored for different categories. To have an impression how classification to each category was done, supporting photos are added where possible. Pressure development could not be put on a photo, but was noticed by indenting the package. When package could be indented slightly it was scored as ‘little pressure’, if much package could not be indented it was scored as “pressure”.

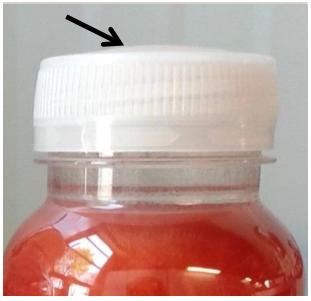
slime formation

start visible mould on surface

Mould at interface surface/headspace



Gas formation



Expansion package

Phase separation

Developed (matured) mould at surface

End phase

Chapter 4

The Gauss-Eyring model: A new thermodynamic model for biochemical and microbial inactivation kinetics

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Abstract

A new primary model has been developed, using Gaussian distributed populations and Eyrings rate constant for the transition state, to describe inactivation kinetics of enzymes and micro-organisms subjected to heat and chemical treatment. The inactivation of both enzymes and micro-organisms could be associated with the irreversible transition to an inactivated state, as suggested by the Lumry-Eyring model for protein denaturation and enzyme inactivation. The characteristic inactivation model parameters, standard activation enthalpy and entropy, are directly related to the reference temperature and *Z*-value commonly used for kinetic analysis in food microbiology. An essential feature of the kinetic model is that its parameters, and hence the transition temperature, are treated as stochastic variables. The characteristic line shape of the primary model is the log-normal distribution. The performance of the model was validated, using literature data for enzyme and microbial inactivation over a wide range of temperature and pH.

4.1 Introduction

Modelling the inactivation kinetics of proteins, enzymes and micro-organisms is an important tool in biochemistry and food science to describe and predict the stability of ingredients, spoilage and the safety of foods. Several different approaches are adapted to model the observed inactivation kinetics. A common, classical approach is to assume first order kinetics (Bigelow, 1921), though this is not always warranted (Van Boekel, 2008). From the observed inactivation data at specific temperatures, a reaction rate constant k (or a D_T-value as its reciprocal, frequently used in microbiology) is first determined. The temperature dependence of chemical and biochemical reactions is usually established via the Arrhenius equation. A D_T-value describes the time, needed to reduce the density of a microbial population by a factor of 10 at a given reference temperature. In a secondary model, the temperature dependence of the D_T-value is analysed, expressed as a Z-value, which describes the temperature needed to reduce or increase the D_T value by a factor 10. The same goes for the inactivation of proteins/enzymes via k-values and activation energies and pre-exponential factors. This method of D_T and Z-values in food microbiology relies on a first order approximation of the observed inactivation kinetics and can resolve sub-populations if sufficient, accurate data are available. One of the implications of a first order approximation is that the plot of the surviving population vs. time should be a straight line to confirm that a first-order approximation holds. Whether this is true in practice is a debatable issue; growing evidence suggests that log-linear survival curves are the exception rather than the rule, both for inactivation of enzymes and micro-organisms (Van Boekel, 2002).

A second approach to model inactivation kinetics is to use an empirical line shape as a mathematical model. Typical line shapes that are used for kinetic modelling are Weibull (Van Boekel, 2002), log-logistic (Anderson et al., 1996) and modifications thereof (Bevilacqua et al., 2015; Metselaar et al., 2013). The choice of a suitable line shape depends on the actual shape of the observed inactivation. In case of suspected heterogeneity (Abee et al., 2016), e.g., when biphasic inactivation curves are observed both for enzymes (e.g., Van Boekel, 2009c) and microbes (e.g. Metselaar et al., 2013), the choice of a specific line shape or the number of assumed sub-populations may differ for a particular subset of the experimental data series, even though the same initial sample was used that was subjected to similar environmental conditions (Metselaar et al., 2016). Nevertheless, parameter estimates in empirical models are extensively used for statistical analysis of the experimental data and to determine confidence levels for predictions (Bevilacqua et al., 2015). The advantage of an empirical line shape is that the primary model is readily available and parameters can be estimated using widespread available, non-linear fitting routines. A major drawback of this method is found in the interpretation of the empirical parameters and the relatively large confidence intervals of parameters and predictions that are usually obtained (Van Boekel, 2009a).

A third approach is modelling by a suitable combination of mass balance-, chemical rate- and thermodynamic equations. Arrhenius' equation and the Eyring model have been used as a basis for secondary models for the thermodynamic modelling of temperature and pressure treated enzymes (Fachin et al., 2006). By using this method, accurate

estimates for inactivation have been obtained with predictions for the combined effect of pressure and temperature (Fachin et al., 2006).

In this chapter we will provide an alternative approach by exploring an *ab initio* stochastic model for biochemical inactivation kinetics. The difficulty to overcome is to define a primary model with physically relevant parameters that accurately and consistently fits to kinetic data series collected over a wide range of environmental conditions. Our point of departure is the Lumry-Eyring model for protein denaturation and inactivation (Lumry & Eyring, 1954). This model provides a rate constant for inactivation that is derived from first principles in Transition State Theory. The rate constant, formulated for a molecular complex in transition, was built on quantum mechanics and statistical physical theory (Eyring, 1935). It has been consistently shown (Ahern & Klibanov, 1985; Lumry & Eyring, 1954; Schokker & Van Boekel, 1997; Yoshioka et al., 1994) that the Lumry-Eyring model for denaturation and inactivation of proteins in solution satisfies the basic scheme:

$$N \underset{k_2}{\overset{k_1}{\rightleftarrows}} U \xrightarrow{k_3} I \qquad \text{(Eq. 4.1)}$$

where the native, active (folded) state (*N*) is in equilibrium with the unfolded (denatured) state (*U*) via its activated state, characterized by the rate constant k_1 for unfolding, k_2 that for refolding and k_3 the one characterizing inactivation. An irreversible transition to the inactive state (*I*) proceeds through a transition state with its energy just above the energy of the unfolded state.

Typical standard activation enthalpies for protein unfolding are in the range of 500–600 kJ/mol while the typical standard activation entropies are also high, in the range of 500–1000 J/mol/K, thus compensating for the high activation enthalpies, as a result of which typical activation free energies are still moderate (because $\Delta^{\ddagger}G^{\Theta} = \Delta^{\ddagger}H^{\Theta} - T\Delta^{\ddagger}S^{\Theta}$, an extensive discussion on these parameters is given in Van Boekel (2009b)).

The equilibrium state that is established before entering the transition state is generally not known. Proteins may switch between a large number of different conformations (Creighton, 1988). The pathway of unfolding and inactivation may therefore proceed through a large number of different intermediate states. This is especially true for complex proteins found in food systems. In practice a large number of pathways have to be considered before kinetic model parameters of unfolding and inactivation can be determined (Schokker, 1997). In the present work we will not assume any specific reaction pathway beforehand, but estimate the standard activation enthalpy $\Delta^{\ddagger}H^{\Theta}$ and entropy $\Delta^{\ddagger}S^{\Theta}$ of the transition state from experimental data. The experimentally observed rate constant is in most cases a lumped parameter, being the resultant of k_1, k_2 and k_3 but always referring to the rate limiting step in the reaction pathway leading to inactivation. If unfolding is rate limiting, then high activation enthalpies and entropies will be found, if the subsequent inactivation step is the rate limiting (chemical) process, then the activation enthalpy will be more in the range of a chemical reaction (typically between 50 – 150 kJ/mol) and activation entropies (typically in the range of 0 – 30 J/mol/K).

Our target is the formulation of a global, thermodynamic model for the inactivation of both enzymes and micro-organisms exposed to heat and chemicals. From a microbiological point of view, it may be unconventional to consider an inactivation model for living cells that has been developed mainly for chemical reactions and isolated proteins. However, proteins contribute to a large extent to the total microbial biomass; typically 55% of the dry weight for an *E. coli* bacterium (Neidhardt & Umbarger, 1996). In this respect, the difference between an isolated protein system and a micro-organism is only found in the number of protein systems present. It has been estimated that the bacterium *E. coli* comprises of 4300 different proteins with standard Gibbs free energies (determining the stability of the native state compared to the unfolded state) in the range of 2 – 50 kJ/mol, which determines the functionality of the organism in a relevant temperature range of interest (Dill, Ghosh & Schmit, 2011). Protein systems found in micro-organisms are complex and highly dynamical. Gene expression in response to environmental induced stress conditions are known to occur within seconds, and an average-size protein can be synthesized within 30 s whereas synthesis of larger proteins, as for example galactosidase, requires 2.5 – 3 min (Ryall, Eydallin & Ferenci, 2012). On the other hand, it is recognized that lethal conditions of temperature for micro-organisms correspond to conditions where the majority of proteins have denatured in an irreversible state (Dill, Gosh & Schmit, 2011). Acknowledging that all individual proteins in the proteome, no matter how many or complex, are subject to the same thermodynamic laws as isolated proteins, and given the crucial role of the proteome in the survival of the organisms, it seems therefore reasonable to explore the static Lumry- Eyring model as a model for microbial inactivation. This approach has been suggested previously (Kemeny & Rosenberg, 1973; Qin et al., 2014; Rosenberg et al., 1971). The approach has, however, also been criticized by, for instance, Peleg, Normand & Corradini (2012), the criticism being that the laws of Arrhenius and Eyring have never been developed for such complicated systems as living cells, only for simple reactions. Whilst assuming that protein/enzyme inactivation is a critical step in death of cells, we will acknowledge this criticism by referring to the activation parameters as apparent, since we do not know which enzymes/proteins are targeted.

This chapter is organised as follows: we will first investigate the correspondence between protein inactivation and the classical approach to model microbial inactivation. Here we link molecular parameters of standard activation entropy and enthalpy in the Lumry-Eyring inactivation model of proteins to the concepts of reference temperature and Z-value for inactivation that are known in microbiology, and occasionally also used in enzyme inactivation (Bigelow, 1921). The second task in our work is devoted to incorporate the stochastic nature of molecular transitions in the model, as to define the new model. Third, the developed survival model, designated as Gauss-Eyring, will be presented and evaluated as a global model for inactivation data of enzymes and micro-organisms.

4.2 Materials and methods

The model parameters were estimated by the methods of Maximum Likelihood Estimation (MLE) and Sum of Least Squares (SSr) using 'R' language and environment

for statistical computing (R, 2008). Models were coded as a single primary model to include all experimental variables and model parameters. The (global) optimisation and parameter estimates were performed using the method of Simulated Annealing using the GenSA package (Xiang et al., 2013). Global optimisation over the entire, relevant domain of parameters was carried out using box constraints, to avoid guessing of starting values and rendering a sub-optimal model.

Confidence intervals, correlation coefficients and fit statistics were calculated by standard procedures using routines and libraries available in 'R'. Selection of redundant parameters and a reduction in the number of model parameters was carried out using the Bayesian Information Criterion (BIC) (Van Boekel, 2009a). Optimized models with a reduction in the value of BIC of more than 3 are considered significantly better in a statistical sense. The models presented in this work were evaluated using published data. Inactivation data of proteinase was obtained by digitizing published graphs (Driessen, 1983; Schokker & Van Boekel, 1997) using GraphClick 3.0.3 (Arizona Software 2012), and for *L. monocytogenes* the original raw data was used (Metselaar et al., 2013 and Metselaar et al., 2016).

4.3 Deriving the Gauss-Eyring model

4.3.1 Temperature-time of inactivation: correspondence relations

The rate constant in Eyring's transition state equation (Van Boekel, 2009b) provides a means to define equivalent temperature-time combinations to reach the same level of inactivation. For an unfolding protein system, characterised by its parameters standard activation enthalpy $\Delta^{\ddagger}H^{\Theta}$ and entropy $\Delta^{\ddagger}S^{\Theta}$, the temperature-time combination for inactivation with temperature T (°C or K) and time t, is given in first order approximation by (Appendix):

$$T(t)=\frac{\Delta^{\ddagger}H^{\Theta}}{\Delta^{\ddagger}S^{\Theta}+R\log\left[\frac{k_b\cdot T_o\cdot\tau_r}{h}\right]}-\frac{\Delta^{\ddagger}H^{\Theta}}{\Delta^{\ddagger}S^{\Theta}+R\log\left[\frac{k_b\cdot T_o\cdot\tau_r}{h}\right]}\frac{R}{\Delta^{\ddagger}S^{\Theta}+R\log\left[\frac{k_b\cdot T_o\cdot\tau_r\cdot e}{h}\right]}\log\left[\frac{t}{\tau_r}\right] \qquad \text{(Eq. 4.2)}$$

where R = 8.3 J/K mol is the gas constant, k_b = 1.4·10^{-23} J/K (Boltzmann's constant), h = 6.63·10^{-34} Js, (Planck's constant), τ_r = 1 min (or 1 s) defining the unit of reference time that is used, T_0 = 300 K is the (arbitrarily chosen) temperature at standard normal conditions (300 K, 100 kPa and pH 7) and e = 2.71828.

Eq. 4.2 is identified as the definition of the reference temperature *Tr* and the *Z*-value known in microbiology (Bigelow, 1921). The *Z*-value is the required increase in temperature relative to the reference temperature needed to achieve the same level of inactivation when decreasing the exposure time, as:

$$T(t) = Tr - Z \log\left[\frac{t}{\tau_r}\right] \qquad \text{(Eq. 4.3a)}$$

with $$Tr(H,S) = \frac{\Delta^{\ddagger}H^{\Theta}}{\Delta^{\ddagger}S^{\Theta} + R\log\left[\frac{k_b \cdot T_o \cdot \tau_r}{h}\right]} \qquad \text{(Eq. 4.3b)}$$

$$Z(H,\ S) = \frac{\Delta^{\ddagger}H^{\Theta}}{\Delta^{\ddagger}S^{\Theta} + R\log\left[\frac{k_b \cdot T_o \cdot \tau_r}{h}\right]} \frac{R}{\Delta^{\ddagger}S^{\Theta} + R\log\left[\frac{k_b \cdot T_o \cdot \tau_r \cdot e}{h}\right]} \qquad \text{(Eq. 4.3c)}$$

Eq. 4.3 are the correspondence relations of the classical reference temperature and *Z*-value (Bigelow, 1921), expressed in the molecular parameters standard activation enthalpy and entropy in the Eyring transition state model (Eyring, 1935). The inverse relations give the standard activation enthalpy and entropy in terms of the classical reference temperature *Tr* and *Z*-value (keeping in mind that the values for enthalpy and entropy are apparent in the case that they do not refer to a specific protein):

$$\Delta^{\ddagger}H^{\Theta}\ (Tr,Z) = \frac{R \cdot Tr^2}{Z} - RTr \qquad \text{(Eq. 4.4a)}$$

$$\Delta^{\ddagger}S^{\Theta}\ (Tr,Z) = \frac{R \cdot Tr}{Z} - R\log\left[\frac{k_b \cdot T_o \cdot \tau_r}{h \cdot e}\right] \qquad \text{(Eq. 4.4b)}$$

The meaning that can be assigned to these correspondence relations is that the classical description of inactivation kinetics in microbiology can be made equivalent to the Lumry-Eyring model that deals with the thermodynamics of protein denaturation and inactivation (or any other chemical reaction for that matter).

4.3.2 Stochastical parameters; probability function for survival

Since at thermal equilibrium the specific, momentary folding state of a protein is subject to random changes, the activation parameters $\Delta^{\ddagger}H^{\Theta}$ and $\Delta^{\ddagger}S^{\Theta}$ should be treated as stochastic variables. This implicates that in an experiment the precise, momentary values of $\Delta^{\ddagger}H^{\Theta}$ and $\Delta^{\ddagger}S^{\Theta}$ are uncertain, and as a result, the temperature and time combinations (Eq. 4.2) where inactivation takes place are no longer exactly defined. If we treat the standard enthalpy and entropy for activation as stochastic variables, there is no longer a need to specify its actual value better than within the limits of uncertainty. Since the temperature-time combination needed to reach a certain level of inactivation is a function of the parameters $(\Delta^{\ddagger}H^{\Theta}, \Delta^{\ddagger}S^{\Theta})$, the temperature has to be treated as a stochastic variable likewise.

In a typical experiment (Metselaar et al., 2016; Schokker & Van Boekel, 1997), the number of micro-organisms or proteins that are inactivated is very large (~10^9 cfu/ml or ~10^{16} proteins/ml). Under these circumstances, the survival function, that is defined by the outcome of repeated sampling of a very large number of random temperature-time combinations leading to inactivation, is well approximated by the normal distribution (Central Limit Theorem).

Since the temperature-time function has a log(t) dependence we can hypothesize the (cumulative) log-normal distribution (Weisstein, 2016a) as the bivariate probability function S (T, t) for the survival of an ensemble of proteins or micro-organisms:

$$S(T,t) = \frac{1}{2}\operatorname{erfc}\left[\frac{T - Tr + Z\log\left[\frac{t}{\tau_r}\right]}{\sqrt{2}\sigma}\right] \qquad \text{(Eq. 4.5)}$$

where erfc(x) is the complementary, cumulative normal (Gaussian) distribution (Weisstein, 2016b) and σ the width (uncertainty in the transition temperature) of the survival function (°C).

4.3.3 Extension to include chemical treatment

The Eyring model is based on fundamental thermodynamics and the rate constant is defined by the standard free activation energy term $\Delta^{\ddagger}G^{\Theta} = \Delta^{\ddagger}H^{\Theta} - T\Delta^{\ddagger}S^{\Theta}$ of the transition state under consideration.

As such, the Gauss-Eyring model can be modified by including the thermodynamic free energy of the system. For the general case of chemical treatment, the standard free energy term at equilibrium yields:

$$\Delta^{\ddagger}G^{\Theta} = -R \cdot T \cdot \log(K) \qquad \text{(Eq. 4.6)}$$

where K is the equilibrium constant of the chemical reaction under consideration (Reiff, 1965).

The Eyring rate equation has been modified for chemical treatment by including the standard thermodynamic free energy for chemical equilibrium (Appendix). Remarkably, the extended temperature-time function defines a unique Z-value to account for changes both in temperature and pH. The temperature change to access the transition state by a change in pH, relative to standard normal conditions, is given by:

$$T(t,\Delta pH) = Tr - Z \cdot \log\left[\frac{t}{\tau_r}\right] - Z \cdot \Delta pH \qquad \text{(Eq. 4.7)}$$

where Z is the commonly known Z-value as defined for thermal treatment as in Eq. 4.3, and ΔpH is the change in pH. Note that the reference state for pH can be chosen arbitrarily, leading to a corresponding shift in the reference temperature.

The validity of this predicted pH dependence was investigated using experimental inactivation data of lysozyme at different pH provided by Ahern & Klibanov (1985). They studied the inactivation of lysozyme at pH 4, 6 and 8 at a fixed temperature of 100 °C and observed first order kinetic response. The standard first order Eyring model, with the addition of the free energy term for the shifted chemical equilibrium by pH, was fitted as a global model to the kinetic data series. It was confirmed (Appendix) that the extension given by Eq. 4.6 can be used to model lysozyme inactivation as a function of pH in the range of 4 – 8. From this we could conclude that the free energy term for chemical equilibrium, as defined by Eq. 4.6, is properly incorporated into

the temperature-time function of the Gauss-Eyring model (Appendix), leading to a temperature shift as in Eq. 4.7.

4.4 Results and discussion

4.4.1 Inactivation model for extracellular protease

To validate the proposed Gauss-Eyring model as given in Eq. 4.5, the model was fitted to reported data sets by Driessen (1983), Schokker (1997), and Schokker & Van Boekel (1997). These authors studied the thermal inactivation of an extracellular protease from *Pseudomonas fluorescens* 22F, together covering the inactivation measured over a temperature range of 48 – 130 °C. Interestingly, inactivation of this protease at reduced temperatures, known as Low Temperature Inactivation (LTI) was discriminated from inactivation at the high temperature regime (90 – 130 °C) (Kroll & Klostermeyer, 1984; Schokker, 1997). By modelling reaction pathways, it was concluded that the mechanism for LTI (auto digestion) could be discriminated from chemical inactivation in the high temperature region (Schokker, 1997).

Schokker & Van Boekel (1997) have investigated inactivation of protease in the temperature range of 90 – 110 °C in detail and observed non-linear inactivation kinetics. Several refined models were investigated, all based on the basic Lumry-Eyring model (Eq. 4.1), to deal with the observed curvature in the data. In the pre-equilibrium models of the unfolding protein, it was required to assume a minimum of two enzyme states in order to obtain a reasonable fit of the model to the data (Schokker & Van Boekel, 1997). The different rate equation models were all based on an equilibrium model for the partly unfolded protein and an irreversible transition to the inactive state, its rate quantitatively defined by Eyrings Transition State Theory.

Model parameters for the activation enthalpy and entropy, that have been determined for a series of models with increasing complexity, show relatively large confidence intervals (Schokker 1997). In the original work of Schokker & Van Boekel (1997) it was concluded that a univocal reaction pathway leading to inactivation could not be determined, but that the thermal inactivation of proteinase from *P. fluorescens* 22F is complex and comprises a sequence of at least two reactions (Schokker & Van Boekel, 1997).

Reactions that trigger irreversible transitions in proteins are highly diverse and include hydrolysis of peptide bonds, reconfiguring of disulfide bonds, destruction of amino acid residues, aggregation and conformational changes of the tertiary structures (Ahern & Klibanov, 1985). Given the large number of possible molecular reactions, modelling the pre-thermal equilibrium of the partial unfolded protein by chemical rate equations, to establish the pathway for inactivation, seems to be a challenging task, especially for complex proteins found in foods. To deal with this nonetheless, we suggest to rely on the stochastic argument that has been made (preceding Eq. 4.5) to account for the uncertainty of the partially unfolded state at equilibrium.

The proposed Gauss-Eyring model for thermal inactivation of extracellular protease is:

$$(T,t)=\begin{cases}\dfrac{a_1}{2}\operatorname{erfc}\left[\dfrac{T-T_1+Z_1\log\left[\dfrac{t}{\tau_r}\right]}{\sqrt{2}\sigma_1}\right]+\dfrac{(1-a_1)}{2}\operatorname{erfc}\left[\dfrac{T-T_2+Z_2\log\left[\dfrac{t}{\tau_r}\right]}{\sqrt{2}\sigma_2}\right] & 48<T<52\ ^{\circ}\text{C (LTI)}\\ \dfrac{1}{2}\operatorname{erfc}\left[\dfrac{T-T_3+Z_3\log\left[\dfrac{t}{\tau_r}\right]}{\sqrt{2}\sigma_3}\right] & 70<T<80\ ^{\circ}\text{C}\\ \dfrac{1}{2}\operatorname{erfc}\left[\dfrac{T-T_4+Z_4\log\left[\dfrac{t}{\tau_r}\right]}{\sqrt{2}\sigma_4}\right] & 90<T<110\ ^{\circ}\text{C (HTI)}\\ \dfrac{1}{2}\operatorname{erfc}\left[\dfrac{T-T_5+Z_5\log\left[\dfrac{t}{\tau_r}\right]}{\sqrt{2}\sigma_5}\right] & 120<T<130\ ^{\circ}\text{C (UHT)}\end{cases} \quad \text{(Eq. 4.8)}$$

where the model parameters, the standard activation enthalpy $\Delta^{\ddagger}H^{\Theta}$ and entropy $\Delta^{\ddagger}S^{\Theta}$ are given in terms of *Tr* and *Z* by Eq. 4.3, σ is the width of the temperature distribution (°C) and where a_1 and $a_2 = 1 - a_1$ are the initial fractions of two iso-enzymes that can be distinguished in the LTI region. The independent variables are temperature (*T*) and time (*t*), while the response is the measured (total) activity of the sample under investigation.

The model in Eq. 4.8 is defined for different temperature domains. It was found that a two iso-enzyme model (7 parameters) is required to fit the data in the LTI region, while the other regimes are best modelled by a single enzyme (3 parameters). This scheme was found after model optimisation of the overall BIC statistic. Optimisation includes the determination of the different temperature domains and number of iso-enzymes. Thus, on the basis of objective selection criteria, it was found that Eq. 4.8 is the best performing model to describe the combined set of data by Driessen (1983), Schokker (1997), and Schokker & Van Boekel (1997) for inactivation of protease in the temperature range of 48 – 130 °C.

The results of the fit to the data are shown in Fig. 4.1. Parameter estimates and 95% confidence intervals are given in Table 4.1. Despite that the optimised Gauss-Eyring model is a single isoenzyme model, the curvature in the observed data is reproduced for the high temperature region. Other models that have been tried were first order Eyring with 1 and 2 subpopulations (Yoshioka et al., 1984). However, alternative models either required a larger number of isoenzymes, or just have poorer performance in BIC statistics (data not shown).

In essence, the model in Eq. 4.8 is a basic Gauss-Eyring model describing the selection of a single, heat stable isoenzyme in the low temperature region, which unfolds in the LTI regime. The rapid decline of activation enthalpy and entropy as a function of temperature (Table 4.1) suggest the unfolding of an enzyme at lower temperature

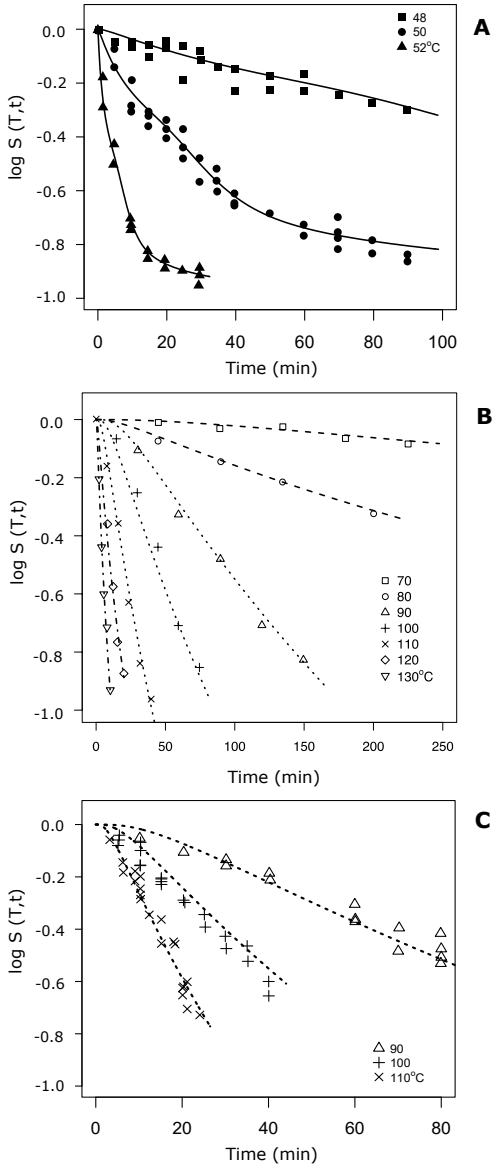

Figure 4.1. Global modelling of the inactivation of extracellular proteinase from *P. fluorescence* 22F in the range of 48–130 °C. A) Inactivation data from Schokker (1997) at temperatures 48–52 °C and the model fit (—) in the LTI regime for a 2- isoenzyme system. B) Inactivation data from Driessen (1983) and model fit for intermediate temperatures 70–80 °C (––), the high temperature regime HTI 90– 110 °C (- -) and at ultra-high temperatures (UHT) 120–130 °C (––) for a single isoenzyme. C) Data from Schokker (1997) and model fit in the high temperature regime HTI 90–110 °C (- -).

(unfolding is here the rate determining process), followed by 'normal' chemical inactivation at higher temperatures (chemical inactivation is the rate determining process), which explains the drastic decrease in value of the standard activation

enthalpies and entropies in going from the LTI region to the UHT region. The standard activation entropy and enthalpy of proteases from *Pseudomonas* spp. in the high temperature regions, shown in Table 4.1, are in good agreement with results typically found for heat resistant proteases (Stoeckel et al., 2016).

The Gauss-Eyring model incorporates two types of heterogeneity. Firstly, heterogeneity that is defined by the presence of multiple iso-enzymes which requires different sets of parameters for activation enthalpy and entropy for each iso-enzyme. Secondly, heterogeneity is defined for a single enzyme system, expressed by the width of the Gaussian temperature distribution for survival. This kind of heterogeneity is assumed to relate to the specific folding state of a single protein. In case that the protein engages in a very specific reaction pathway, the activation enthalpy and entropy are well defined and hence, the temperature width is relatively small. If, on the other hand, the enzyme can access a large number of conformations, the activation enthalpy and entropy are uncertain and a broader temperature distribution can be expected.

Table 4.1 Model parameter estimates for inactivation of extracellular proteinase of *Pseudomonas fluorescence* 22F for temperatures in the range of 48 °C to 130 °C according to the Gauss-Eyring model. The 95% confidence intervals of the parameter estimates are given in square brackets.

Model parameter	**LTI region 48-52°C (Schokker, 1997)**	**Intermediate 70-80°C (Driessen, 1983)**	**HTI region 90-110°C (Driessen, 1983; Schokker, 1997)**		**UHT region 120-130°C (Driessen, 1983)**
			(Driessen, 1983)	**(Schokker, 1997)**	
a_1 (%)	79 [76-83]	100 [-]	100 [-]	100 [-]	100 [-]
$\Delta^{\ddagger}H_1^{\Theta}$ (kJ/mol)	916.5 [916.4 - 916.6]	185.5 [185.3-185.6]	112.4 [112.2–112.6]	111.2 [111.1 - 111.3]	111.4 [111.3–111.6]
$\Delta^{\ddagger}S_1^{\Theta}$ (J/K mol)	2565 [2564 - 2566]	232 [231-233]	24.4 [24.1–24.8]	23.3 [23.0-23.6]	22.6 [22.2–23.0]
σ_1 (°C)	1.24 [1.16-1.31]	8.7 [8.3- 9.2]	11.2 [12.6-13.5]	12.0 [11.3-12.7]	14.3 [13.8-14.9]
Z_1 (Eq.4.3b) (°C)	2.16	15.8	30	31	31
a_2 (%)	21 [17-24]	not detected / not resolved			
$\Delta^{\ddagger}H_2^{\Theta}$ (kJ/mol)	604.6 [604.4– 605.0]				
$\Delta^{\ddagger}S_2^{\Theta}$ (J/K mol)	1597.9 [1597.0- 1598.7]				
σ_2 (°C)	0.34 [0.21-0.50]				
Z_2 (Eq.4.3b) (°C)	3.31				

In the LTI region, the Gauss-Eyring model was able to discriminate for two iso-enzymes (Table 4.1) on the basis of differences in the standard activation entropy for inactivation i.e., two rate determining processes could be resolved. Global modelling and direct parameter estimation resulted in an activation entropy and enthalpy that are apparently highly precise (Table 4.1). The direct inactivation pathway in the LTI

region that proceeds directly from the native to the inactive state (Schokker, 1997) can be identified with the presence of a temperature sensitive isoenzyme in the LTI region (Table 4.1). Schokker (1997) has reported a standard activation entropy and enthalpy for inactivation of $\Delta^{\ddagger}H^{\Theta}$ = 504 ± 51 kJ/mol and $\Delta^{\ddagger}S^{\Theta}$ = 1232 ± 165 J/K mol. This result was obtained by kinetic modelling of the inactivation by rate equations and subsequent thermodynamic analysis (Arrhenius plot). The activation enthalpy and entropy estimates for the heat labile enzyme, according to the Gauss-Eyring model, yield respectively, $\Delta^{\ddagger}H^{\Theta}$ = 604.6 kJ/mol and $\Delta^{\ddagger}S^{\Theta}$ = 1597.9 J/K mol (Table 4.1). These numbers are in reasonable agreement with the parameter estimates for the rate determining, direct inactivation pathway made by Schokker (1997).

From the unfolding and folding reaction constants, determined by chemical rate modelling, the equilibrium constant for the $\mathrm{N} \underset{\leftarrow}{\rightarrow} \mathrm{U}$ (unfolding) reaction could be quantified as a function of temperature (Schokker, 1997). The estimated enthalpy and entropy for the unfolding reaction is ΔH^{Θ} = 1128 kJ/mol and ΔS^{Θ} = 3481 J/K mol and denaturation temperature T_d = 324 K (Schokker, 1997). Note that these are not activation parameters but refer to the differences in thermodynamical state of the native and unfolded state. These results come with the remark that the procedure of secondary modelling (van 't Hoff plot) is prone to large errors. Notwithstanding, these thermodynamic parameter estimates for (rate determining) unfolding are consistent to the parameter estimates for the activation energy of the heat stable isoenzyme complex in the LTI region (Table 4.1) yielding $\Delta^{\ddagger}H^{\Theta}$ = 916.5 kJ/mol and $\Delta^{\ddagger}S^{\Theta}$ = 2565 J/K mol and a reference temperature defined by Eq. 4.3b of 326 K.

A relatively large temperature width (σ = 1.24 °C) was found (Table 4.1) for the survival of the heat stable iso-enzyme when compared to the heat labile directly inactivated iso-enzyme (σ =0.34 °C) in the LTI region. This is consistent with the view that a large number of changes in the conformational state of the protein is required to reach an unfolded pre-equilibrium transition state prior to inactivation.

To summarise: the Gauss-Eyring model provides a consistent estimation for standard activation enthalpy and entropy for thermal inactivation of extracellular protease from *P. fluorescence* 22F over the temperature range of 48 – 130 °C. Upon quantitative comparison of the standard activation parameters for the molecular inactivation pathways known to date, the sub-populations in the Gauss-Eyring model that are coined as 'iso-enzymes', are likely to represent two alternative pathways that lead to inactivation.

4.4.2 Inactivation of *Listeria monocytogenes* by heat and pH

As a second test, the Gauss-Eyring model was tested as the primary model for inactivation of a microbial population as a function of temperature, time and pH by combining Eq. 4.5 and Eq. 4.7. Highly accurate kinetic inactivation data for *Listeria monocytogenes* at different temperatures and pH, were provided by Metselaar et al. (2013), Metselaar et al. (2016). Metselaar et al. (2013) have studied the inactivation of *L. monocytogenes* LO28 wild type (WT) and an acid resistant variant 14, at two different growth stages (late exponential and stationary phase) towards combinations of heat and acidification. They have modelled the observed (non-linear) inactivation kinetics

piecewise using a biphasic model with a shoulder and a reduced model (Metselaar et al., 2016). Following this approach, the observed non-linear inactivation kinetics could be associated to heterogeneities in the initial population.

The Gauss-Eyring model was fitted as a global model to the inactivation data of Metselaar et al. (2013), Metselaar et al. (2016) using temperature, pH and time as the independent variables and assuming two Gaussian subpopulations (8 parameters) according to:

$$S(T,t,pH) = \frac{a_1}{2}\operatorname{erfc}\left[\frac{T - T_1 + Z_1\left[\log\left[\frac{t}{\tau_r}\right] - m\,\Delta pH\right]}{\sqrt{2}\,\sigma_1}\right] + \frac{1-a_1}{2}\operatorname{erfc}\left[\frac{T - T_2 + Z_2\left[\log\left[\frac{t}{\tau_r}\right] - m\,\Delta pH\right]}{\sqrt{2}\,\sigma_2}\right] \quad \text{(Eq. 4.9)}$$

where m is the order of pH equilibrium (supplement).

The result of the global fit of the Gauss-Eyring model and the parameter estimates are shown in Fig. 4.2 and Table 4.2, respectively. The reference level for pH was chosen at pH = 0. When choosing an intermediate reference level e.g. pH = 4.0 we observed numerical instabilities as the fitting algorithm needs to cross a zero point during the optimization routine. This could be avoided by choosing the reference state for pH either higher than 7.3 or less than 2.5. Since the region of interest is the survival in the low pH range, pH = 0 was chosen as the reference level in this work.

Table 4.2. Parameter estimates of the Gauss-Eyring model for *L. monocytogenes* when exposed to heat and pH. The estimates are for a global fit to the inactivation data (Metselaar et al., 2013 and Metselaar et al., 2016) for LO28-WT and the variant 14 in the late exponential (LE) and stationary phase (SP). In each isolate two sub-populations were resolved that differ in reference temperature *Tr* and *Z*-value. The fit parameters are given for pH = 0 as the (arbitrary chosen) reference level. The 95% confidence intervals of the parameter estimates are given in square brackets.

Model parameter	LE-LO28-14	SP-LO28-14	LE-LO28-WT	SP-LO28-WT
T_1 (°C)	33.9 [33.8 - 34.0]	38.7 [38.6 - 38.8]	35.0 [34.9 - 35.1]	34.5 [34.4 - 34.7]
T_2 (°C)	12.8 [12.5 - 13.0]	7.3 [7.1 - 7.5]	20.9 [20.7 - 21.1]	0.2 [0.1 - 0.3]
Z_1 (°C)	3.53 [3.52 - 3.54]	4.45 [4.43 - 4.47]	4.6 [4.5 - 4.7]	3.74 [3.72 - 3.77]
Z_2 (°C)	6.21 [6.17 - 6.25]	11.1 [11.0 - 11.2]	5.2 [5.1 - 5.3]	8.71 [8.67 - 8.76]
σ_1 (°C)	0.53 [0.52 - 0.56]	0.37 [0.34 - 0.38]	0.42 [0.40 - 0.43]	0.44 [0.40 - 0.47]
σ_2 (°C)	1.14 [1.07 - 1.20]	1.04 [1.01 - 1.07]	2.75 [2.72 - 2.78]	1.29 [1.24 - 1.34]
a_1 (%)	87 [82 - 94]	66 [55 - 79]	62 [53 - 71]	48 [29 - 69]
a_2 (%)	13 [7 - 21]	34 [23 - 47]	38 [29 - 47]	52 [33 - 73]
m	1.30 [1.29 - 1.31]	0.858 [0.855 - 0.861]	0.939 [0.936 - 0.942]	1.237 [1.234 - 1.241]

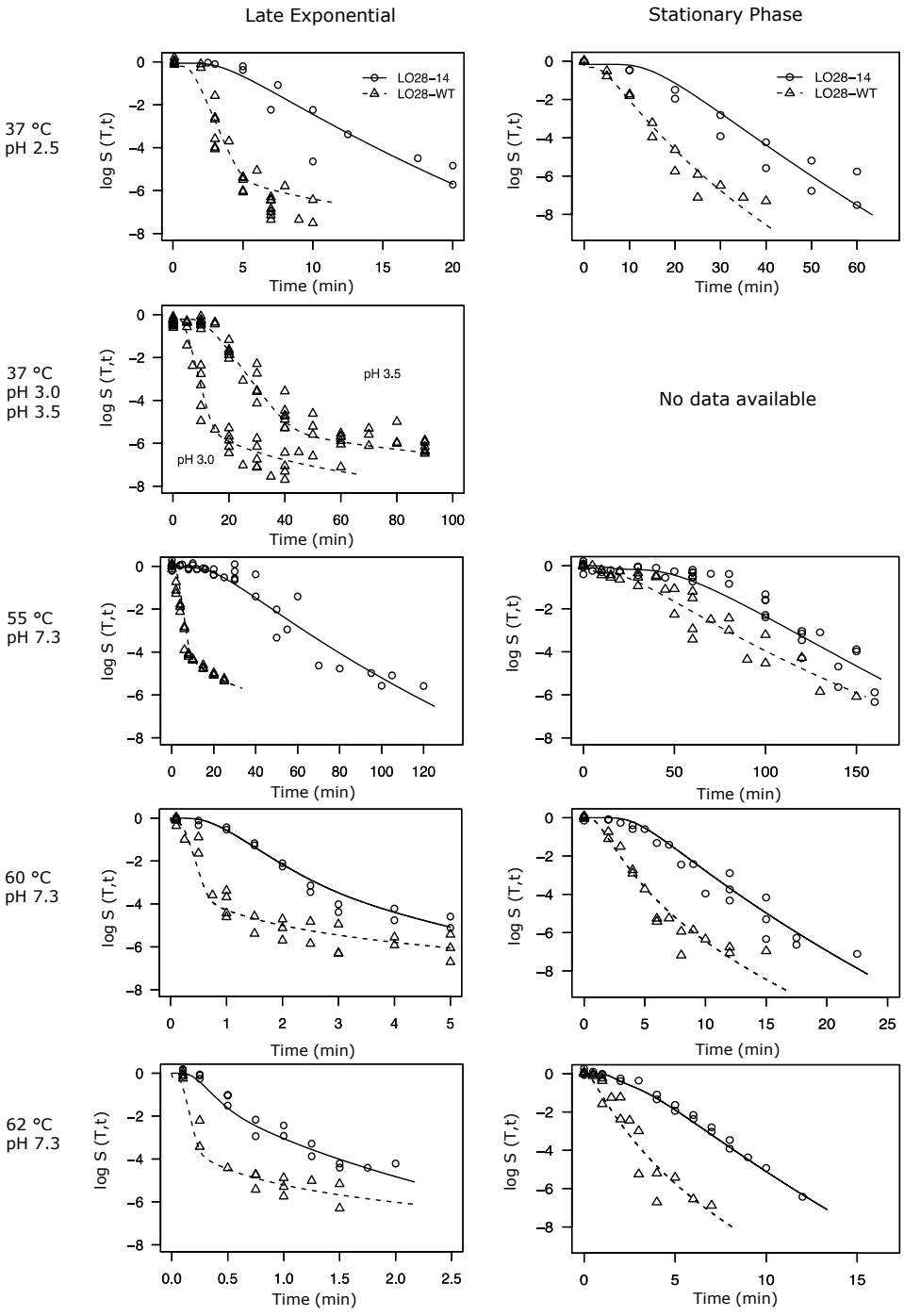

Figure 4.2 Global fit of the Gauss-Eyring model to the inactivation data of *L. monocytogenes* (Metselaar et al., 2013 and Metselaar et al., 2016) for different temperature, pH, exposure time and growth phase. The estimates of the model parameters and the 95% confidence intervals are shown in Table 4.2.

It can be observed in Fig. 4.2, that the Gauss-Eyring model fits both to the inactivation data of *L. monocytogenes* LO28-WT as well to the data for the acid-resistant variant 14, either harvested in the late exponential or stationary growth phase. Remarkably, the model according to Eq. 4.9, which is defined by a single line shape, fits to the data for all combinations of pH and temperature under consideration.

The striking difference of the empirical model of Metselaar et al. (2016) in comparison to the Gauss-Eyring model is that the empirical model accounts for a majority (99.99%) and a minority (0.01%) population. Instead, the Gauss-Eyring model consistently fits to an optimal global solution for two sub-populations that have nearly equal concentrations (Table 4.2). Comparison of the biphasic model with shoulder (Metselaar et al., 2013) (15 parameters, BIC = 247) and the Gauss-Eyring model (8 parameters, BIC = 201) for a series of three different pH's at a fixed temperature of 37 °C, showed that the Gauss-Eyring model performs considerably better (ΔBIC = -46). An even more relevant difference is found in the number of model parameters; the Gauss-Eyring model has 8 parameters, whereas the piecewise biphasic model requires 30 parameters. Another important observation is that the (relative) confidence intervals for parameter estimates for the Gauss-Eyring model (~5%) are significantly smaller than for the biphasic model (~25%). A smaller number of fit parameters is always preferred (Occam's razor principle) and smaller confidence intervals indicate that critical model parameters are more precisely determined. Based on statistical arguments, it is therefore concluded that the performance of the global Gauss-Eyring model is better than for the biphasic model on this data set.

However, a decisive preference for one model over an alternative is found in the ability to incorporate basic mechanisms that explain the observed dynamics. The key question that is brought to attention by the results of Metselaar et al. (2013), Metselaar et al. (2016) is whether or not the observed non-linear inactivation kinetics originates from a possible selection of a sub-population that is initially present, or is the result of adaptation of the population during treatment as a whole (Abee et al., 2016). In the case of selection, the observed inactivation can be decomposed into two, static sub-populations of micro-organisms, whereas in the case of adaptation, the model should include dynamical effects (Corradini & Peleg, 2009), invoked by the treatment that adjusts the properties of a single population during the time of exposure. The empirical biphasic model with a shoulder was assumed to contain two sub-populations, each including an explicit time shift (Metselaar et al., 2013). Therefore, both static and dynamical aspects are accounted for. The Gauss-Eyring model does not require an explicit time delay to mimic a shoulder. Remarkably, the Gauss-Eyring model is able to deal with both the observed shoulder and tail (Fig. 4.2), without introduction of an additional model parameter or other modification. To understand this phenomenon, the intrinsic time delay in the Gauss-Eyring model was studied in more detail.

In Fig. 4.3, the dynamics of inactivation in the Gauss-Eyring model are exemplified by decomposition of the two underlying Gaussian sub-populations. Decomposition was carried out for the inactivation of late exponential cells of LO28-WT treated at 37 °C, pH 3.5. In Fig. 4.3a, the Gaussian distributions for the two subpopulations are shown, using the fit parameters given in Table 4.2.

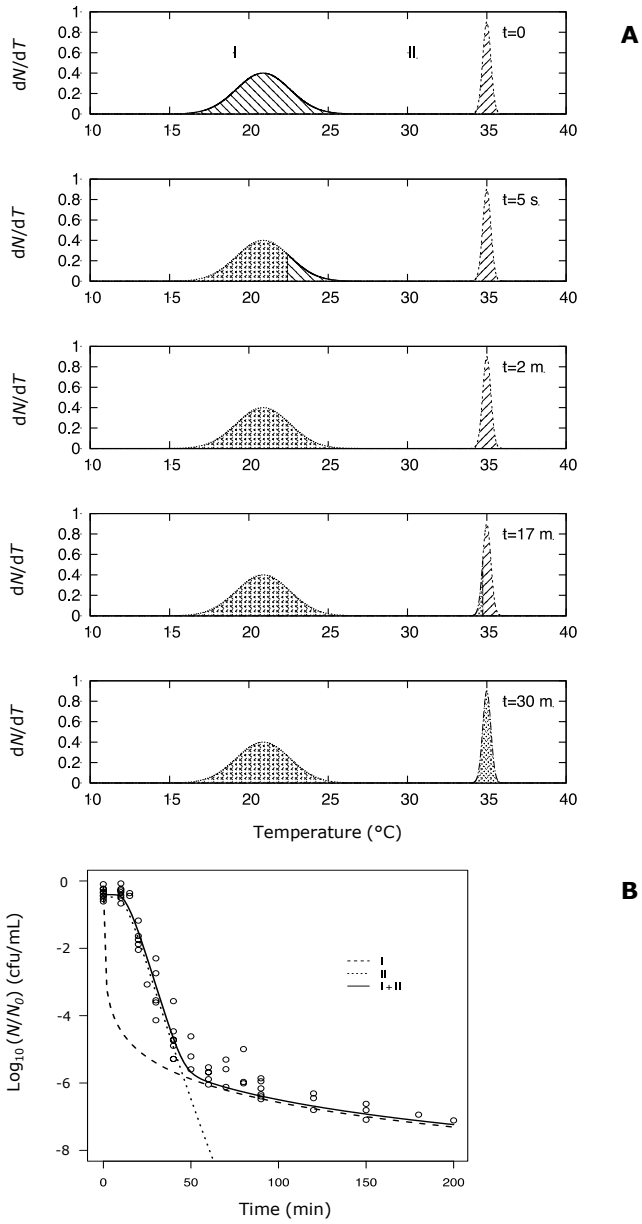

Figure 4.3. Inactivation dynamics by acid treatment of two sub-populations resolved in *L. monocytogenes* LO28WT by the Gauss-Eyring model. A). The initial culture consists of two Gaussian distributed subpopulations with distinct *T* and *Z*-values. Subpopulation I has a reference temperature of 20.9 °C and *Z*-value of 5.2 °C. The acid resistant subpopulation II has a reference temperature of 35.0 °C and *Z*-value of 4.6 °C. Inactivation (indicted as blackened area) in subpopulation I is initiated immediately after exposure to pH=3.5 at 37 °C. B). Inactivation of cells in subpopulation II is delayed for 17 min but proceeds thereafter at a higher rate than for organisms in subpopulation I. The survivors that are found in the tail originating from subpopulation I, are getting less sensitive during exposure and therefore can survive for treatment times longer than 50 min.

Subpopulation I is characterised by Tr_1 = 19.7 °C, Z_1 = 5.5 °C, σ_1= 1.8 °C and subpopulation II by Tr_2 = 31.1 °C, Z_2 = 3.9 °C, σ_2 = 0.8 °C. The different values of Tr and σ for the subpopulations I and II, clearly show their contribution into the Gaussian distribution in Fig. 4.3a, with Tr as the mean of the 'bell-shaped' curve and σ as the width of the distribution. The Gaussian distribution is directly related to the survival function (cumulative distribution) given by Eq. 4.5, and represents the number of organisms initially present in a (small) temperature interval $[T,T + dT]$.

The dynamics of inactivation in the Gauss-Eyring model is determined by its three parameters, Tr, Z and σ. The onset for inactivation occurs for the subpopulation with the lowest reference temperature. A shoulder is the result of a time delay in inactivation that is defined by the term $Tr - Z \cdot \log(t)$ in the survival curve (Eq. 4.5). A large reference temperature, or a small Z value, leads to a broader shoulder. The tailing of the inactivation curve is determined by the ratio of Z/σ where σ is the temperature width of the Gaussian distribution. A large temperature width, or small Z-value leads to a longer tail. The behaviour at intermediate times (between the onset and tailing), and hence the shape of the survival curve, depends on the actual values of the three model parameters. In the case that multiple sub-populations are present, the observed kinetics for the total inactivation is the sum of multiple survival curves (superposition principle), which may appear quite complex (Figs. 4.1a, 4.2 and 4.3).

As in the case of heat inactivation of protease, which was discussed in the previous section, the Gauss-Eyring model entails two types of heterogeneity. For the inactivation of *L. monocytogenes*, heterogeneity has been attributed to the presence of distinct sub-populations that have different reference temperatures and Z-values. Using the corresponding relations of the model parameters according to Eq. 4.3, a difference in reference temperature may be associated to differences in the denaturation temperature of certain proteins, that are included in the proteome and are critical for survival. The survival of micro-organisms with a distinct, static feature resembles the principle of selection.

A second kind of heterogeneity originates from the uncertainty in the transition temperature that is contained within the Gaussian distribution representing the subpopulation. As discussed throughout this work, the width of this associated Gaussian distribution reflects the uncertainty of the reference temperature and Z-value for an ensemble of micro-organisms. For proteins it was hypothesised that an uncertainty in the standard activation entropy and enthalpy for the activated complex leaves more choice to proceed along alternative reaction pathways. So, this kind of heterogeneity suggests that a system is subject to dynamical changes which resemble the principle of adaptation. Clearly, the mathematical and basic thermodynamic framework of the Gauss-Eyring model has potential to falsify and validate predictions made for thermal and chemical inactivation of protease and *L. monocytogenes*. This is impossible, or at least very difficult, in the case that phenomenological models are considered. Although the Gauss-Eyring model was successfully demonstrated to include complex inactivation kinetics for both enzymes and micro-organisms, it should be noted that only a limited amount of data available in literature has been considered. Therefore, the Gauss-Eyring model should be, for the moment at least, hailed as nothing more than a concept to deal with fundamental uncertainties in biochemistry and microbiology; further evaluation of the performance of the model is definitely needed.

4.5 Conclusion

A new primary model has been formulated for complex inactivation kinetics of proteins and micro-organisms. It has been shown that a one-to-one correspondence exists between the standard activation enthalpy and entropy in the Lumry-Eyring model for protein inactivation and the reference temperature and *Z*-value defined in the kinetic heat inactivation model used in food chemistry and classical microbiology. The temperature-time combination for inactivation is identified as a stochastic variable that reflects the uncertainty in the momentary folding state of proteins in thermal equilibrium. The primary line shape for inactivation is given by the cumulative probability distribution of the temperature-time function of inactivation.

As the model is the result of merging the Eyring rate constant for the transition state with the Gaussian distribution, the model is denominated Gauss-Eyring. The thermodynamics covered by the Gauss-Eyring model are primarily based on heat treatment, but can be extended to any treatment by adding the proper free energy term and recalculation of the temperature-time combination for inactivation.

The validity of the Gauss-Eyring model has been evaluated on the basis of a global fit to experimental data. Performance of the model was tested for inactivation of both enzymes and micro-organisms. Complex inactivation kinetics have been accurately modelled as a single primary model over a broad range of temperatures and pH. In both cases it was demonstrated that the Gauss- Eyring model has improved statistical performance over state-of the art kinetic models for inactivation and in both cases more accurate estimates for model parameters were obtained.

4.6 Appendix

The rate constant in Eyring's Transition State Theory (Eyring, 1935) is given by:

$$k(T) = \frac{k_b T}{h} \exp\left[-\frac{\Delta^{\ddagger}H^{\Theta} - T\Delta^{\ddagger}S^{\Theta}}{RT}\right] \qquad \text{(Eq. S4.1)}$$

with k_b = 1.38 x 10^{-23} J/K (Boltzmann's constant), R = 8.31 J/K mol (gas constant) and h = 6.63 x 10^{-34} J s (Planck's constant). The variable is temperature (*T*) and model parameters are $(\Delta^{\ddagger}H^{\Theta}, \Delta^{\ddagger}S^{\Theta})$ the standard activation enthalpy and entropy of the transition state under consideration.

The – well known – solution of the first-order kinetic model for the survival of a population at time *t* held at temperature *T* is:

$$N(t,T) = N(0)\exp\left[-k(T)t\right] \qquad \text{(Eq. S4.2)}$$

where it is assumed that the inactivation is proportional to the number of molecules or cells present. The characteristic time for inactivation is given by $\tau_c = 1/k$.

An alternative approach for this reaction rate equation method, is to adapt a stochastic model to obtain a survival function (main article). The preparatory step is to define the temperature-time function *T(t)* that stands for all combinations that lead to inactivation.

By definition, inactivation occurs when:

$$k(T)\ t = 1 \qquad \text{(Eq. S4.3)}$$

This equation is a non-linear equation for the function $T(t)$ under investigation. The analytical solution of Eq. S4.3 is (Wolfram, 2016):

$$T(t) = \frac{\Delta^{\ddagger}H^{\ominus}}{R}\frac{1}{W_0\left[\frac{k_b\Delta^{\ddagger}H^{\ominus}}{hR}\exp\left[\frac{\Delta S^{\ddagger}}{R}\right]t\right]} \qquad \text{(Eq. S4.4)}$$

where $W_o(x)$ is Lamberts W function defined on the principal branch.

For a linear approximation of $T(t)$, the calculation proceeds by taking the logarithm of Eq. S4.3 after substituting $k(T)$ according to S4.2:

$$\log\left[\frac{k_bT\tau}{h}\right] - \frac{\Delta H^{\ddagger}}{RT} + \frac{\Delta S^{\ddagger}}{R} = -\log\left[\frac{t}{\tau_r}\right] \qquad \text{(Eq. S4.5)}$$

where the unit of time, $\tau_r = 1\ \mathrm{s}$, is introduced to account for the proper units after splitting the factor under the logarithm.

When Eq. S4.3 is to be solved in lowest order approximation in T, it is convenient to make the substitution:

$$T \to T_1\left[1 + \frac{T - T_1}{T_1}\right] \qquad \text{(Eq. S4.6)}$$

followed by a first order Taylor expansion of the *1/T* term in Eq. S4.5.

For $\frac{T - T_1}{T_1} \ll 1$:

$$\frac{1}{T} \approx \frac{1}{T_1}\left[1 - \frac{T - T_1}{T_1}\right] \qquad \text{(Eq. S4.7)}$$

and

$$\log\left[\frac{k_bT_r\tau_r}{h}\right] \approx \log\left[\frac{k_bT_1\tau_r}{h}\right] + \frac{T - T_1}{T_1} \qquad \text{(Eq. S4.8)}$$

When the approximations S4.7 and S4.8 are substituted in Eq. S4.5 the following equation needs to be solved:

$$\log\left[\frac{k_bT_1\tau_r}{h}\right] + \frac{T - T_1}{T_1} - \frac{\Delta^{\ddagger}H^{\ominus}}{RT_1}\left[1 - \frac{T - T_1}{T_1}\right] + \frac{\Delta^{\ddagger}S^{\ominus}}{R} = -\log\left[\frac{t}{\tau_r}\right] \qquad \text{(Eq. S4.9)}$$

After recombining the terms proportional to T,

$$\left[\log\left[\frac{k_bT_1\tau_r}{h}\right] - \frac{2\Delta^{\ddagger}H^{\ominus}}{RT_1} + \frac{\Delta^{\ddagger}S^{\ominus}}{R} - 1\right] + T\left[\frac{1}{T_1} + \frac{\Delta^{\ddagger}H^{\ominus}}{RT_1^2}\right] = -\log\left[\frac{t}{\tau_r}\right] \qquad \text{(Eq. S4.10)}$$

further rearranging and approximation of the k_bT_1 term by ~ k_bT_o with T_o = 300 K, the temperature (arbitrarily) chosen at standard normal conditions 300 K, 1.0 10^5 Pa), the temperature-time combination for inactivation is obtained:

$$T(t) = \left[\frac{RT_1^2}{\Delta^{\ddagger}H^{\Theta} + RT_1}\right]\left[-\log\left[\frac{t}{\tau_r}\right] - \left[\log\left[\frac{k_bT_0\tau_r}{h}\right] - \frac{2\Delta^{\ddagger}H^{\Theta}}{RT_1} + \frac{\Delta^{\ddagger}S^{\Theta}}{R} - 1\right]\right] \quad \text{(Eq. S4.11)}$$

The resulting Eq. S4.11 is identified as the definition of the classical *Z*-value in microbiology (Bigelow, 1921) that defines the required change in temperature, when increasing exposure time, to achieve the same level of inactivation:

$$T(t) = T_r - Z\,\log\left[\frac{t}{\tau_r}\right] \quad \text{(Eq. S4.12)}$$

with

$$Z = \left[\frac{RT_1^2}{\Delta^{\ddagger}H^{\Theta} + RT_1}\right] \quad \text{(Eq. S4.13)}$$

$$Tr = \left[\frac{RT_1^2}{\Delta^{\ddagger}H^{\Theta} + RT_1}\right]\left[\frac{2\Delta^{\ddagger}H^{\Theta}}{RT_1} - \frac{\Delta^{\ddagger}S^{\Theta}}{R} - \log\left[\frac{k_bT_0\tau_r}{h}\right] + 1\right] \quad \text{(Eq. S4.14)}$$

Since T_1 was chosen as an arbitrary temperature, but close to where inactivation is observed, T_1 can be chosen to equal *Tr* , which is defined as the transition temperature at unit holding time.

After rearranging the terms in Eq. S4.14 the following expression is obtained that defines the reference temperature:

$$Tr = \frac{\Delta^{\ddagger}H^{\Theta}}{\Delta^{\ddagger}S^{\Theta} + R\,\log\left[\frac{k_bT_o\tau_r}{h}\right]} \quad \text{(Eq. S4.15)}$$

which is the ratio of activation enthalpy and entropy for the transition state. The two entropy terms in the denominator relate, respectively, the entropy for molecular structure (internal energy) and the entropy of the ideal gas that's yields a constant 245 J/K mol at 300 K.

Incidentally, the reference temperature defined in Eq. S4.15 has a close relationship with the melting temperature (T_m) for proteins, defined as the temperature where the free energy is zero or equivalent, $T_m = \Delta H / \Delta S$ where ΔH and ΔS are the enthalpy and entropy differences between the native and unfolded state.

By combining Eq. S4.12 with Eq. S4.15 the explicit expression of the temperature-time function in terms of $(\Delta^{\ddagger}H^{\Theta}, \Delta^{\ddagger}S^{\Theta})$, is obtained, with *e* = 2.718:

$$T(t) = \frac{\Delta^{\ddagger}H^{\Theta}}{\Delta^{\ddagger}S^{\Theta} + R\log\left[\frac{k_bT_o\tau_r}{h}\right]} - \frac{\Delta^{\ddagger}H^{\Theta}}{\Delta^{\ddagger}S^{\Theta} + R\log\left[\frac{k_bT_o\tau_r}{h}\right]}\,\frac{R}{\Delta^{\ddagger}S^{\Theta} + R\log\left[\frac{k_bT_o\tau_r\,e}{h}\right]}\,\log\left[\frac{t}{\tau}\right] \quad \text{(Eq. S4.16a)}$$

Eq. S4.16a defines the temperature-time combination for inactivation of a molecular complex through a transition state by thermal treatment.

The *Z*-value is given by:

$$Z = \frac{\Delta^{\ddagger}H^{\Theta}}{\Delta^{\ddagger}S^{\Theta} + R\log\left[\frac{k_b T_o \tau_r}{h}\right]} \frac{R}{\Delta^{\ddagger}S^{\Theta} + R\log\left[\frac{k_b T_o \tau_r e}{h}\right]} \quad \text{(Eq. S4.16b)}$$

Eq. S4.15 shows that the reference temperature is defined by the ratio of enthalpy and entropy. This makes sense from a thermodynamic point of view: more cross linking in proteins – or stronger bonds – that hold up the tertiary structure, implicate higher transition temperatures for unfolding. In addition, one can deduct from Eq. S4.16b that for a fixed activation enthalpy, the *Z*-value decreases with increasing activation entropy. This suggests that proteins (as a polymer structure) with more degrees of freedom are more sensitive at higher temperature.

The backward conversion of the parameters enthalpy and entropy $(\Delta^{\ddagger}H^{\Theta}, \Delta^{\ddagger}S^{\Theta})$, in the Eyring model to the classical *Z*-value and transition temperature (Z, Tr) is given by:

$$\Delta^{\ddagger}H^{\Theta} = \frac{RTr^2}{Z} - RTr \quad \text{(Eq. S4.17)}$$

$$\Delta^{\ddagger}S^{\Theta} = \frac{RTr}{Z} - R\log\left[\frac{k_b T_o \tau_r}{he}\right] \quad \text{(Eq. S4.18)}$$

$$\Delta^{\ddagger}G^{\Theta} = RTr\log\left[\frac{k_b T_o \tau_r}{he^2}\right] \quad \text{(Eq. S4.19)}$$

Chemical treatment

The free energy term in Eq. S4.5 can be modified to include all treatments with a known thermo-dynamical potential. For the general case of a chemical equilibrium, the change in free energy is given by:

$$\Delta G_c^{\Theta} = -RT\log(K) \quad \text{(Eq. S4.20a)}$$

where K is the equilibrium constant of the chemical reaction under consideration. K is given by the activities of active species. For an (complex) equilibrium reached by acidification, the pH is determined by the free hydrogen activity and the equilibrium constant is proportional to:

$$K \sim \left[H_3 0^+\right]^m$$

where *m* is the order of the reaction.

For a first order reaction (i.e., $m = 1$), the activation change is assumed to change with pH according to:

$$\Delta^{\ddagger}G_{pH}^{\Theta} = 2.3\,RT\ pH \quad \text{(Eq. S4.20b)}$$

which yields 5.9 kJ/mol per pH point at standard normal conditions.

The predicted thermodynamic shift in activation energy evaluated using the observed

inactivation of lysozyme at different pH. In Fig. S4.1 the experimental data (Ahern & Klibanov, 1985) for the inactivation of lysozyme at pH 4, 6, 8 at 100 °C are shown.

For a pH series at fixed temperature, the Eyring model is reduced to a linear model:

$$\log\left[\ S(t,pH)\right] = -\log\left[\frac{k_b T}{h}\right]\exp\left[-\frac{\Delta^{\ddagger}G^{\Theta}}{RT} - 2.3\cdot m\cdot pH\right] \qquad \text{(Eq. S4.20c)}$$

The model according to Eq. S4.20C has been fitted to the observed data and the result is shown in Fig. S4.1. It was found that Eyring's rate constant for the transition state – extended with chemical shift as in Eq. S4.20a – is a proper, global model for the inactivation for lysozyme at different pH. The order of the equilibrium reaction found is m = 0.5. Rate constants reported by Ahern & Klibanov (1985) – that were obtained by piece wise regression (D_T -value method) – are, within the experimental error, in good agreement with Eq. S4.20c.

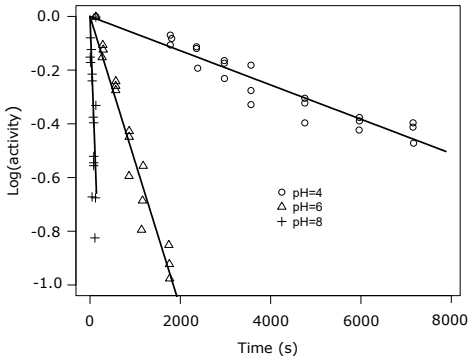

Figure S4.1. Lysozyme inactivation at pH 4, 6, 8 at 100 °C. Data is from Ahern and Klibanov (1985). The global model is derived from Eyring's transition state equation after extension of the free energy term by $\Delta^{\ddagger}G^{\Theta} = m\cdot R\cdot T\cdot 2.3\cdot pH$, shown by a fit (lines) of Eq. S4.20c to the data.

A change in equilibrium constant K leads to a chemical temperature shift $T_c(t)$ inactivation. This temperature shift can be calculated using Eq. S4.16 by making a direct substitution for the entropy:

$$\Delta^{\ddagger}S^{\Theta} \rightarrow \Delta^{\ddagger}S^{\Theta} - R\,\log(K) \qquad \text{(Eq. S4.21)}$$

For dissociated systems that satisfy $Rlog[K] \ll \Delta^{\ddagger}S^{\Theta} + R\log\left[\frac{k_b T_o \tau_r}{h}\right]$, e.g. for polymer systems where the entropy change is large, the chemical temperature shift T_c can be approximated in first order of $\log(K)$:

$$T_c = \frac{R\Delta^{\ddagger}H^{\Theta}}{\left[\Delta^{\ddagger}S^{\Theta} + R\log\left[\frac{k_b T_o \tau_r}{h}\right]\right]^2}\log(K) \qquad \text{(Eq. S4.22)}$$

Upon comparison of S4.22 and S4.16b, the temperature change to access the transition state by a change in pH is in good approximation given by:

$$T_{pH} = \mathrm{Z}\,\Delta pH \qquad \text{(Eq. S4.23)}$$

where Z is the commonly known Z-value as defined for thermal treatment Eq. S4.12.

Chapter 5

Evaluation of the Gauss-Eyring model to predict thermal inactivation of micro-organisms at short holding times

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Abstract

Application of mild (non)-thermal processing technologies have received considerable interest as alternative to thermal pasteurisation, because of its shorter holding time and lower temperature aiming for an improved product quality. To understand and develop these alternative technologies, like pulsed electric fields, a proper comparison between the conventional thermal and alternative process is necessary. Up to recent, no suitable models were available to predict the inactivation of micro-organisms by a thermal process at a chosen short holding time, due to non-linearity. The recently developed Gauss-Eyring model with two variables temperature and time has the properties to be a suitable model to apply for short holding times, and was tested for this purpose.

Therefore, this study aims to validate if the Gauss-Eyring model can be used to describe non-linear isothermal (a fixed temperature with varying holding time) and isotime (a fixed holding time with varying temperature) thermal inactivation data, and if it is a suitable model to predict the thermal inactivation as a function of temperature for short holding times. Inactivation data of *Escherichia coli, Listeria monocytogenes, Lactobacillus plantarum, Salmonella* Senftenberg and *Saccharomyces cerevisiae* in orange juice were collected via isothermal and isotime inactivation kinetics. Survival of the tested micro-organisms was modelled with the Gauss-Eyring model, which contains three parameters σ, Tr and Z. The transition of 'no inactivation' to 'inactivation' (i.e. the 'shoulder' in inactivation curves) can be characterised as the temperature-time (T, t) combination where $T = Tr - Z \cdot log_{10}(t)$, with Tr as the reference temperature defined for 1 s treatment, Z as the temperature needed for a 10-fold increase or decrease of the holding time t, and σ as the temperature width of the distribution. The Gauss-Eyring model fitted well to the experimental data, and revealed different sensitivity for the tested micro-organisms. Based on the parameter estimations, survival curves for the desired short holding times were predicted.

5.1 Introduction

Thermal pasteurisation of food products is widely used to eliminate a desired number of micro-organisms and to inactivate enzymes. In order to achieve a sufficient level of inactivation, the process has to be carried out at sufficient high temperatures and for a sufficient amount of time. Although inactivation of micro-organisms and enzymes increases with temperature and duration of the heating process, the use of high temperatures for a longer time, will also (partly) destroy compounds responsible for fresh flavour and nutrients in the product. Therefore, alternative milder preservation processes have received considerable interest over the last decades, as they allow processing at lower temperatures and/or shorter holding times compared to conventional thermal processes, aiming for an improved, yet safe, product quality. Alternative methods can be either optimised thermal processes, such as ohmic heating and microwave heating, or non-thermal processes, such as pulsed electric fields (PEF) and high hydrostatic pressure. These non-thermal techniques can both be used as single technology, or in a combination with mild heat, to optimise for microbial inactivation and quality retention. In an industrial setting, PEF processing is usually combined with mild heat to enhance inactivation, while impact on product attributes is limited (Raso, Condón & Álvarez, 2014; Timmermans et al., 2014).

To better understand the electroporation and/or thermal effect on the microbial inactivation in a combined process of PEF and mild heat, a comparison to a conventional thermal process is important. However, comparison of the two processes is difficult, or not feasible, as different set-ups and time scales are used to measure microbial inactivation kinetics. The most common method to measure thermal inactivation of a microbial population is based on isothermal kinetics. This method of isothermal kinetics is based on fast heating of a microbial sample to a desired temperature, followed by sampling at regular (holding) times, to enumerate to the number of survivors. Contrary, in emerging technologies like continuous PEF processes, the microbial inactivation kinetics cannot be characterised by a holding time series, as the temperature holding section is absent. The PEF treated product is immediately cooled after leaving the treatment zone where electric pulses are received, at the same point where the highest temperature is reached. By the design of a continuous flow PEF process, kinetic inactivation series can be made by variations of electric field strength, duration of the pulse and/or number of pulses applied to the product, resulting in different maximum temperatures (Raso, Condón & Álvarez, 2014). As the residence time in the treatment chambers is fixed and no holding time is used, inactivation kinetics of a PEF treatment can be characterised as series with different maximum temperatures and a fixed 'holding' time. Often the intensity of the PEF treatment is expressed in electric energy applied to the system, but this can be converted to maximum temperatures using the specific heat capacity of the matrix (Siemer, Toepfl & Heinz, 2014; Timmermans et al., 2014).

For a proper comparison between the conventional thermal process and a PEF treatment combined with mild heat, temperature-time conditions should be equal for both processes. Since the temperature-time exposure in a PEF process involves a short time (~1 second) to leave the treatment chamber and enter the cooling section, an accurate estimation of the thermal inactivation at this temperature-time combination is

essential. It is therefore important to define a method and model to describe 'isotime' inactivation: a single holding time with varying temperatures. As a preparatory step we need to consider a two variable (temperature and time) thermodynamic model for inactivation to compare data that is obtained either as constant temperature or at fixed holding time.

Thermal inactivation kinetics is often described by first-order kinetic models, with parameters D_T and Z. D_T, the decimal reduction time, is defined as the time needed to reduce the number of viable micro-organisms in suspension with a factor 10, at temperature T. The Z-value is defined as the change in temperature required to change D_T by a factor of 10. The parameter D_T has been widely used in the calculation of the efficacy of pasteurisation and sterilisation processes, conceivably because it is so simple (Peleg & Normand, 2004; Van Boekel, 2008). Although there are micro-organisms that show linear or approximately linear semi-logarithmic survival curves, most microbial survival curves are not linear in practice (Van Boekel, 2002).

In order to account for the usually observed non-linearity, (empirical) models have been proposed to describe curves with a shoulder, curves with a tail (or biphasic curves) or curves including both a shoulder and a tail (sigmoid curves). Examples of these models include the Weibull model (Peleg & Cole, 1998; Van Boekel, 2002), biphasic linear model (Cerf, 1977), biphasic logistic model (Whiting, 1993), log-normal distribution (Arago et al., 2007), reparameterized Gompertz model (Den Besten et al., 2006) and Geeraerd model (Geeraerd, Herremans & Van Impe, 2000). Although these non-linear models fit very well to the survival data, most of these kinetic models are kinetic models with time as a single variable, and not both temperature and time as variables. The temperature dependence of the D_T-value is analysed using secondary models (Arrhenius-like) to obtain a Z-value. Secondary models based on polynomials accounting for additional variables (T, pH, water activity) have been studied (Gil et al., 2016), but it is questionable if and how these models can be used to describe isotime inactivation, which requires extrapolation to very short holding times.

Recently, a thermodynamic model (Gauss-Eyring) for enzyme inactivation was presented, with model parameters reference temperature Tr and Z-value that are directly linked to standard activation enthalpy and entropy values of proteins (Mastwijk, Timmermans & Van Boekel, 2017). This model was also explored as global model for the microbial inactivation of *Listeria monocytogenes* exposed to combined heat and pH stress. Interestingly, this primary model expresses the inactivation kinetics as a function of both temperature and time. The characteristics of this model allow to handle data sets where inactivation is observed as a single holding time with temperature as a variable, i.e. to handle datasets where treatment time cannot be varied, and to make predictions for processes with very short holding times. The objective of this study was to examine the Gauss-Eyring model to describe non-linear isothermal as well as 'isotime' thermal inactivation data of *Escherichia coli, Listeria monocytogenes, Lactobacillus plantarum, Salmonella* Senftenberg and *Saccharomyces cerevisiae*, and to predict the thermal inactivation of these five micro-organisms as a function of temperature at short, fixed holding times.

5.2 Material and methods

5.2.1 Micro-organisms and culture conditions

Fresh cultures of *Escherichia coli* ATCC 35218 were prepared by plating from frozen stock culture on TSB (Oxoid) agar plates. Plates were incubated overnight (o/n) at 37 °C. A single colony isolate was used to inoculate a 100 mL flask with 10 mL TSB broth and cultivated for 24 h at 20 °C in an Innova shaking incubator (180 rpm) (2nd o/n). From this culture, 200 µL was used to inoculate 19.8 mL fresh TSB broth supplemented with 1% glucose (Sigma-Aldrich) (100 mL flask) and incubated for 24 h at 20 °C and 180 rpm (3rd o/n). The cultivation of *Salmonella* Senftenberg ATCC 43845 was similar to the protocol above, for the other micro-organisms specific agar composition and different incubation temperatures for the first o/n cultivation were used: *Listeria monocytogenes* NV8 was incubated at 30 °C using BHI agar (Oxoid), *Lactobacillus plantarum* ATCC 14917 was incubated at 30 °C using MRS broth (Merck) and *Saccharomyces cerevisiae* CBS 1544 was incubated at 25 °C using glucose-peptone-yeast agar, containing 40 g glucose, 5 g peptone (Fluka), and 5 g yeast extract (Oxoid) per 1 L distilled water. 1% glucose was added to the medium during the third o/n cultivation to mimic sugar content in fruit juice, resulting in a reduction of the pH for *E. coli*, *S.* Senftenberg and *L. monocytogenes,* when compared to the pH of the medium without extra glucose (Table 5.1). After the 3rd o/n incubation, microbial cells were pelleted by centrifugation (4000 rpm, 5 min) at 20 °C. Pellet was resuspended in 20 mL sterile peptone physiological salt diluent (PSDF, Tritium) and centrifuged again at same conditions. This step was repeated once, and after the third centrifugation step, pellet was suspended in 20 mL pasteurised (30 min kept at 98 °C) orange juice (Minute Maid, original) to a cell density of 10^7 – 10^8 cfu/mL (yeast) or 10^8 – 10^9 cfu/mL (bacteria).

Table 5.1. Effect of 1% glucose addition on the pH of the medium during the third overnight incubation. t = 0 is the pH measured at the start of the overnight cultivation, when the glucose is added. t = 24 h is at the end of the third overnight cultivation.

Species/strains used	pH at t = 0 h	pH at t = 24 h without 1% glucose	pH at t = 24 h with 1% glucose
Escherichia coli	7.1	8.1	5.1
Lactobacillus plantarum	5.5	4.5	4.5
Listeria monocytogenes	7.3	5.9	5.1
Saccharomyces cerevisiae	5.0	4.5	4.5
Salmonella Senftenberg	7.1	8.2	5.1

5.2.2 Heat treatment

For the thermal treatment, 130 µL of the inoculated orange juice was transferred into a 200 µL glass capillary (1.6 mm diameter, 125 mm length, Blaubrand® Intramark, Wertheim, Germany). Both ends were sealed in a gas flame, leaving a sufficient air column to avoid heating of the microbial suspension during sealing. Capillaries were heat treated in a circulating water bath. Before and immediately after the heat treatment samples were stored in ice-water. Temperature was measured in a capillary with open end, filled with 130 µL suspension, by using a mini-hypodermic thermocouple (HYP-O-T-type, Omega) to determine come-

up time, holding time and cooling time. The total treatment can be characterised by a come-up time of 6 s (which is the time needed from 0 °C to > 95% of the desired temperature), a holding time (chosen for the specific treatment) and a cooling time of 3 s (from desired temperature to temperatures < 25 °C) (Fig. 5.1). Only the 'holding times' at the desired temperature were used for calculations, come-up time and cooling time were not used. The reason why this is sufficient will be explained in the next section, after introduction of the Gauss-Eyring model. Isothermal survival curves were taken with chosen, fixed temperatures [52 – 65 °C] and varying holding times [14 – 3004 s], and isotime survival curves were taken for selected, fixed holding times [4 – 184 s] and varying temperatures [46 – 73 °C].

Capillaries were aseptically opened and serially diluted in sterile PSDF and plated on suitable agar plates (medium supplemented with 15 g agar/L (Oxoid) and 0.1% sodium pyruvate (Sigma Aldrich)) to enhance outgrowth of sub-lethally damaged cells (McDonald, Hackney & Ray, 1983; Sharma et al., 2005). Surviving cells were enumerated after 4-5 days at 25 °C (*S. cerevisiae*), 30 °C (*L. monocytogenes* and *L. plantarum*) and 37 °C (*E. coli* and *S.* Senftenberg).

The total number of temperature-time conditions was too large to test all combinations of each strain on a single day. Therefore, experiments were executed over multiple days starting with cultivation of the same microbial stock culture, kept in 15% (v/v) glycerol and stored at -80 °C. This stock was thawed and frozen before the start of each cultivation, and biological variability for the different cultivations of the same culture could be evaluated as well.

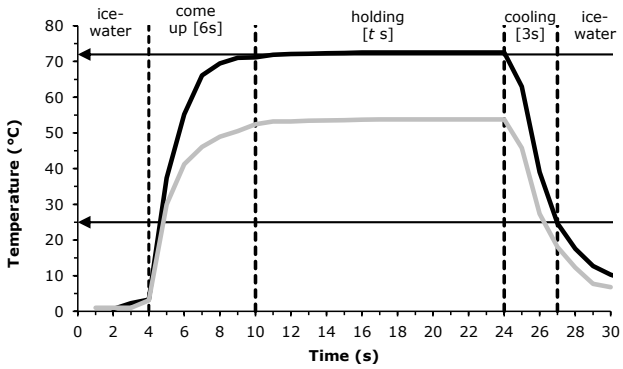

Figure 5.1. Example of heating profile of a microbial suspension in orange juice in a capillary heated for 14 s at 54 °C (grey line) and 72 °C (black line).

5.2.3 Inactivation model

To describe the thermal inactivation of the vegetative micro-organisms as a function of temperature and time, the Gauss-Eyring model was used (Mastwijk, Timmermans & Van Boekel, 2017). This Gauss-Eyring model is based on the Cumulative Distribution Function (CDF) for the standard normal distribution (Gauss) with μ for mean and σ_y for standard deviation. The function (F) of the CDF is given in Eq. 5.1, with $erf(x)$ as the error function, being the integral of the Gaussian probability distribution.

$$F(x) = \Phi\left(\frac{x-\mu}{\sigma_y}\right) = \frac{1}{2}\left[1 + \mathrm{erf}\left(\frac{x-\mu}{\sigma_y\sqrt{2}}\right)\right] \quad \text{(Eq. 5.1)}$$

For inactivation studies, where the degree of inactivation is often plotted as $\log_{10}(N/N_0)$, it is appropriate to use the complementary function of the CDF, Erfc, given in Eq. 5.2.

$$\mathrm{Erfc}(x) = 1 - \mathrm{Erf}(x) \quad \text{(Eq. 5.2)}$$

Although the Weibull CDF could be used as an alternative model, this would miss some of the important features of using the erfc (Aragao et al., 2007).

To describe the degree of inactivation, $\log_{10}(N/N_o)$, as a function of temperature and time, parameters μ and x in Eq. 5.1 were replaced by *Tc(t)* and *T,* respectively, in Eq. 5.3a, where $N(t,T)$ is the number of surviving micro-organisms after exposure to a temperature *T* for a holding time *t* (s), N_0 is the initial number of micro-organisms at *t = 0* s, *T* is the temperature of the treatment (°C), *Tc(t)* is the critical temperature, describing the transition of 'no inactivation' to 'the start of inactivation' (°C) (shoulder) at a specific time *t*, and σ the temperature width (the uncertainty in the transition temperature) of the survival function (°C), a characteristic of the population under study.

$$\log_{10}\left(\frac{N(t,T)}{N_0}\right) = \log_{10}\left(\frac{1}{2}\left[\mathrm{erfc}\left(\frac{T - Tc(t)}{\sigma\sqrt{2}}\right)\right]\right) \quad \text{(Eq. 5.3a)}$$

The critical temperature, *Tc(t)*, is a function of the holding time *t* (s) and *Z* (°C), which is the temperature needed to reduce or increase the holding time by a factor of 10 to reach the same level of inactivation, τ_r= 1 s a chosen unit of time and *Tr* is the reference temperature in °C defined for a treatment of 1 s, given in Eq. 5.3b. Note that the model parameter reference time τ_r is fixed by the particular choice of the unit of time (s or min) used for inactivation studies and the corresponding reference temperature *Tr* is defined by the outcome of the inactivation experiment (Mastwijk, Timmermans & Van Boekel, 2017).

$$Tc(t) = Tr - Z \cdot log_{10}(t/\tau_r) \quad \text{(Eq. 5.3b)}$$

The variables in the model are temperature and time (*T, t*) and the model parameters are the reference temperature*, Z*-value and temperature width (*Tr, Z,* σ). The relation between these three model parameters is visualised in Fig. 5.2(A,B). In Fig. 5.2A, the Gauss-Eyring model is presented for three different values of σ, with same critical temperature *Tc(t)* at the same *Tr, Z* and chosen, fixed time *t*. *Tc(t)* represents a characteristic temperature at which 50% of the population is inactivated, corresponding to $\log_{10}(N/N_0)$ = -0.30, regardless of the value of parameter σ. A smaller value of σ induces a faster inactivation (steeper inactivation curve), meaning more inactivation $\log_{10}(N/N_0)$ at temperature $T > Tc(t)$. The relation between parameter *Z* and *Tc(t),* given in Eq. 5.3b, is illustrated in Fig. 5.2B. Increasing or decreasing the holding time *t* will change the value of *Tc(t),* resulting in a temperature shift (shoulder) for the start of the inactivation curve. The *Z*-value indicates the temperature shift of this *Tc(t),* when the holding time is changed by a factor 10. This definition is identical to the definition of the *Z*-value in first-order kinetics, where *Z* is the temperature change required for a ten-fold change in the D_T-value, visualised in Fig. 5.2D. This D_T-value is

the time required to inactivate the number of micro-organism by a factor 10 at a given temperature T (D_T-value, visualised in Fig. 5.2C).

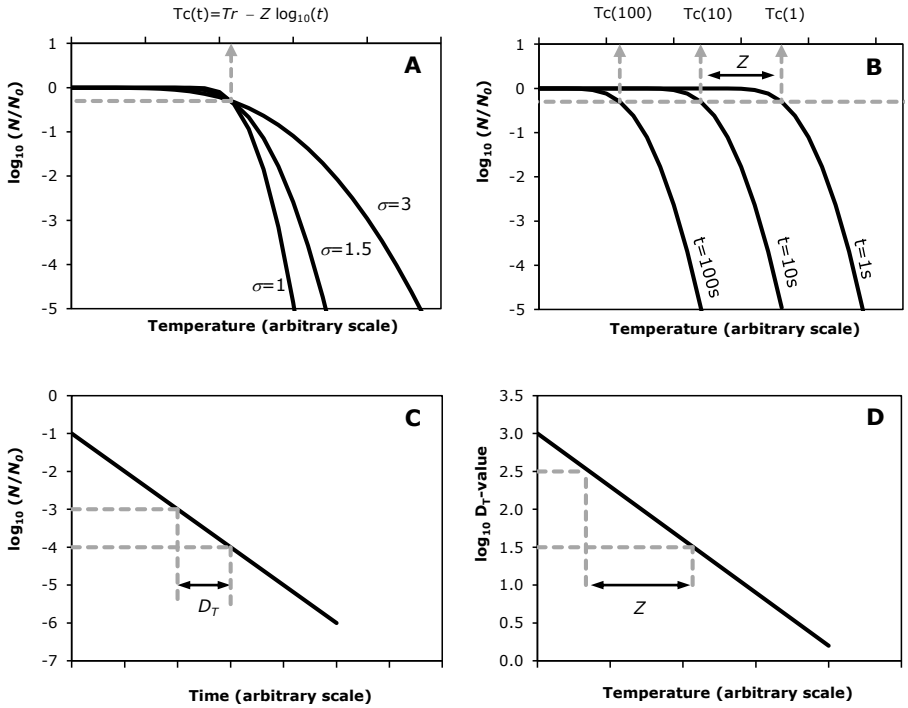

Figure 5.2. Schematic plot of survival curves according to Gauss-Eyring model (A, B) and to first-order kinetic model (C, D). (A) Gauss-Eyring model for three different values of σ, with same critical temperature *Tc* at a fixed time *t*. The dashed line indicates the critical temperature *Tc(t)*. (B) Gauss-Eyring model illustrating the relation between *Z*-value, holding time *t*, and *Tc(t),* using one σ. (C) First-order kinetics showing the D_T-value concept. (D) First order kinetics illustrating the *Z*-value concept.

The Gauss-Eyring model presented is a static model i.e. the temperature-time combination is defined for a stationary treatment temperature. In an experimental setting using a capillary technique a sample cannot be instantaneously heated to reach a stationary temperature. This suggests that in principle, a dynamical model should be used to account for the coming up and cooling times. However, in the case of the Gauss-Eyring model the static approach for the reported experimental temperature-time combinations is an accurate approximation. The reason is that the mathematical line shape for the inactivation rate of the Gauss-Eyring model, dS/dt, is a sharp peaked function around $Tr - T(t) - Z \cdot log_{10}(t) = 0$. This means that the integral with respect to time to obtain S(T(t),t)) only contributes significantly for temperature-time combinations $T(t) = Tr - Z \cdot log_{10}(t)$ within the temperature interval of [T-σ, T+σ]. This is a property of sharp peaked distributions (Dirac delta function) (Reiff, 1965). The definition of the stationary temperature according to Fig. 5.1 is where the stationary temperature is defined by the 95% level of the come-up time. The static model therefore provides an excellent approximation for the dynamical situation, even for come-up times that are relatively long with respect to the (short) holding time.

5.2.4 Mathematical modelling and statistical analysis

The Gauss-Eyring model was fitted to the data by means of non-linear least squares, using the software package Athena Visual Studio version 14.2 (www.athenavisual.com). Outcome and statistics of non-linear least squares fit routines were validated against Maximum Likelihood Estimates (MLE) fitting estimated with software package R (R Core Team, 2016) with domain optimization for parameter estimates using generalized simulated annealing (Xiang et al., 2013). Although the definition of the error function *erf(x)* was different among the software programs, it was found that fit results of both methods were identical, both for quantitative and qualitative purposes.

The model given in Eq. 5.3 was fitted to the inactivation data collected for each individual overnight culture, taken from the same frozen stock culture. For data series where only the temperature was varied at a single fixed time, no *Z*-value can be determined. In this case the model was reduced to a model with parameters *Tr* and σ (Eq. 5.3a). The reference temperature *Tr* is given for the fixed time used. When the exposure times were varied for an individual cultivation, Eq. 5.3a and 5.3b were fitted to complete (*T, t*) data sets, and an additional *Z*-value was obtained. In this case, the reference temperature *Tr* is defined for t = 1 s. In this way 2 or 3 model parameters were obtained for each individual cultivation.

Alternatively, Eq. 5.3 was fitted to the inactivation data of all overnight cultures, i.e. one fit to the data sets of all cultivations at once. With this method, only three parameters *Tr*, *Z* and σ were determined as an average of all cultivations.

The goodness-of-fit was determined by evaluation of the distribution of the residuals and Sum of Squared Residuals (SSr). Comparison of the approach to fit the model to each individual cultivation or the single fit to all the cultivations at once, was done by comparison of the Bayesian Information Criterion (BIC) values. In Eq. 5.4 the definition for BIC is given, wherein p is the number of fitted parameters and n is the number of data points.

$$BIC = n \cdot \ln\left(\frac{SS_r}{n}\right) + p \cdot \ln(n) \qquad \text{(Eq. 5.4)}$$

The first term in Eq. 5.4 indicates that a better fit of the model to the data is obtained for smaller BIC values, whereas the second term is a penalty for the introduction of too many parameters. One BIC value could be determined for the individual fits for each cultivation. Therefore, the sum of all SSr values for the individual cultivations was taken, p was the total number of model parameters, being 2 (σ, *Tr*) or 3 (σ, *Tr*, *Z*) for each cultivation and n the total number of data points. For the other approach, a fit to the data of all cultivations at once, a single value for SSr was determined. In this case the number of model parameters is p = 3, and n has the same value as the for sum of the individual fits.

Comparison of the BIC values determined how a representative parameter estimation for *Tr*, *Z* and σ for each micro-organism could be obtained. *t*-statistics was carried out to calculate the mean and standard deviation of the fit results of the individual cultivations. Weighted means and weighted standard deviations were calculated to compensate for the number of data points of each individual cultivation.

The 95% Confidence Interval (95% CI) of the *Tc*(*t*) function with representative parameters was determined by Monte Carlo simulations (n = 5000 simulations, Excel), where the 5% and 95% quantiles of the observed data define the parameter interval around the Maximum Likelihood. This method accounts for possible unsymmetrical confidence intervals occurring from non-linear regression, in contrast to linear approximation methods used in most software packages.

5.3 Results and discussion

5.3.1 Validation of the model on *E. coli* data-set

Thermal inactivation kinetics with variables *T* and *t* was carried out for individual overnight cultures of *E. coli* prepared at 8 different days, but cultivated from the same frozen stock culture. The data set of the different cultures was based on either isothermal or isotime inactivation kinetics. A representation of the measured inactivation data with model fit of the individual data is shown in Fig. 5.3 (upper panel), and fit results of all cultivations are shown in Table 5.2. It can be observed that the Gauss-Eyring model fits well to both isothermal and isotime inactivation data, as residuals are randomly distributed, and a low value of SSr was found. This resulted in a high value for R^2 (determination coefficient, calculated as the $1 - SSr/SS_{total}$) and small 95% confidence interval for the parameter estimates. Comparison of the parameter estimates for the different cultures showed some (small) deviations, suggesting that variability is introduced by cultivation of the different individual precultures, as the individual fits were all good.

Next, the Gauss-Eyring model was fitted to the combined inactivation data of the 8 individual cultures at once. Parameter estimates are shown in Table 5.2 indicated as 'total', together with the 95% confidence interval for the fit parameters. The small confidence intervals are the result of the relative large number of data points used (n = 304). However, it is clear that the sum of the residuals is large in this fit, describing the variance of the individual measured data points to the predicted model. This variation might be ascribed to the variability found between different individually grown cultures. This could have resulted from heterogeneity in the stock culture, introducing different single colonies to start the different cell cultivations, but it could also result from spontaneous mutation during cultivation (Nahku et al., 2011).

Comparison of the two models, describing the inactivation for individual cultures or describing the inactivation as single fit to the sum of the cultures at once, was done using the BIC value. The model describing the inactivation per culture (p = 24, n = 304, BIC = -462.2) is preferred over the model describing the sum of the cultures (p = 3, n = 304, BIC = -258.7), as the BIC value is significantly lower.

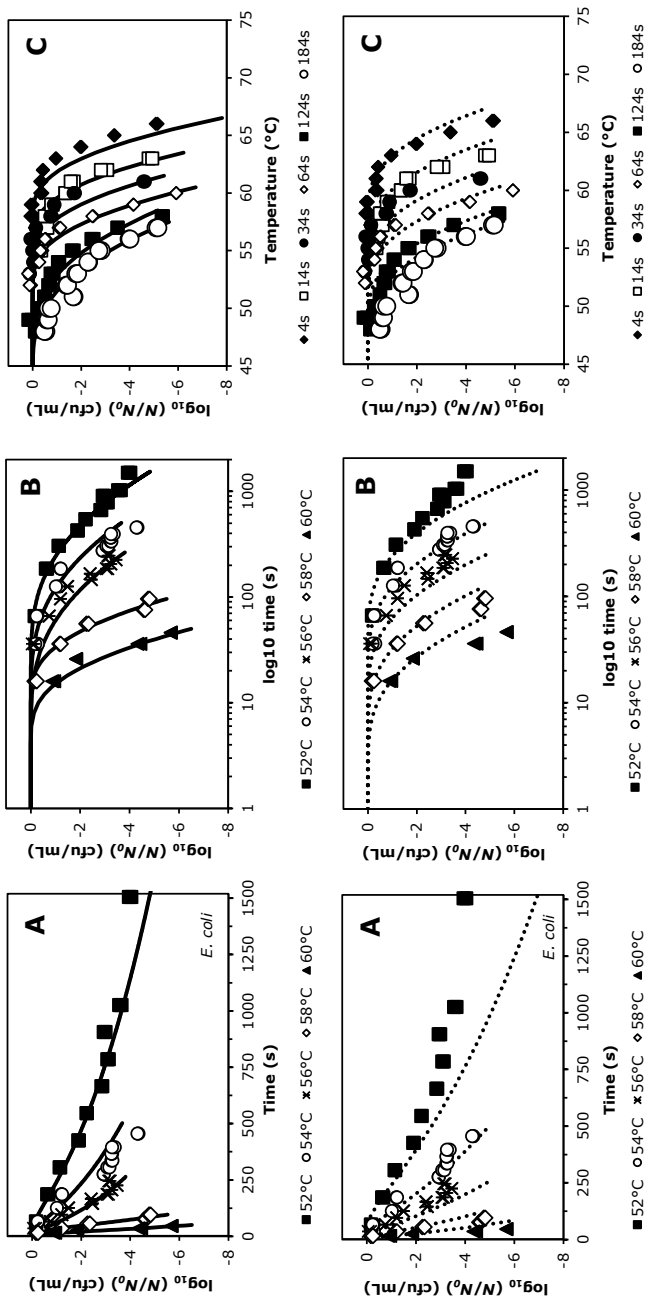

Figure 5.3. Thermal inactivation of *E.coli* in orange juice at isothermal (A, B) and isotime (C) conditions. Data points represent the experimental values obtained from individual overnight cultures from the same frozen stock. Upper panel: continuous lines present the fit of the Gauss-Eyring model fitted to each individual culture. Lower panel: dotted lines present the fit of the Gauss-Eyring model as single model with parameter estimations based on the average of cultivation.

As the objective of this paper was to predict inactivation for an arbitrary chosen short (holding) time, one parameter estimate for *Tr*, *Z* and σ is needed to calculate the level of inactivation at chosen time *t.* Since the model describing the inactivation per culture was preferred over the model describing the sum of the cultures, the parameter estimates *Tr*, *Z* and σ should be based on the results for the individual cultures. Because the estimation of the individual parameters *Tr*, *Z* and σ for the different cultures is rather precise (small 95% confidence interval), the average value for *Tr*, *Z* and σ can be obtained by calculating the average of the estimates of the 8 individual cultures. As the parameter estimates of the cultures are based on a different number of data points (*n*), a weight for each culture was calculated, with n/n_{total}.

A weighted average and weighted variance were calculated for *Tr*, *Z* and σ, and results of the mean and 95% confidence interval are shown in Table 5.2, with average for *Tr* = 66.5 °C, *Z* = 6.9 °C and σ = 1.41 °C. A model fit of these weighted parameter estimates to the measured inactivation is shown in Fig. 5.3 (lower panel). In general, the single model with weighted parameter estimates (lower panel) fitted well to the observed data, including the data with shortest holding time. The deviations between the model and observed data can be attributed to the variability between the individual cultures, which is also incorporated in the 95% confidence interval of the parameter estimates. Predictions based on these parameter estimates should therefore include this variability as well, for instance by indicating the 95% CI.

A prediction by the function of *Tc(t)* based on the calculated average values of *Tr* and *Z* is shown in Fig. 5.4A. It can be seen that a short (holding) time corresponds to a high value of *Tc(t)*, and that a longer (holding) time corresponds to a lower *Tc(t)*-value, which reduces slowly to a temperature around 45 °C. Interestingly, this relative low temperature - long time relation is typically used as holding time for sous-vide cooking, excluding the extra time needed to heat up the product to this temperature, product specific characteristics and safety margins (Baldwin, 2012). The 95% confidence interval of the *Tc*(*t*) function was determined using Monte Carlo simulations, and bands are shown in Fig. 5.4A. It can be seen that the 95% confidence interval is relatively large and increasing over time. The width of this interval is determined by the variation in the parameters *Tr* and *Z,* which is relatively large in our example due to variability between the individual cultures. The increase of this interval over time could be explained by the function itself, where *Z* is multiplied by $\log_{10}(t)$. The uncertainty in the parameter *Z* will therefore be propagated with a factor $\log_{10}(t)$, leading to wider confidence bands at longer times.

In Fig. 5.4B, the predicted inactivation as a function of temperature is shown for different (holding) times. The inactivation is determined with the calculated parameter estimates and the Gauss-Eyring model. It can be seen that the curvature of the inactivation is the same, and that the inactivation curve only shifts with the temperature-axis by changing the holding time, also for short holding times.

Table 5.2. Parameter estimations of thermal inactivation of *E. coli* using the Gauss-Eyring model. Parameter estimations are shown for individual fits to each of the 8 individual cultures from the same frozen stock culture, indicated per Ec-number, and for the fit to all the inactivation data, indicated as Ec-1-8 total. Finally the weighted average of the individual fits is shown, indicated as Ec-average. Values between brackets show the 95% confidence interval estimated for the parameters.

Cell cultivation	***n***	***t* (s)**	***T*** **(°C)**	σ (°C)	***Tr*** **(t=1) (°C)**	***Z*** (°C)	**R^2**	**SSr**	**BIC**
Ec 1-8, per cultivation	304						0.92	42.3	-462.2
Ec-1, isotime	40	4,14	[56:66][55:63]	1.10 [1.01-1.20]	64.5 [64.1-65.0]	5.3 [4.9-5.7]	0.98	2.5	
Ec-2, isotime	64	34,64	[53:61][52:60]	1.16 [1.04-1.32]	66.4 [65.7-67.1]	6.5 [6.1-6.8]	0.93	17.4	
Ec-3, isotime	66	14,34,124,184	[54:61][57:60] [48:58][48:57]	1.83 [1.67-2.02]	68.2 [67.0-69.4]	8.4 [7.7-9.1]	0.94	8.2	
Ec-4, isothermal	14	[34:244][34:64]	55,58	1.11 [0.94-1.35]	67.4 [66.4-68.4]	7.0 [6.4-7.7]	0.99	0.4	
Ec-5, isothermal	18	[34:364][34:124]	54,56	1.20 [0.96-1.60]	63.7 [62.1-65.3]	5.9 [4.8-6.9]	0.96	1.9	
Ec-6, isothermal	24	[34:294][34:144]	52,56	1.14 [0.98-1.36]	70.8 [69.0-72.5]	8.4 [7.6-9.1]	0.96	0.8	
Ec-7, isothermal	18	[14:94][14:44]	58,60	0.81 [0.68-1.01]	65.7 [64.7-66.7]	5.7 [5.1-6.4]	0.96	2.6	
Ec-8, isothermal	60	[64:1504][64:454][34:244]	52,54,56	1.83 [1.58-2.18]	65.4 [65.3-65.5]	6.6 [6.0-7.2]	0.91	8.5	
Ec 1-8, total	304			1.32 [1.23-1.42]	64.5 [64.1-64.9]	5.8 [5.7-6.0]	0.86	122.7	-258.7
Ec-average of cultivations	8			1.41 [0.65-2.17]	66.5 [62.9-70.1]	6.9 [4.7-9.0]	-		

n: number of *t*-*T* data points within a cell culture

σ, Tr and *Z:* parameter estimations according to Gauss-Eyring model (Eq. 5.3)

R^2: determination coefficient

SSr: residual sum of squares

BIC: Bayesian Information Criterion

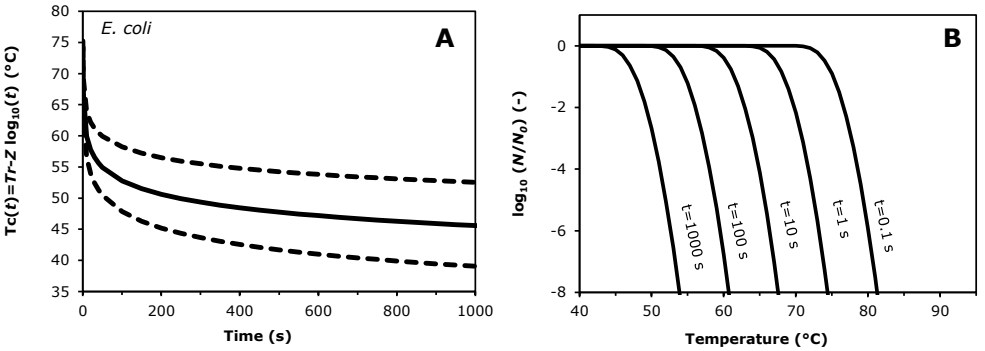

Figure 5.4. A) Relation of parameters *Tc* and *t* of the Gauss-Eyring model, with parameter estimates, *Tr* = 66.5 °C and *Z* = 6.9 °C, calculated as weighted average value of the various cell cultures of *E. coli* in orange juice. Continuous line representing the mean and dashed line the approximate 95% confidence intervals as calculated via Monte Carlo simulation. B) Isotime survival curve of *E. coli* in orange juice calculated for different holding times using the Gauss-Eyring model and weighted parameter estimates of 8 cultures, σ = 1.41 °C, *Tr* = 66.5 °C and *Z* = 6.9 °C.

5.3.2 Validation of the model on other species

The approach described in the previous section for *E. coli* in orange juice, was tested for *L. monocytogenes, L. plantarum, S.* Senftenberg, and *S. cerevisiae* strains in orange juice as well. Individual overnight cultures were made at different days, and isothermal and isotime survival kinetics were determined. For some of the cultivations, in particular for *L. monocytogenes,* only one isotime condition for a cultivation was measured. This resulted in an estimated parameter *Tc* for the specific *t,* without estimations for *Tr* at *t* = 1 s and *Z*. Results for the individual fits to the measured data for each individual culture and strain are shown in Fig. 5.5. Parameter estimates and fit results for the individual cultures are shown in the appendix Tables A1-A4, and a summary of the results is shown in Table 5.3. Generally, a proper fit of the model to the data of each individual culture was found, with a random distribution of the residuals and a low value for the SSr. This resulted in a small confidence interval for the estimated parameters.

Heterogeneity within some individual cultures was observed, i.e. *L. plantarum* at 52 °C, and could be described by the presence of two subpopulations, as discussed and illustrated in Mastwijk, Timmermans & Van Boekel (2017).

As the fit to one population (performed in this study using Eq. 5.3) was not bad and residuals were distributed randomly, further quantification and characterisation of the inactivation data to two subpopulations, as demonstrated in Mastwijk, Timmermans & Van Boekel (2017), was not included in this study.

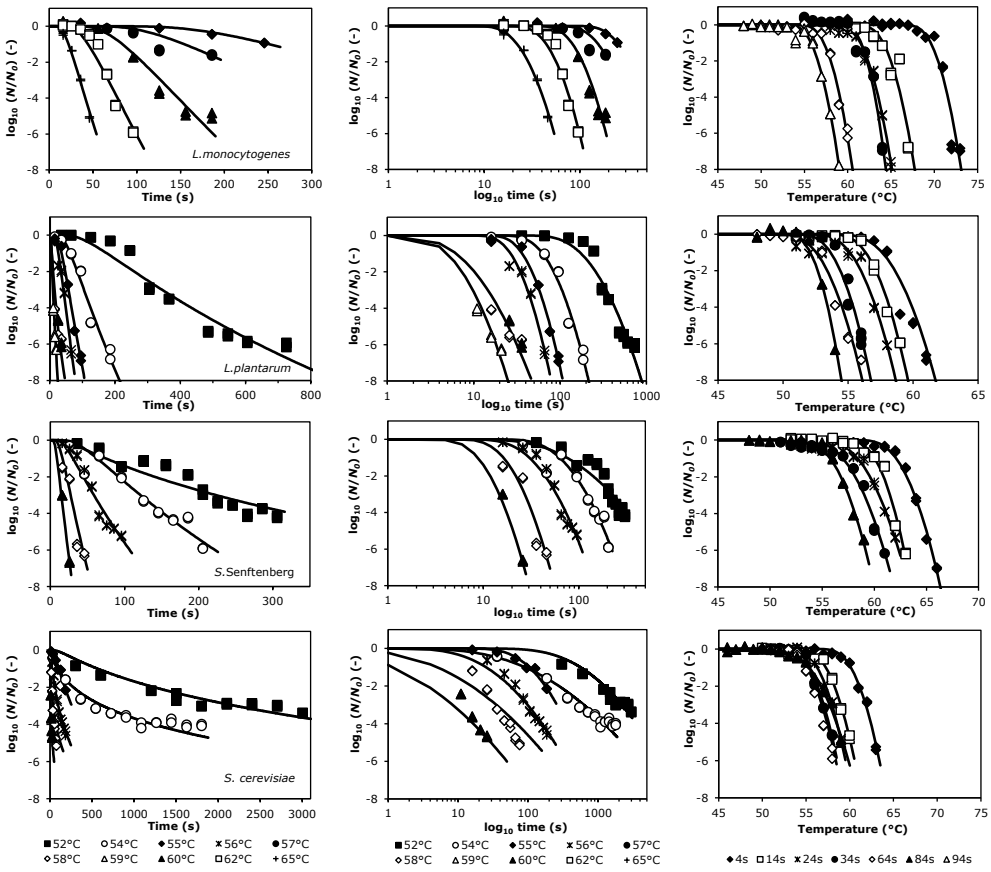

Figure 5.5. Thermal inactivation of *L. monocytogenes, L. plantarum, S.* Senftenberg and *S. cerevisiae* in orange juice at isothermal (left and middle panels) and isotime (right panels) conditions. Data points represent the experimental values obtained from individual overnight cultures from the same frozen stock, continuous lines present the fit of the Gauss-Eyring model fitted to each individual culture.

Variability between the individual cultures was observed as well, leading to differences in the parameter estimates. This variability was also observed when the Gauss-Eyring model was fitted to all inactivation data of a strain. The total SSr increased dramatically, and consequently also the BIC value (Table 5.3). Therefore, the weighted average mean and standard deviation were calculated from the parameter estimates of the individual cultures. Note that this average is based on only 4 values for *L. monocytogenes,* as datasets for this strain contained a relatively high number of single (holding) time tested in the individual culture, lacking parameter estimates of *Tr* and *Z.* A fit of the Gauss-Eyring model as single model with parameter estimations based on the weighted average of cultivations is shown to the observed data (Fig. 5.6). Although this single model did show some deviation from the observed data, these deviations were relatively small at the shortest holding times measured, and therefore this single model could be used for predictions at even shorter holding times.

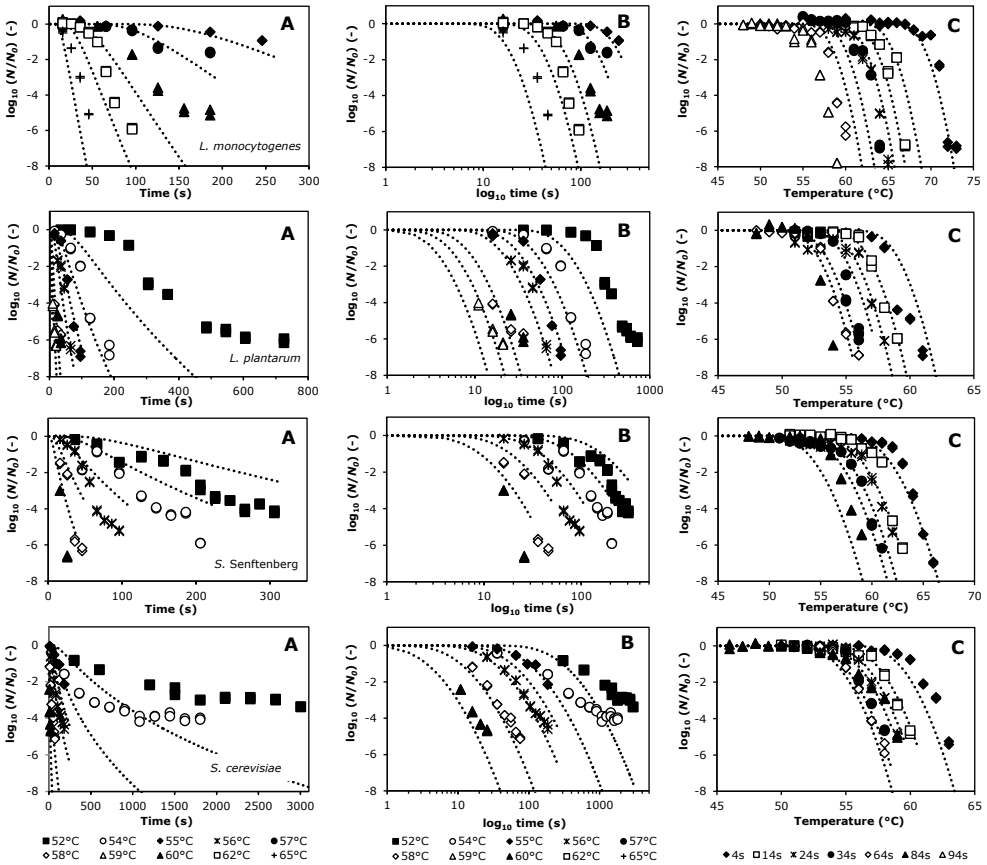

Figure 5.6 Thermal inactivation of *L. monocytogenes, L. plantarum, S.* Senftenberg and *S. cerevisiae* in orange juice at isothermal (A and B) and isotime (C) conditions. Data points represent the experimental values obtained from individual overnight cultures from the same frozen stock, dotted lines present the fit of the Gauss-Eyring model as single model with parameter estimations based on the average of cultivations.

A plot of the *Tc(t)* function based on the calculated weighted parameter estimations and prediction of the isotime inactivation curves for various holding times is shown in Fig. 5.7. The differences between the parameter estimates for the different species show a clear effect on the *Tc(t)* function (Fig 5.7A) and inactivation curves (Fig. 5.7B). Comparison of the parameter estimates for the different species, including those for *E. coli*, showed that the estimated value of parameter σ is comparable for all species, varying between 0.79 and 1.41. This means that at the same critical temperature-time combination *Tc(t)*, all strains have the same degree of inactivation, under the same experimental circumstances tested. Nevertheless, the parameter estimations of *Tr* and *Z* are not the same for all species, and resistance tested in this study follows the order *L. monocytogenes* > *E. coli* > *S.* Senftenberg > *L. plantarum* > *S. cerevisiae.* This is also shown in Fig 5.7B, where inactivation curves of *L. monocytogenes* start at higher temperatures compared to those of other micro-organisms, due to higher *Tr*-values. Furthermore, larger temperature distances between the different inactivation curves with variable holding times are shown for *L. monocytogenes*, showing the effect of a larger *Z*-value.

The mentioned *Z*-value in the Gauss-Eyring model is comparable to the *Z*-value reported for linear inactivation kinetics in literature, as they both represent the temperature required to change the D_T or *Tr* value by a factor of 10. The value for *Z* is dependent on the species, strains, cultivation protocol and medium characteristics (Mazzotta, 2001; Smelt & Brul, 2014; Van Asselt & Zwietering, 2006). Direct comparison of the *Z*-values obtained in this study to literature is therefore not possible. However, the *Z*-values in our study are comparable to results of Mazzotta (2001), who reported average *Z*-values of 6.1 ± 0.3 °C in fruit juices for mixtures of *L. monocytogenes*, 5.8 ± 0.3 °C for *Salmonella* mixtures and 5.3 ± 0.4 °C *E. coli* O157:H7 mixtures. Strains known for higher thermal resistance as for example *S.* Senftenberg showed higher resistance than other *Salmonella* strains (Ng, Bayne, Garibaldi, 1969). Doyle & Mazzotta (2000) reported *Z*-values of *S.* Senftenberg in buffer between 6.8 - 7.7 °C, which is similar to the *Z*-value in orange juice calculated in this study.

Table 5.3. Parameter estimations of thermal inactivation of *L. monocytogenes, L. plantarum. S.* Senftenberg and *S. cerevisiae* using the Gauss-Eyring model. Parameter estimations are shown for calculated weighted average of the individual fits per cell cultures (average), and for the fit to the sum of all cultures (sum). Values between brackets show the 95% confidence interval estimated for the parameters. Results of individual fits per culture are shown in the Appendix.

strain	*n*	σ (°C)	*Tr* (*t* = 1) (°C)	*Z* (°C)	R^2	SSr	BIC
L. monocytogenes							
Fit to 10 cultures	273				0.96	47.2	-344.2
average of cultures	4	0.89 [0.58-1.21]	74.7 [72.1-77.2]	9.0 [6.2-11.7]			
Fit to sum of cultures	273	1.38 [1.23-1.57]	70.8 [69.9-71.6]	7.6 [7.3-8.0]	0.65	404.2	123.4
L. plantarum							
Fit to 12 cultures	266				0.95	71.3	-160.4
average of cultures	10	0.79 [0.30-1.28]	61.8 [58.2-65.3]	5.3 [2.9-7.8]			
Fit to sum of cultures	266	1.29 [1.16-1.45]	58.8 [58.1-59.6]	4.5 [4.2-4.8]	0.65	527.4	198.2
S. Senftenberg							
Fit to 11 cultures	309				0.95	61.7	-320.0
average of cultures	9	1.23 [0.55-1.91]	64.5 [60.4-68.7]	6.4 [3.9-8.9]			
Fit to sum of cultures	309	1.49 [1.37-1.64]	64.2 [63.5-64.9]	6.9 [6.6-7.2]	0.74	312.4	20.6
S. cerevisiae							
Fit to 11 cultures	318				0.96	20.2	-697.3
average of cultures	9	1.00 [0.55-1.45]	61.0 [57.5-64.6]	4.2 [2.1-6.3]			
Fit to sum of cultures	318	1.36 [1.27-1.47]	59.1 [58.8-59.5]	3.3 [3.2-3.4]	0.85	130.8	-265.2

n: number of *t-T* data points within a cell culture
σ, *Tr* and *Z:* parameter estimations according to Gauss-Eyring model (Eq. 5.3)
R^2: determination coefficient
SSr: residual sum of squares
BIC: Bayesian Information Criterion

5.4 Conclusion

In this study the Gauss-Eyring model was exploited to describe non-linear thermal inactivation for vegetative micro-organisms, and as a model suitable to predict inactivation at short (holding) times. Due to the short come up time and cooling time in the set-up of the experimental part, only holding time was incorporated as time in the Gauss-Eyring model. Parameter estimates σ, *Tr* and *Z*-value were determined from experimental isothermal

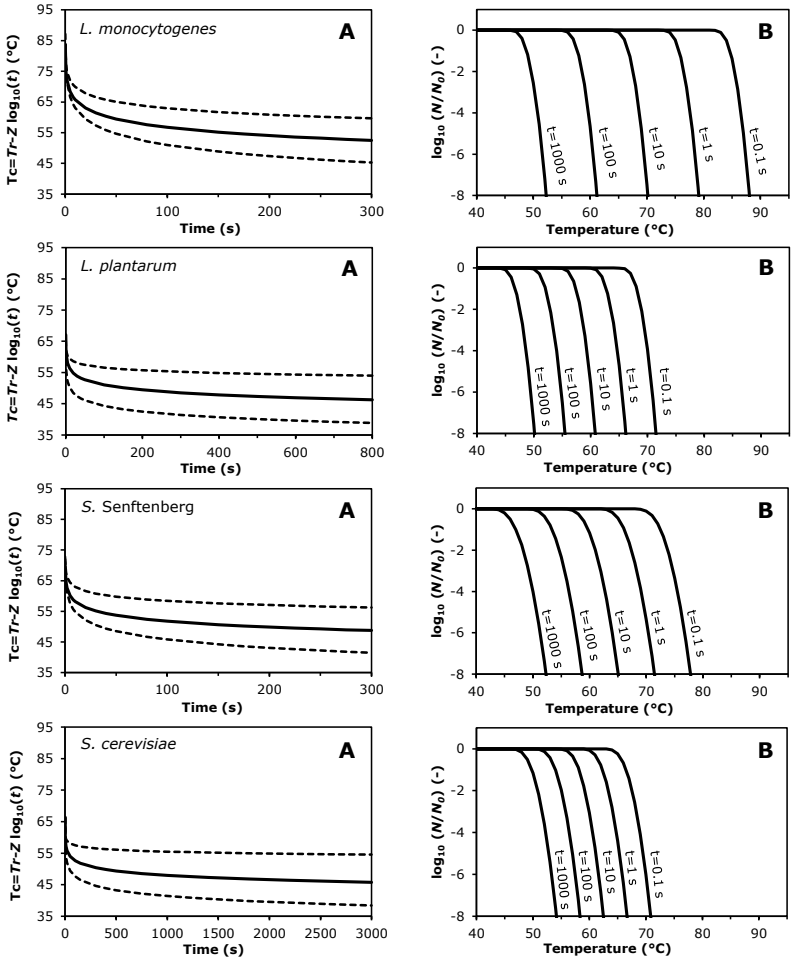

Figure 5.7. A) Relation of parameters *Tc* and *t* of the Gauss-Eyring model, with parameter estimates calculated as weighted average value of the various cell cultures of *L. monocytogenes, L. plantarum, S.* Senftenberg and *S. cerevisiae* in orange juice. Continuous line represents the mean and dashed line the approximate 95% confidence intervals as calculated via Monte Carlo simulation. B) Isotime survival curve of *L. monocytogenes, L. plantarum, S.* Senftenberg and *S. cerevisiae* in orange juice calculated for different holding times using the Gauss-Eyring model and weighted parameter estimates of the cultures.

and isotime data and fitted well to the individual cultures. Variability between the different cultures of the five tested micro-organisms was observed. Therefore, an average of the individual cultures was calculated, and used to predict inactivation as a function of temperature for chosen (short) holding times. The Gauss-Eyring model showed to be a suitable model to predict inactivation at a single holding time with varying temperatures. This predicted inactivation curve will be used in chapter 6 to compare thermal inactivation to an alternative non-thermal inactivation process with short holding time.

Table A1. Parameter estimation of thermal inactivation of *Listeria monocytogenes* using the Gauss-Eyring model. Parameter estimations are shown for individual fit to each of the 10 individual cultures from the same frozen stock culture, indicated per Lm-number, and for the fit to all the inactivation data, indicated as Lm-1-10 total. Finally the weighted average of the individual fits is shown, indicated as Lm-average. Values between brackets show the 95% confidence interval estimated for the parameters. Values of *Tr(t)* show estimation of *Tr* at holding time *t*, estimated for series with one fixed time.

Cell cultivation	*n*	*t* (s)	*T* (°C)	σ (°C)	*Tr* (t) (°C)	*Tr* (*t* = 1) (°C)	*Z* (°C)	R^2	SSr	BIC
Lm 1-10, per cultivation	273							0.96	47.2	-344.2
Lm-1, isotime	28	4	[60:73]	0.81 [0.68-1.00]	68.5 [67.8-69.3]			0.93	10.1	
Lm-2, isotime	14	14	[60:69]	1.09 [1.05-1.13]	63.7 [63.5-63.9]			1.00	0.1	
Lm-3, isotime	20	34	[55:64]	0.69 [0.59-0.84]	60.5 [60.0-61.0]			0.96	3.7	
Lm-4, isotime	32	14,24	[60:67][58:65]	0.84 [0.73-0.99]		75.9 [74.1-77.6]	11.2 [9.8-12.6]	0.94	10.1	
Lm-5, isotime	20	24	[55:64]	0.66 [0.55-0.81]	60.7 [60.2-0.53]			0.96	3.8	
Lm-6, isotime	21	34	[50:60]	0.77 [0.67-0.89]	56.3 [55.8-56.7]			0.98	2.1	
Lm-7, isotime	23	64	[48:59]	0.76 [0.70-0.83]	54.8 [54.5-55.1]			0.99	1.8	
Lm-8, isothermal	54	[14:244][14:184] [14:94][14:64][14:44]	55,57,60,62,65	1.06 [0.97-1.17]		75.6 [74.6-76.5]	9.1 [8.7-9.6]	0.95	3.8	
Lm-9, isothermal	29	[14:184][14:94]	60,62	0.73 [0.61-0.92]		72.6 [71.3-74.0]	7.1 [6.3-7.9]	0.95	6.1	
Lm-10, isothermal	32	[34:544][34:304]	55,57	0.77 [0.65-0.95]		73.8 [71.7-75.8]	8.1 [7.2-8.9]	0.95	4.9	
Lm 1-10, total	273			1.38 [1.23-1.57]		70.8 [69.9-71.6]	7.6 [7.3-8.0]	0.65	404.2	123.4
Lm-average of cultivation	4			0.89 [0.58-1.21]		74.7 [72.1-77.2]	9.0 [6.2-11.7]			

n: number of *t-T* data points within a cell culture

σ, Tr and *Z:* parameter estimations according to Gauss-Eyring model (Eq. 5.3)

R^2: determination coefficient

SSr: residual sum of squares

BIC: Bayesian Information Criterion

Table A2. Parameter estimations of thermal inactivation of *Lactobacillus plantarum* using the Gauss-Eyring model. Parameter estimations are shown for individual fit to each of the 12 individual cultures from the same frozen stock culture, indicated per Lp-number, and for the fit to all the inactivation data, indicated as Lp-1-12 total. Finally the weighted average of the individual fits is shown, indicated as Lp-average. Values between brackets show the 95% confidence interval estimated for the parameters. Values of *Tr(t)* show estimation of *Tr* at holding time *t*, estimated for series with one fixed time.

Cell cultivation	*n*	*t* (s)	*T* (°C)	σ (°C)	*Tr* (*t*) (°C)	*Tr* (*t* = 1) (°C)	*Z* (°C)	R^2	SSr	BIC
Lp 1-12, per cultivation	266							0.95	71.3	-160.4
Lp-1, isotime	27	64,84	[49:55][48:54]	0.55 [0.50-0.61]		64.2 [62.0-66.4]	6.7 [5.5-7.8]	0.98	2.0	
Lp-2, isotime	20	24,184	[51:58][52:53]	0.82 [0.66-1.07]		62.5 [61.6-63.4]	6.1 [5.6-6.5]	0.95	6.9	
Lp-3, isotime	12	4	[55:61]	0.95 [0.78-1.24]	56.4 [55.4-57.3]			0.94	5.4	
Lp-4, isotime	17	14	[51:59]	0.75 [0.68-0.84]	55.4 [55.0-55.7]			0.99	0.9	
Lp-5, isotime	26	14,34	[51:56][55:59]	0.72 [0.63-0.83]		62.5 [61.6-63.5]	6.4 [5.8-7.0]	0.96	6.0	
Lp-6, isotime	24	34,64	[54:56][48:56]	0.82 [0.70-0.98]		58.5 [56.4-60.6]	3.9 [2.6-5.2]	0.95	7.3	
Lp-7, isothermal	18	[14:124][14:34][24:34]	55,58,60	1.45 [1.06-2.29]		62.8 [59.1-66.5]	6.7 [4.8-8.5]	0.79	18.0	
Lp-8, isothermal	24	[14:184][14:184]	52,54	0.48 [0.40-0.58]		60.2 [58.7-61.6]	3.8 [3.0-4.6]	0.98	2.5	
Lp-9, isothermal	12	[14:64][14:34]	56,58	1.23 [0.84-2.27]		60.5 [57.8-63.2]	5.7 [3.4-8.0]	0.86	6.2	
Lp-10, isothermal	18	[14:94][24:64]	55,56	0.80 [0.64-1.09]		65.0 [62.9-67.1]	7.2 [5.6-8.7]	0.98	2.4	
Lp-11, isothermal	28	[34:724][9:19]	52,59	0.73 [0.64-0.86]		60.8 [60.1-61.4]	4.4 [4.2-4.5]	0.94	9.4	
Lp-12, isothermal	40	[24:184][9:19][9:14] [24:74][14:19]	54,56,58,59,60	0.74 [0.68-0.80]		61.3 [60.9-61.7]	4.4 [4.3-4.4]	0.96	4.4	
Lp 1-12, total	266			1.29 [1.16-1.45]		58.8 [58.1-59.6]	4.5 [4.2-4.8]	0.65	527.4	198.2
Lp-average of cultivation	10			0.79 [0.30-1.28]		61.8 [58.2-65.3]	5.3 [2.9-7.8]			

n: number of *t*-*T* data points within a cell culture
σ, Tr and *Z:* parameter estimations according to Gauss-Eyring model (Eq. 5.3)
R^2: determination coefficient
SSr: residual sum of squares
BIC: Bayesian Information Criterion

Table A3. Parameter estimations of thermal inactivation of *Salmonella* Senftenberg using the Gauss-Eyring model. Parameter estimations are shown for individual fit to each of the 11 individual cultures from the same frozen stock culture, indicated per SS-number, and for the fit to all the inactivation data, indicated as SS-1-11 total. Finally the weighted average of the individual fits is shown, indicated as SS-average. Values between brackets show the 95% confidence interval estimated for the parameters. Values of *Tr(t)* show estimation of *Tr* at holding time *t*, estimated for series with one fixed time.

Cell cultivation	***n***	***t* (s)**	***T*** (°C)	σ **(°C)**	***Tr* (*t*) (°C)**	***Tr* (*t* = 1) (°C)**	***Z*** **(°C)**	**R^2**	**SSr**	**BIC**
SS 1-11, per cultivation	309							0.95	61.7	-320.0
SS-1, isotime	22	84	[48:59]	0.98 [0.91-1.06]	54.5 [54.2-54.8]			0.99	0.7	
SS-2, isotime	24	14	[52:63]	0.81 [0.71-0.93]	59.0 [58.6-59.5]			0.97	2.6	
SS-3, isotime	44	24,34	[52:63][51:61]	1.13 [1.05-1.24]		67.7 [65.2-70.2]	7.3 [5.7-9.0]	0.97	10.1	
SS-4, isotime	28	4,14	[53:67][58:61]	1.53 [1.42-1.64]		63.0 [62.2-63.8]	3.7 [3.0-4.4]	0.99	0.2	
SS-5, isotime	22	4,34	[58:66][59:60]	0.98 [0.90-1.07]		64.8 [64.5-65.2]	6.6 [6.4-6.8]	0.99	1.5	
SS-6, isothermal	26	[14:184][14:124]	54,56	0.83 [0.69-1.06]		61.5 [59.9-63.1]	4.5 [3.5-5.5]	0.91	7.7	
SS-7, isothermal	24	[14:184][14:44][14:24]	52,58,60	0.92 [0.81-1.08]		65.1 [64.1-66.0]	7.1 [6.7-7.6]	0.96	4.8	
SS-8, isothermal	22	[14:124][14:94]	54,56	1.61 [1.16-2.67]		67.8 [63.0-72.5]	8.1 [5.2-11.0]	0.86	1.9	
SS-9, isothermal	24	[14:184][14:44][14:24]	52,58,60	1.03 [0.93-1.15]		64.7 [63.9-65.4]	6.6 [6.3-7.0]	0.98	1.5	
SS-10, isothermal	37	[34:304][14:74]	52,58	1.87 [1.51-2.46]		62.4 [59.8-64.9]	6.9 [5.9-7.9]	0.75	22.4	
SS-11, isothermal	36	[24:224][14:94]	54,56	1.04 [0.84-1.38]		63.7 [62.6-64.9]	6.2 [5.4-6.9]	0.94	8.3	
SS 1-11, total	309			1.49 [1.37-1.64]		64.2 [63.5-64.9]	6.9 [6.6-7.2]	0.74	312.4	20.6
SS-average of cultivation	9			1.23 [0.55-1.91]		64.5 [60.4-68.7]	6.4 [3.9-8.9]			

n: number of *t-T* data points within a cell culture

σ, *Tr* and *Z:* parameter estimations according to Gauss-Eyring model (Eq. 5.3)

R^2: determination coefficient

SSr: residual sum of squares

BIC: Bayesian Information Criterion

Table A4. Parameter estimations of thermal inactivation of *Saccharomyces cerevisiae* using the Gauss-Eyring model. Parameter estimations are shown for individual fit to each of the 11 individual cultures from the same frozen stock culture, indicated per Sc-number, and for the fit to all the inactivation data, indicated as Sc-1-11 total. Finally the weighted average of the individual fits is shown, indicated as Sc-average. Values between brackets show the 95% confidence interval estimated for the parameters. Values of *Tr(t)* show estimation of *Tr* at holding time *t*, estimated for series with one fixed time.

Cell cultivation	***n***	***t* (s)**	***T* (°C)**	σ **(°C)**	***Tr* (*t*) (°C)**	***Tr* (*t* = 1) (°C)**	***Z* (°C)**	**R^2**	**SSr**	**BIC**
Sc 1-11, per cultivation	318							0.97	20.2	-697.3
Sc-1, isotime	24	4,14,34,64	[52:60][52:60] [50:58][48:56]	0.91 [0.85-0.99]		61.1 [60.8-61.5]	4.4 [4.3-4.6]	0.99	0.3	
Sc-2, isotime	46	4,14	[52:63][50:60]	0.94 [0.88-1.40]		62.1 [61.8-62.4]	5.2 [5.0-5.5]	0.99	1.5	
Sc-3, isotime	18	64	[50:58]	0.96 [0.89-1.04]	53.9 [53.6-54.2]			0.99	0.6	
Sc-4, isotime	32	24,34	[52:59][51:58]	0.93 [0.87-1.00]		63.7 [62.4-65.0]	6.2 [5.3-7.1]	0.99	1.1	
Sc-5, isotime	28	84	[46:59]	1.29 [1.19-1.41]	53.7 [53.3-54.1]			0.99	0.9	
Sc-6, isothermal	38	[14:604][14:184][14:44]	50,55,60	0.93 [0.85-1.04]		62.7 [61.9-63.4]	4.1 [3.7-4.4]	0.97	2.1	
Sc-7, isothermal	16	[14:184][14:34]	55,58	0.83 [0.74-0.95]		61.2 [60.8-61.7]	3.6 [3.5-3.8]	0.98	0.2	
Sc-8, isothermal	26	[14:184][14:104]	54,56	0.71 [0.59-0.90]		60.9 [59.6-62.1]	3.4 [2.7-4.0]	0.91	1.4	
Sc-9, isothermal	10	[14:44][14]	58,60	0.95 [0.77-1.23]		61.6 [60.7-62.5]	4.6 [4.0-5.3]	0.97	0.6	
Sc-10, isothermal	44	[14:1804][14:74][9:24]	54,58,60	1.49 [1.25-1.84]		58.2 [57.3-59.2]	3.1 [3.0-3.3]	0.85	9.1	
Sc-11, isothermal	36	[304:3004][24:184]	52,56	0.97 [0.87-1.09]		58.8 [58.4-59.2]	2.9 [2.8-3.0]	0.94	2.6	
Sc 1-11, total	318			1.36 [1.27-1.47]		59.1 [58.8-59.5]	3.3 [3.2-3.4]	0.85	130.8	-265.2
Sc-average of cultivation	9			1.00 [0.55-1.45]		61.0 [57.5-64.6]	4.2 [2.1-6.3]			

n: number of *t*-*T* data points within a cell culture

σ, *Tr* and *Z:* parameter estimations according to Gauss-Eyring model (Eq. 5.3)

R^2: determination coefficient

SSr: residual sum of squares

BIC: Bayesian Information Criterion

Chapter 6

Effect of Moderate and High Intensity Pulsed Electric Fields (PEF) on microbial inactivation in fruit juice

Submitted as:

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Abstract

A systematic evaluation of the effect of moderate and high intensity pulsed electric field (PEF) parameters electric field strength (E) and pulse width (τ) in combination with heat on the inactivation of *E. coli, L. monocytogenes, L. plantarum, S.* Senftenberg and *S. cerevisiae* in orange juice was carried out in continuous flow to evaluate if moderate intensity PEF conditions could be used as alternative (PEF) process for classical thermal pasteurisation. A wide range of conditions has been evaluated, and both E and τ were shown to be important in the efficacy to inactivate micro-organisms. Inactivation kinetics of the tested conditions were compared to an equivalent thermal reference process. For three specific sets of conditions an additional electroporation effect was observed next to the thermal element responsible for the microbial inactivation. These conditions were at standard high intensity PEF treatment at E = 15 or 20 kV/cm and τ = 2 µs pulses, and remarkably also at moderate intensity PEF at E = 2.7 kV/cm and τ = 1000 µs. The effectivity for these moderate intensity PEF conditions was evaluated for *E. coli* and *L. monocytogenes* in watermelon juice and coconut water, varying in pH and conductivity. Interestingly, these moderate intensity PEF conditions showed the same effectivity for all evaluated matrices in the pH range of 3.8 – 6.0, wheras high intensity PEF conditions did show a strong dependence of microbial inactivation on product pH, suggesting that another underlying mechanism is responsible for the inactivation.

6.1 Introduction

Novel processes that avoid extensive heating times and over-processing as alternative to thermal pasteurization and sterilization have received considerable interest, because these processes better retain food quality. High intensity pulsed electric fields, often referred to as 'Pulsed Electric Fields' (Toepfl, Heinz & Knorr, 2007b; Vega-Mercado et al., 2007) is one of these alternative processes, suitable for the preservation of liquid food products, such as fruit juices. Typical process conditions are in the range of an electric field strength (E) of 15 – 40 kV/cm, and total pulse time of 20 µs up to 2000 µs (Álvarez et al., 2003a; Toepfl, Heinz & Knorr, 2007b). The capacity of high intensity PEF to induce cell electroporation to inactivate micro-organisms was demonstrated first by Sale & Hamilton (1967). Later, studies on the membrane electroporation effect of high intensity PEF reported to occur at the lipid domain (Teissié, Golzio & Rols, 2005; Weaver & Chizmadzhev, 1996), in the protein channel (Tsong, 1990) or cell wall (Pillet et al., 2016). Moderate intensity PEF conditions, defined in this study as conditions with an electric field strength $E < 5$ kV/cm, have been extensively studied as a pre-step in mass transfer phenomena in food and biotechnological processes, aiming to mildly disintegrate plant cells (Donsì, Ferrari & Pataro, 2010). Although the electroporation effect in plant cells was observed, these process conditions have received no attention for the application as alternative preservation process to inactivate micro-organisms, probably due to their small size (1 – 10 µm) compared to the size of eukaryotic plant cells (40 – 200 µm) (Raso, 2016). The use of moderate intensity PEF conditions has been studied in biology applications for gene transfer, and showed electroporation effects in micro-organisms, when exposure time was long enough (Eynard et al., 1998). However, in most studies the application of the pulses took up to hours, as a temperature increase of the sample was avoided (El Zakhem et al., 2006a; El Zakhem et al., 2007). Although high intensity PEF treatment aims for minimal thermal effects, the temperature of the treated medium increases as the result of Ohmic, also called Joule, heating. Contrarily, an 'ohmic heating process' with moderate electric field strength (typically at $E < 0.5$ kV/cm) aims at the conversion of electrical energy into thermal energy that allows rapid heating of the food (Jaeger et al. 2016). In practice, the differences between high intensity PEF and ohmic heating are fading, as high intensity PEF processing is increasingly combined with heat to improve its efficacy (Buckow, Ng & Toepfl, 2013) and ohmic heating processing is sometimes associated with an additional electroporation effect (Lebovka et al. 2005, Loghavi & Sastry, 2009).

Understanding of the effect of moderate and high intensity pulse conditions on microbial inactivation, requires a systematic evaluation of process parameters, microbial characteristics and culture conditions, and treatment medium characteristics, as these factors all contribute to microbial inactivation (Raso, Condón & Álvarez, 2014). Improved understanding of the effect of these parameters could help to design an optimal pasteurization process, with minimal energy input and maximal microbial inactivation.

The research question of this study was to investigate if a combined process of moderate intensity pulsed electric fields and heat could be used as alternative PEF condition to inactivate micro-organisms and serve as an alternative, mild preservation process for fruit juices.

To answer this question, high intensity PEF conditions (E = 10 – 20 kV/cm and pulse width τ = 2 µs) and moderate intensity PEF conditions (E = 0.9 and 2.7 kV/cm and variable pulse width of 15 – 1000 µs) were compared in orange juice for three pathogenic (*Escherichia coli, Salmonella* Senftenberg and *Listeria monocytogenes*) and two spoilage micro-organisms (*Lactobacillus plantarum* and *Saccharomyces cerevisiae*). The inactivation kinetics for the tested conditions was modelled and is compared to the inactivation kinetics of an equivalent thermal process to distinguish the electroporation effect from the thermal effect. The most promising moderate intensity PEF conditions were evaluated in other fruit juices as well to evaluate the potential of these conditions to act as alternative preservation process.

6.2 Material and methods

6.2.1 Product matrices

Three different fruit juices varying in pH and conductivity were selected: orange juice (Minute Maid, original), coconut-water (HealthyPeople) and watermelon juice (extracted manually). Watermelon juice was extracted according to the method described in Timmermans et al., 2014, and stored at -20 °C upon treatment. Juices were sieved (pore size 0.225 mm) to remove large fibres and particles, and pasteurized (30 min. 98 °C) prior to inoculation.

Electric conductivity (Greisinger GMH 3430) and pH (Metrohm 744) of the juices was measured before and after inoculation with target micro-organisms. Temperature dependency of the conductivity before inoculation is shown in Fig. 6.1 for the three juices.

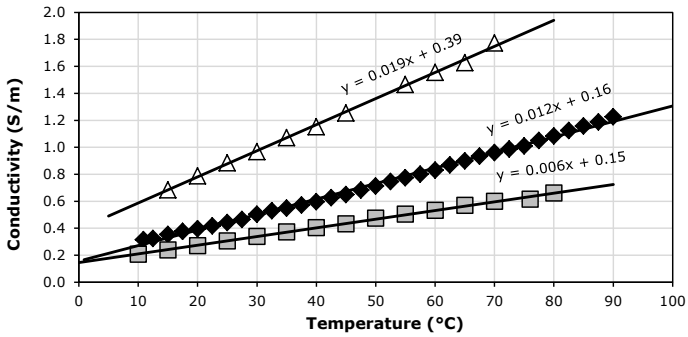


Figure 6.1. Temperature dependence of the electrical conductivity of the selected juices. Orange juice, pH = 3.8 (black diamonds), coconut water, pH = 5.0 (open triangles) and watermelon juice, pH = 6.0 (grey squares).

6.2.2 Micro-organisms and culture conditions

Pathogenic and spoilage micro-organisms were selected based on differences in morphology and its relevance in fruit juices (Table 6.1). Additionally, PEF-resistance or heat-resistance of the strains was used as criteria for selection.

Fresh cultures of *Escherichia coli* were prepared by plating from frozen stock culture on TSB (Oxoid) agar plates. Plates were incubated overnight (o/n) at 37 °C. A single

colony was used to inoculate a 100 mL flask with 10 mL TSB broth and cultivated for 24 h at 20 °C in an Innova shaking incubator (180 rpm) (2nd o/n). From this culture, 1 mL was used to inoculate 95 mL fresh TSB broth supplemented with 1% glucose (4 mL) (Sigma-Aldrich) in a 500 mL flask, and incubated for 24 h at 20 °C and 180 rpm (3rd o/n). The cultivation of *Salmonella* Senftenberg was similar to this protocol, for the other micro-organisms specific agar composition and different incubation temperatures for the first o/n cultivation were used: *Listeria monocytogenes* was incubated at 30 °C using BHI agar (Oxoid), *Lactobacillus plantarum* was incubated at 25 °C using MRS broth (Merck) and *Saccharomyces cerevisiae* was incubated at 25 °C using glucose-peptone-yeast agar, containing 40 g glucose, 5 g peptone (Fluka) and 5 g yeast (Oxoid) per 1 L distilled water. 1% glucose was added to the medium during the third o/n cultivation to mimic sugar content in fruit juice. After the 3rd o/n incubation, microbial cells were pelleted by centrifugation (4000 rpm, 5 min) at 20 °C. Pellet was resuspended in 20 mL sterile peptone physiological salt diluent (PSDF, Tritium) and centrifuged again at same conditions. This step was repeated once, and after the third centrifugation step, pellet was suspended in 2 L pasteurised juice to a cell density of 10^6 – 10^7 cfu/mL (yeast) or 10^7 – 10^8 cfu/mL (bacteria).

Table 6.1. Bacterial strains and yeast strain used in this study.

Species/strains used	Cell wall structure	Size*	Reference
Escherichia coli ATCC 35218	Gram-negative	1.1 - 1.5 µm x 2 - 6 µm	Gurtler et al. (2011)
Lactobacillus plantarum ATCC 14917	Gram-positive	0.9 - 1.2 µm x 3 - 8 µm	Campos & Cristianini (2007)
Listeria monocytogenes NV8	Gram-positive	0.4 - 0.5 µm x 0.5 - 2 µm	Van der Veen et al. (2009)
Saccharomyces cerevisiae CBS 1544	-	3 - 15 µm x 2 - 8 µm	Put et al. (1976)
Salmonella enterica subsp. *enterica serovar* Senftenberg ATCC 43845	Gram-negative	0.7 - 1.5 µm x 2 - 5 µm	Ng, Bayne & Garibaldi (1969)

ATCC: American Type Culture Collection, USA
CBS: Centraal Bureau voor Schimmelcultures (Fungal Biodiversity Centre, Utrecht, The Netherlands)
* Characteristic dimensions taken from Bergey, 1986

6.2.3 Pulsed Electric field processing

The effect of PEF processing conditions was studied in a continuous-flow system using three different configurations. The key information of the process settings used is given below according to guidelines established by the precursor of the International Society for Electroporation-Based Technologies and Treatments (Raso et al., 2016). The three configurations varied in pulse shape, pulse intensity, pulse width and number of treatment chambers used (Table 6.2). In short, in configuration I monopolar pulses of a short width of 2 µs with high intensity (E = 10 − 20 kV/cm) were used, in configuration II bipolar pulses of long width (τ = 100 − 1000 µs) with moderate intensity (E = 0.9 − 2.7 kV/cm) were used, and in configuration III bipolar pulses of moderate width (τ = 15 − 100 µs) with moderate intensity (E = 2.7 kV/cm) were used. To vary in electric field strength, treatment chambers with different dimensions were used, as indicated in Table 6.2. Insulators were made of polyetherimide (PEI, Ultem™ resin), and electrodes consisted of stainless steel (SS-316) for configuration I and of titanium for configuration II and III.

The set-up for the experiments was identical for the three configurations, with sterilisation, start-up and cleaning procedures as described previously in Timmermans et al. (2014). A detailed description and overview of the set-up is shown in the Appendix. The inoculated suspension was pumped at a flowrate of 13 ± 1 mL/min, and preheated in a heating spiral (SS-316) to 36 °C prior to the PEF treatment. Next, the suspension entered either two or three vertical positioned co-linear treatment chambers for electrical treatment. Due to the variation in the intensity of the treatment conditions (electric field strength, pulse duration and frequency) the juice suspension heated up to variable maximum temperatures. No holding section was included, so directly after leaving the treatment chambers (typically within 2.3 seconds), juice suspension was cooled down via a cooling spiral (SS-316) that was immersed in an ice-water bath. At the exit, samples were collected aseptically. An example of a typical temperature-time profile of the PEF treatment using two treatment chambers is shown in Fig. 6.2. Temperature before and directly after the treatment chambers was measured using HYP-O T-type thermocouples (Omega). Additionally, maximum temperature was measured indirectly at the exit of the last treatment chamber using a NTC-resistor. Pulse shape, voltage and current realized in the treatment chamber were recorded with a digital oscilloscope (Rigol DS1102E), and an example of come up time and fall time of the pulses is given in Fig. 6.3. Fall time of the square wave pulses was calculated as time to reduce the intensity x 1/e, which is similar to the calculated fall time for exponential decay pulses calculated as a 37% reduction of the peak value (Raso et al., 2016), being 60 µs in configuration II, and 1.5 µs in configuration III.

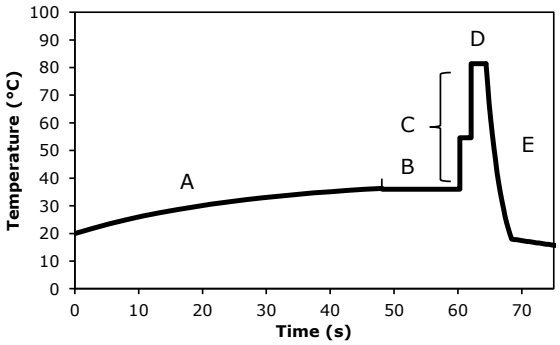


Figure 6.2. Example of a temperature-time profile during PEF treatment, using two treatment chambers and heating to a maximum temperature of 81 °C. Inoculated juice is preheated from room temperature to 36 °C in 48 s (A), maintained at 36 °C for 12 s (B) before entering the treatment chambers, heated up in the first treatment chamber, followed by a pause of 1.7 s, the time to go to the next treatment chamber, and heated up to a desired maximum temperature in the second treatment chamber, i.e. 81 °C (C). Then juice is transferred to the cooling section in 2.3 s (D) and cooled down for 122 s (E, partly displayed).

The electrical energy input was calculated by numerical integration of the voltage and current traces (Eq. 6.1), and equals to the caloric power measured in the bulk (Eq. 6.2) within experimental error (< 5%), with absolute deviations of 0.5 – 3 °C based on maximum temperature. Specific energy, w (kJ/kg), is calculated with $P_{electric}$ as the electrical power (W), φ the flow rate (m^3/s), ρ is the density of the juice, 1020 kg/m^3, f is the frequency (Hz), U is the voltage signal (V), I is the current signal (A), T_{out} as the outlet temperature (°C), T_{in} is the inlet temperature (°C) and c_p is the specific heat capacity, which is 3.8 kJ/kg·K for fruit juice and 4.1 kJ/kg·K for coconut water (Fontan et al., 2009).

$$w = \frac{P_{electric}}{\phi \cdot \rho} = \frac{f \cdot \int U(t) I(t) \mathrm{d}t}{\phi \cdot \rho} \quad \text{(Eq. 6.1)}$$

$$w = (T_{out} - T_{in}) \cdot c_p \quad \text{(Eq. 6.2)}$$

The technical design of the treatment chamber is described by Mastwijk et al. (2007), and all treatment chambers were scaled to this design. As a result of the variation in length, and diameter, the residence times within the treatment chambers was not the same for every tested condition (Table 6.2). However, the frequency could be adjusted as well here to obtain the desired specific heat and maximum temperature. The number of pulses (n) applied was calculated by Eq. 6.3, where V is the volume of the high electric field region approximated by the volume defined by the distance of the electrodes and diameter of the gap (Mastwijk et al., 2007) (m^3).

For calculations with bipolar pulses this number has to be multiplied with 2, as both a positive and negative pulse are given within one cycle.

$$n = \frac{V \cdot f}{\phi} \quad \text{(Eq. 6.3)}$$

For each experiment, electric field strength and pulse duration were pre-set, and a kinetics series with variation in chosen frequency led to variable maximum temperatures. Variation in chosen process conditions electric field strength (E) and pulse duration (τ) and matrix characteristic conductivity (σ) led to a different energy density per pulse (Eq. 6.4), with u as the energy density per pulse (J/m^3). To obtain the same specific energy (w) and maximum temperatures (T_{out}) in each experiment, the frequency was adjusted (Eq. 6.5).

$$u = \sigma \cdot E^2 \cdot \tau \quad \text{(Eq. 6.4)}$$

$$w = \frac{f \cdot V}{\phi \cdot \rho} \int \kappa \cdot E^2 \mathrm{d}t \quad \text{(Eq. 6.5)}$$

Table 6.2 shows the range of frequencies and number of pulses applied for each setting.

6.2.4 Experimental design

Selected juice was inoculated just before the start of the experiment, and a sample was taken to determine the exact starting concentration. Directly after inoculation, the inactivation kinetics series was started and samples were collected at each setting, and kept on ice until plating. At the end of a kinetic series, PEF equipment was turned off and a control without treatment was taken at the exit of the equipment. The number of viable cells was determined by plating 100 µL of a serially diluted sample in sterile peptone physiological salt diluent in duplicate on suitable agar plates (medium supplemented with 15 g/L agar (Oxoid) and 0.1% sodium pyruvate (Sigma Aldrich)) to enhance outgrowth of sub-lethally damaged cells (Timmermans et al., 2014). Surviving cells were enumerated after 3– 5 days incubation at 25 °C (*S. cerevisiae),*

Table 6.2. Pulse conditions and characterizations for the different PEF configurations I-III used in this study.

Configuration	Pulse shape	Number of treatment chambers	Pulse width (τ)(µs)	Electric field strength (E) (kV/cm)	Dimensions of treatment chamber (diameter x length)(mm)	Residence time (ms)	Matrix	Frequency (Hz)	Number of pulses
I	Monopolar square wave	2	2	10	2.8 x 4.0 (U, L)	227	Orange juice	0 - 964	0 - 220
				15	1.5 x 3.0 (U, L)	49	Orange juice	0 - 964	0 - 204
				20	1.0 x 2.0 (U, L)	14	Orange juice	0 - 964	0 - 14
II	Bipolar square wave	2	1000	0.9	4.0 x 6.0 (U, L)	696	Orange juice	0 - 55	0 - 77
			1000	2.7	1.0 x 2.0 (U, L)	14	Orange juice	0 - 255	0 - 7.4
							Coconut water	0 - 100	0 - 2.9
							Watermelon juice	0 - 238	0 - 6.8
			100	2.7	1.0 x 2.0 (U, L)	14	Orange juice	0 - 1040	0 - 30
III	Bipolar square wave	3	100	2.7	2.0 x 4.0 (U, L) 1.0 x 2.0 (M)	123	Orange juice	0 - 270	0 - 39
			15	2.7	2.0 x 4.0 (U, L) 1.0 x 2.0 (M)	123	Orange juice	0 - 1700	0 - 247

U = upper treatment chamber
M = middle treatment chamber
L = lower treatment chamber

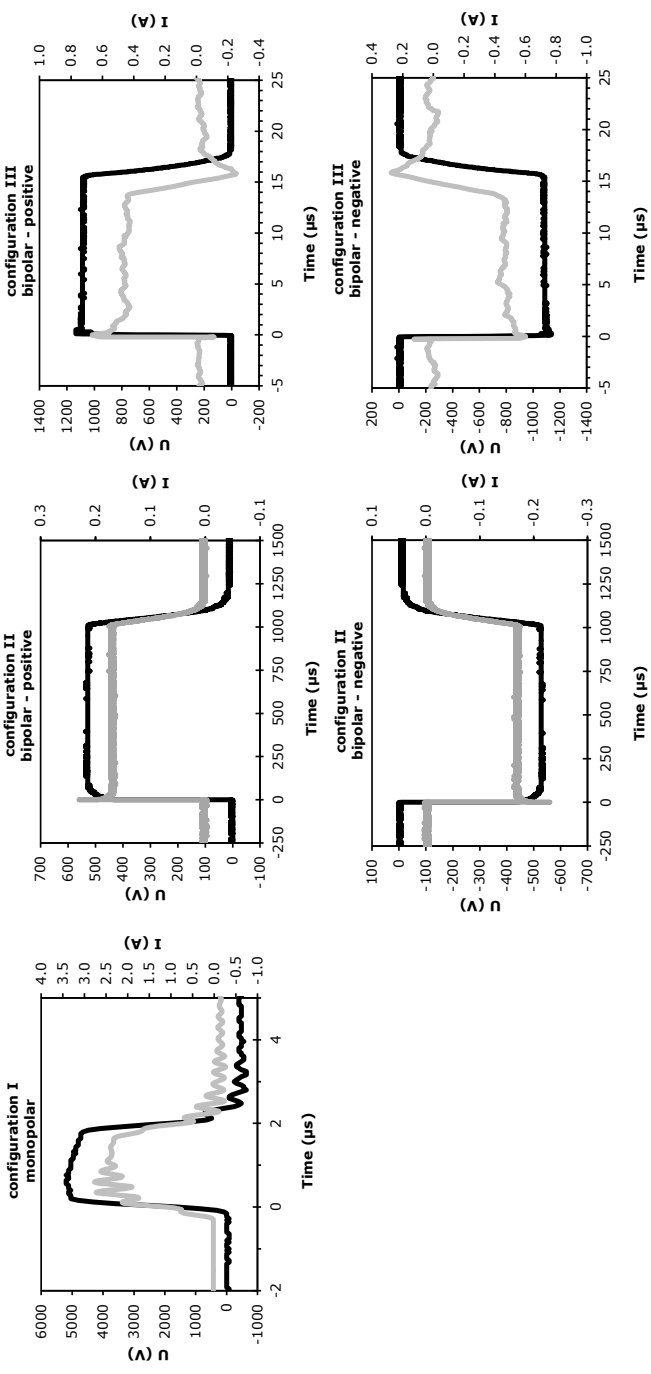


Figure 6.3. Typical voltage-current characteristics of pulses applied in configuration I-III. Configuration I: monopolar square wave pulses of τ = 2 µs, configuration II: bipolar square wave pulses of τ = 1000 µs and configuration III: bipolar square wave pulses of τ = 15 µs. Black line corresponds to voltage and grey line corresponds to current.

30 °C (*L. monocytogenes, L. plantarum*) and 37 °C (*S.* Senftenberg, *E. coli*), and plates with counts between 10 and 300 colony forming units (cfu) per plate were used for calculations.

Experiments were carried out on two different days to include biological variability. Inactivation kinetics of thermal treatment only was determined in capillary tubes in a water bath as described in Chapter 5, and is considered as configuration 0.

6.2.5 Modelling and statistical analysis

Microbial inactivation is expressed as $\log_{10}(N/N_0)$, where N is the number of surviving micro-organisms at a specific PEF-condition in cfu/mL, and N_0 is the average number of surviving micro-organisms at start and control, in cfu/mL.

The Gauss-Eyring model for inactivation kinetics (Mastwijk, Timmermans & Van Boekel, 2017) was used to fit to the survival data $\log_{10}(N/N_0)$ as a function of maximum temperature (T) according to Eq. 6.6, with $Tc(t)$ describing the critical temperature when 50% of the population is inactivated (°C) and σ is the width of the temperature distribution (°C).

$$\log_{10}\left(\frac{N(t,T\mid \mathrm{E})}{N_0}\right)=\log_{10}\left(\frac{1}{2}\left[\operatorname{erfc}\left(\frac{T-Tc(t)}{\sqrt{2}}\right)\right]\right) \qquad \text{(Eq. 6.6)}$$

Note that $Tc(t)$ is defined for a specific process time, which is assumed to be t = 2.3 s for the PEF treatments in this study (time between exit treatment chamber and entrance cooling section, that corresponds to section D in Fig. 6.2). Parameter estimation was done via nonlinear least-squares regression using Athena Visual Studio, version 14.2 (www.athenavisual.com).

The Gauss-Eyring model was fitted to the inactivation data collected for single experiments. *t*-statistics was used to calculate the mean and standard deviation of the duplicate experiments. To compensate for the number of data points used in each individual experiment, weighted means and weighted standard deviations were calculated.

Parameter estimates σ, Tr and Z of the Gauss-Eyring model applied to thermal inactivation of the same strains in orange juice were taken from Chapter 5. The Z value in Eq. 6.7 expresses the temperature increase/decrease required to reduce/raise the holding time value by a factor of ten, and is identical to the expression used in linear kinetics, and τ_r = 1 s a chosen unit of time.

$$Tc(t)=Tr-Z\cdot log_{10}(t/\tau_r) \qquad \text{(Eq. 6.7)}$$

Tc was recalculated to correspond to a reference time of t = 2.3 s, using Eq. 6.7, as

$$Tc(2.3)=Tr-Z\cdot log_{10}(2.3) \qquad \text{(Eq. 6.8)}$$

The critical temperatures found in a thermal series at holding time of 2.3 seconds can be compared to the critical temperatures for PEF treatment at 2.3 s holding time.

The 95% Confidence Interval (95% CI) of the predicted inactivation curves after PEF treatment was given based on parameter estimates. For the predictions after thermal treatment with estimations for $Tc(t = 2.3)$ and σ, Monte Carlo simulations were used to predict the 95% CI (n = 1000 simulations, Excel), where the 5% and 95% quantiles of the observed data define the parameter interval around the Maximum Likelihood. This method accounts for possible unsymmetrical confidence intervals occurring from nonlinear regression, in contrast to linear approximation methods used in most software packages (Poschet et al., 2003; Van Boekel, 2009a).

For each single experiment carried out, the temperature required to obtain a 5 log reduction of the target micro-organism was predicted, based on the parameter estimates of the Gauss-Eyring model at t = 2.3 s. Data of the single experiments was combined per treatment condition to calculate the mean value, and an independent sample t-test was conducted (Excel) to compare the mean values between two treatments at 5 log reduction.

6.3 Results and discussion

6.3.1 Influence of electric field strength

PEF inactivation experiments were performed in orange juice that was preheated to 36 ± 1 °C before PEF treatment. Dependent on the chosen electric field strength and single pulse duration, a specific number of pulses was given to reach the selected maximum temperature. Inactivation of *E. coli*, *L. monocytogenes*, *L. plantarum*, *S.* Senftenberg and *S. cerevisiae* was measured for monopolar pulses of 2 µs at different high electric field strengths between 10-20 kV/cm (Fig. 6.4, left panel) and the inactivation kinetics of the individual experiments were modelled using the Gauss-Eyring model (Eq. 6.6). Other data in Fig. 6.4 will be discussed in the next section. Weighted average values of the parameter estimates σ and Tc (t = 2.3 s) of the duplicate experiments were calculated as representative parameter estimates for the specific treatment condition used. Model fit and parameter estimates for the different treatments are shown in Fig. 6.4 and Table 6.3, respectively.

A decrease in the intensity of the electric field strength from 20 kV/cm to 10 kV/cm led to a reduced efficiency in inactivation for all tested bacteria, meaning that a higher electrical energy input (kJ/kg) and corresponding maximum temperature is required to obtain the same level of inactivation. Table 6.3 shows an increase of the value of parameter Tc (t = 2.3 s), indicating the temperature necessary to inactivate 50% of the population. This observation is in agreement with results of Grahl & Märkl (1996), Heinz et al. (2002), Hülsheger, Potel & Niemann (1983), and Sale & Hamilton (1967), who described increased inactivation at elevated electric field strength. Interestingly, parameter estimates Tc (t = 2.3 s) and σ (the temperature width of the distribution) of the yeast *S. cerevisiae* are similar for conditions at E = 10 kV/cm and E =20 kV/cm, indicating that an additional elevation of the electric field strength from E = 10 kV/cm to E = 20 kV/cm for this species does not enhance inactivation.

Table 6.3. Parameter estimates for inactivation of *E. coli, L. monocytogenes, S. cerevisiae, S.* Senftenberg and *L.* plantarum using the Gauss-Eyring model (Eq. 6.6). Parameter estimates are shown for the calculated weighted average of the individual fits per experiment. Note that 'holding time' in the PEF equipment is estimated at t = 2.3 s, and therefore Tc is given for t = 2.3 s. Data of thermal inactivation (configuration 0) at t = 2.3 s is calculated according to Eq. 6.7 and 6.8, based on parameter estimates given in Chapter 5. Predictions were made to calculate the temperature required to have a 5 $\log_{10}$ reduction. Prediction for *L. monocytogenes* at E = 10 kV/cm was not made, as virtually no inactivation was observed. Values between brackets show the 95% confidence interval estimated for the parameters and for the predictions to obtain 5 $\log_{10}$ reduction.

Micro-organism	Electric field strength (kV/cm)	Pulse width (µs)	Medium	Data shown in Figure
E. coli	0 (thermal)	0	Orange juice	Fig. 6.4
E. coli	20	2	Orange juice	Fig. 6.4
E. coli	15	2	Orange juice	Fig. 6.4
E. coli	10	2	Orange juice	Fig. 6.4
E. coli	2.7	1000	Orange juice	Fig. 6.4, 6.6, 6.7
E. coli	2.7	1000	Coconut water	Fig. 6.7
E. coli	2.7	1000	Watermelon juice	Fig. 6.7
E. coli	2.7	100	Orange juice	Fig. 6.5
E. coli	2.7	100	Orange juice	Fig. 6.5, 6.6
E. coli	2.7	15	Orange juice	Fig. 6.6
E. coli	0.9	1000	Orange juice	Fig. 6.4
L. monocytogenes	0 (thermal)	0	Orange juice	Fig. 6.4
L. monocytogenes	20	2	Orange juice	Fig. 6.4
L. monocytogenes	10	2	Orange juice	Fig. 6.4
L. monocytogenes	2.7	1000	Orange juice	Fig. 6.4, 6.6, 6.7
L. monocytogenes	2.7	1000	Coconut water	Fig. 6.7
L. monocytogenes	2.7	1000	Watermelon juice	Fig. 6.7
L. monocytogenes	2.7	100	Orange juice	Fig. 6.6
L. monocytogenes	2.7	15	Orange juice	Fig. 6.6
L. monocytogenes	0.9	1000	Orange juice	Fig. 6.4
S. cerevisiae	0 (thermal)	0	Orange juice	Fig. 6.4
S. cerevisiae	20	2	Orange juice	Fig. 6.4
S. cerevisiae	10	2	Orange juice	Fig. 6.4
S. cerevisiae	2.7	1000	Orange juice	Fig. 6.4, 6.6
S. cerevisiae	2.7	100	Orange juice	Fig. 6.6
S. cerevisiae	2.7	15	Orange juice	Fig. 6.6
S. cerevisiae	0.9	1000	Orange juice	Fig. 6.4
S. Senftenberg	0 (thermal)	0	Orange juice	Fig. 6.4
S. Senftenberg	20	2	Orange juice	Fig. 6.4
S. Senftenberg	10	2	Orange juice	Fig. 6.4
S. Senftenberg	2.7	1000	Orange juice	Fig. 6.4
S. Senftenberg	0.9	1000	Orange juice	Fig. 6.4
L. plantarum	0 (thermal)	0	Orange juice	Fig. 6.4
L. plantarum	20	2	Orange juice	Fig. 6.4
L. plantarum	10	2	Orange juice	Fig. 6.4
L. plantarum	2.7	1000	Orange juice	Fig. 6.4
L. plantarum	0.9	1000	Orange juice	Fig. 6.4

Configuration	n (exp.)	n (data points)	σ (°C)	*Tc* (*t* = 2.3 s) (°C)	Predicted Tmax (°C) necessary for 5 $\log_{10}$ reduction
0	8	304	1.4 [0.7-2.2]	64.0 [60.5-67.7]	69.5 [64.7-74.3]
I	2	20	3.2 [2.9-3.5]	36.9 [36.5-37.3]	50.6 [49.3-51.8]
I	2	36	6.4 [5.7-7.1]	38.6 [35.8-41.3]	65.9 [65.3-66.4]
I	1	16	3.5 [2.9-4.4]	59.2 [57.3-61.2]	74.0
II	2	24	1.0 [0.7-1.2]	59.1 [56.7-61.5]	63.1 [61.5-64.7]
II	2	30	0.8 [0.7-1.0]	63.4 [62.1-64.6]	66.7 [64.0-69.4]
II	2	34	1.0 [0.9-1.1]	62.9 [59.3-66.5]	67.1 [61.6-72.6]
II	2	38	2.5 [2.1-2.9]	61.2 [60.6-61.7]	70.4 [64.6-76.1]
III	2	32	2.3 [0.7-3.9]	61.9 [59.0-64.1]	72.0 [65.3-78.8]
III	2	32	1.0 [0.4-1.7]	62.3 [61.7-62.9]	66.4 [63.0-69.8]
II	3	50	2.1 [1.0-3.1]	71.4 [67.4-75.3]	80.0 [72.0-88.1]
0	4	147	0.9 [0.6-1.2]	71.4 [68.8-74.2]	72.0 [61.0-83.0]
I	1	14	4.5 [4.1-5.1]	39.8 [38.7-40.8]	59.1
I	2	34	17.3 [13.3-21.3]	56.9 [52.4-61.4]	X
II	2	30	1.1 [1.0-1.2]	60.4 [60.1-60.7]	65.2 [64.1-66.3]
II	1	18	1.4 [1.3-1.4]	60.4 [60.1-60.7]	66.2
II	2	32	1.6 [1.4-1.8]	58.2 [55.2-61.2]	64.7 [59.2-70.1]
III	2	36	1.4 [0.8-2.0]	66.1 [64.8-67.4]	72.1 [70.0-74.2]
III	2	40	1.6 [0.9-2.3]	65.1 [63.8-66.4]	72.1 [70.0-74.2]
II	2	34	2.3 [1.7-2.9]	72.1 [70.4-73.8]	81.9 [76.1-87.6]
0	9	272	1.0 [0.6-1.5]	59.5 [56.0-63.1]	63.8 [61.6-66.0]
I	2	28	5.0 [4.3-5.7]	40.5 [36.4-44.5]	61.7 [51.8-71.6]
I	2	44	5.5 [4.0-7.0]	41.3 [39.3-43.3]	64.6 [58.3-70.9]
II	2	22	1.0 [0.5-1.5]	49.9 [47.0-52.8]	54.1 [53.0-55.3]
III	2	43	1.7 [1.6-1.8]	55.9 [55.8-56.0]	63.1 [62.5-63.6]
III	2	40	1.7 [0.9-2.7]	54.6 [52.2-56.9]	62.3 [60.4-64.3]
II	2	34	2.2 [1.6-2.8]	62.5 [59.8-65.3]	72.0 [71.9-72.1]
0	9	263	1.2 [0.6-1.9]	62.2 [58.0-66.5]	67.3 [62.8-71.8]
I	2	28	5.5 [5.2-5.7]	34.0 [32.8-35.2]	57.3 [53.9-60.7]
I	2	36	3.5 [0.7-7.9]	49.6 [46.8-52.3]	68.3 [50.2-86.4]
II	2	30	1.5 [1.3-1.6]	55.7 [55.2-56.2]	62.0 [61.8-62.1]
II	2	36	2.5 [2.3-2.6]	60.7 [60.3-61.1]	71.4 [69.9-72.8]
0	10	237	0.8 [0.3-1.3]	59.8 [56.2-63.5]	63.4 [59.6-67.2]
I	2	28	3.3 [3.1-3.4]	40.5 [39.4-41.6]	54.4 [51.9-57.0]
I	2	36	3.7 [1.5-10.8]	55.2 [51.7-58.7]	75.8 [47.2-104.4]
II	2	23	0.9 [0.8-1.0]	52.6 [52.1-53.1]	56.6 [55.2-58.0]
II	2	32	1.9 [1.3-2.4]	65.0 [62.7-67.3]	72.7 [66.2-79.1]

Next, it can be seen that the response to the PEF treatment is species dependent. Comparison of the parameter estimates σ and *Tc* (t = 2.3 s) for the different micro-organisms showed highest values for *L. monocytogenes* indicating a higher resistance to PEF compared to the other species tested. Especially at E = 10 kV/cm, a significantly larger σ value was observed for *L. monocytogenes* compared to the other micro-organisms. To facilitate the comparison among different species and treatment conditions, the temperature required to obtain a 5 $\log_{10}$ reduction was calculated, based on parameter estimates *Tc* (t = 2.3 s) and σ given in Table 6.3. Most values had to be extrapolated from the measured data, because observed microbial inactivation did not exceed 4 $\log_{10}$ reduction. Conditions at E = 10 kV/cm showed a higher variance between the duplicate experiments than conditions at E = 20 kV/cm, resulting in a larger confidence interval (Table 6.3). The calculation of *L. monocytogenes* at E = 10 kV/cm was not included, as virtually no inactivation was observed at the maximum temperature of 70 °C and parameter estimates could not make a sound prediction.

The most PEF resistant species followed the order *L. monocytogenes* > *E. coli* > and *L. plantarum, S.* Senftenberg and *S. cerevisiae*. Differences between the latter three species are small and deviate in order for the different electric field strength applied. High PEF resistance of *L. monocytogenes* was also observed by Grahl & Märkl (1996), Hülsheger, Potel & Niemann (1983), and Toepfl, Heinz & Knorr (2007b), who concluded that smaller sized cells and Gram-positive bacteria require a higher energy input for inactivation compared to larger sized and Gram-negative micro-organisms.

The same species were treated in orange juice preheated to 36 ± 1 °C using bipolar pulses of τ = 1000 µs at an moderate electric field strength of E = 0.9 or 2.7 kV/cm (Fig. 6.4, middle panel). Inactivation could be modelled using the Gauss-Eyring model, model fits (Fig. 6.4) and parameter estimates (Table 6.3) are provided. Also for this configuration, a higher electric field strength led to more inactivation at constant energy input or maximum temperature with the degree of inactivation, also for *S. cerevisiae.* Resistance of the species at moderate intensity (E = 0.9 and 2.7 kV/cm) and long duration (τ = 1000 µs) followed the same order as high intensity pules (E = 10 − 20 kV/cm) of short duration (τ = 2 µs): *L. monocytogenes* > *E. coli* > and *L. plantarum, S.* Senftenberg and *S. cerevisiae*.

Comparison of the inactivation kinetics for each individual strain showed that an increase of electric field strength resulted in a temperature reduction for a given degree of inactivation. Interestingly, inactivation at an electric field strength of 2.7 kV/cm with bipolar pulses with a pulse duration of 1000 µs, was higher when compared to inactivation kinetics at E = 10 kV/cm with monopolar pulses with a pulse width of 2 µs, at the same energy input. This effect was observed for all species tested. Comparison of the maximum temperature required to reach a 5 $\log_{10}$ reduction (Table 6.3), showed a significant difference between these two conditions, for all species tested. This suggests that not only the electric field strength, but also the duration of a single pulse (pulse width) plays a role in the effectivity of the PEF process.

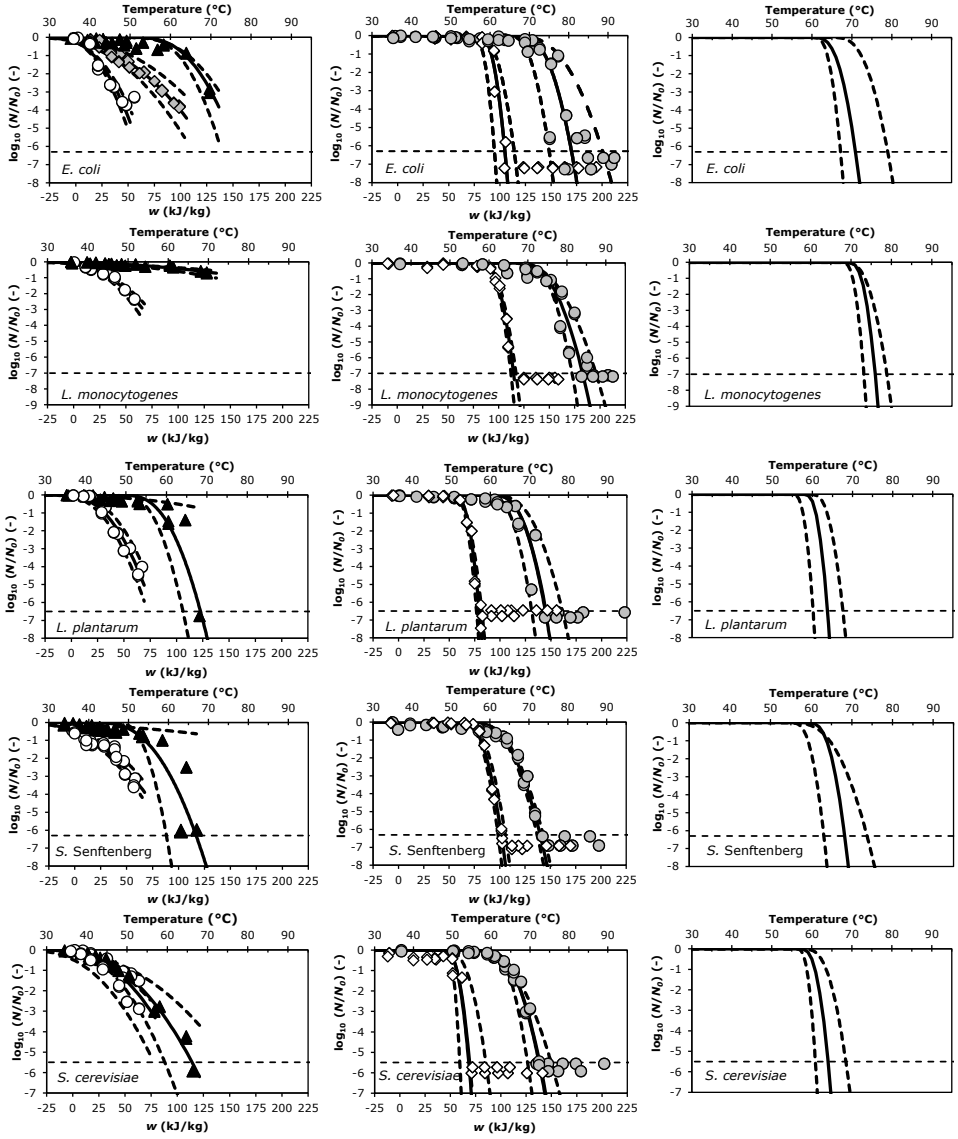


Figure 6.4. Reduction of viable counts $\log_{10}(N/N_0)$ of *E. coli, L. monocytogenes, L. plantarum, S.* Senftenberg and *S. cerevisiae* in orange juice after PEF (left or middle panel) or thermal treatment (right panel). Inactivation after PEF treatment is expressed as a function of maximum temperature (°C) or electrical energy applied (kJ/kg). Symbols represent measured data, continuous lines represent the mean value estimated with the Gauss-Eyring model and dashed lines the 95% confidence interval.

Left panel: PEF treatment using monopolar pulses of τ = 2 µs at E = 20 kV/cm (open circles), E = 15 kV/cm (grey diamonds) or E =10 kV/cm (black triangles) and preheating at 36 °C. Part of the data at E = 20 kV/cm was published in Timmermans et al., 2014.

Middle panel: PEF treatment using bipolar pulses of τ = 1000 µs at E = 0.9 kV/cm (grey circles) and E = 2.7 kV/cm (white diamonds) and preheating at 36 °C.

Right panel: predictions using thermal reference data from Chapter 5. The Gauss-Eyring model was fitted to the data and the parameter estimates were recalculated for t = 2.3 s holding time, according to Eq. 6.8. Horizontal dashed lines represent the detection limit.

Although a relationship between electric field strength and minimal total treatment time was noticed before and empirically determined for *E. coli* by Hülsheger, Potel & Niemann (1981), the effect of the pulse width, as shown in Fig. 6.4, has not been investigated before, and will be discussed in the next section.

6.3.2 Influence of pulse width

To study the effect of a single pulse duration at an electric field strength of 2.7 kV/cm, the PEF equipment was adapted, to reduce the fall time of the pulse from τ = 60 µs to 1.5 µs (Fig. 6.3, configuration II and III). As a consequence of this adaption, the number of treatment chambers was increased from 2 to 3. To evaluate if this change in lay-out and fall time influenced the degree of inactivation, an inactivation experiment with *E. coli* exposed to bipolar pulses of τ = 100 µs at E = 2.7 kV/cm was conducted at both configurations II and III. Comparison of the inactivation data (Fig. 6.5) and parameter estimates of the Gauss-Eyring model fitted to the inactivation data (Table 6.3) showed similar inactivation curves and parameter estimates, indicating that the data collected in configuration II and III were comparable, and that this adaption did not influence the degree of inactivation.

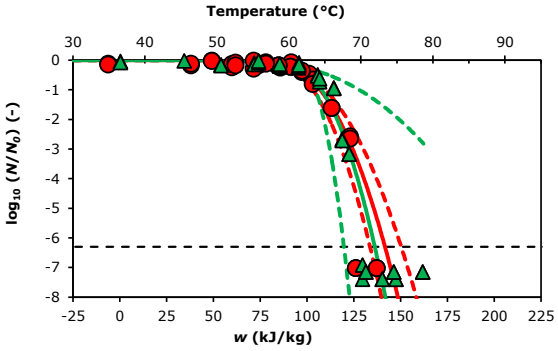


Figure 6.5. Reduction of viable counts of *E. coli* in orange juice, preheated at 36 °C and exposed to a PEF treatment with bipolar pulses of τ = 100 µs at E = 2.7 kV/cm, using 2 treatment chambers in configuration II (red circles) or 3 treatment chambers in configuration III (green triangles). Inactivation is measured as a function of maximum temperature (°C) or electrical energy applied (kJ/kg). Symbols represent measured data, continuous lines represent the mean value estimated with Gauss-Eyring model and dashed lines the 95% confidence interval. Horizontal dashed line indicates the detection limit.

The effect of a variable pulse width at identical electric field strength of 2.7 kV/cm was studied for *E. coli, L. monocytogenes* and *S. cerevisiae* in orange juice. A clear distinction could be observed when the inactivation kinetics (curves) of the long pulse width of 1000 µs was compared to the inactivation kinetics of the shorter pulse width of 100 and 15 µs (Fig. 6.6, and Table 6.3). Statistical comparison between the temperatures required for 5 $\log_{10}$ reduction, showed no significant difference between the pulse width of 100 µs and 15 µs, for all three species tested. Comparison of these shorter pulses to a pulse width of 1000 µs, showed a significant difference for *L. monocytogenes* and *S. cerevisiae.*

Evaluation of the pulse duration at a constant electric field strength of 2.7 kV/cm showed that a longer pulse width (τ = 1000 µs) was more effective than a short pulse width (τ = 100 or 15 µs).

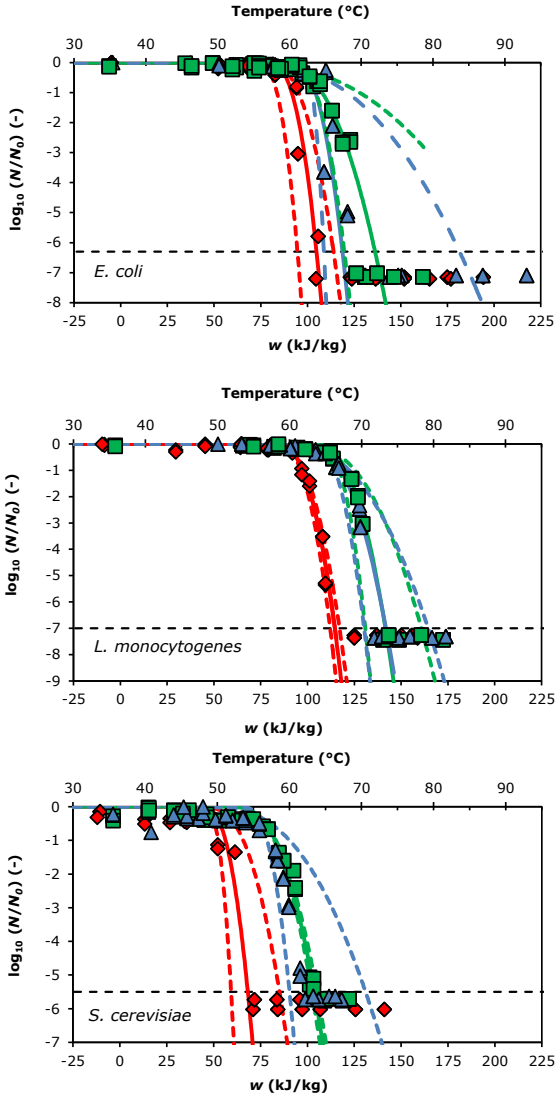


Figure 6.6. Reduction of viable counts of *E. coli, L. monocytogenes* and *S. cerevisiae* in orange juice, preheated at 36 °C and exposed to a PEF treatment with bipolar pulses at E = 2.7 kV/cm, with a duration of τ = 1000 µs (red diamonds), τ = 100 µs (blue triangles) or τ = 15 µs (green squares). Inactivation is measured as a function of maximum temperature (°C) or electrical energy applied (kJ/kg). Symbols represent measured data, continuous lines represent the mean value estimated with Gauss-Eyring model and dashed lines the 95% confidence interval. Horizontal dashed lines indicates the detection limit.

6.3.3 Comparison of PEF process towards a thermal process

To evaluate if, in addition to thermal effects, an electroporation effect in the inactivation kinetics described in the previous sections was involved, a comparison of these inactivation curves to a thermal reference process was made. At equivalent temperature-time exposure for both electrical and thermal processes, inactivation kinetics could be compared. The temperature-time profile of the PEF process included an exposure of t = 2.3 s to a selected maximum temperature (see Fig. 6.2, section D). Consequently, the thermal reference process should have the same exposure-time of t = 2.3 s to this temperature, which is too short to determine accurately experimentally. Therefore inactivation at t = 2.3 s was predicted, using Eq. 6.6 and Eq. 6.7 of the previously reported Gauss-Eyring model (Mastwijk, Timmermans & Van Boekel, 2017). Estimations of the model parameters *Tr, Z* and σ for the thermal inactivation of *E. coli, L. monocytogenes, L. plantarum, S.* Senftenberg and *S. cerevisiae* in similar orange juice were obtained from inactivation kinetics using different temperature-time combinations determined previously (Chapter 5). Calculated inactivation at t = 2.3 s is shown in Fig. 6.4 (right panel) and parameter estimates of the thermal process are given in Table 6.3 (E = 0 kV/cm). Since the PEF inactivation data is predicted with the same model as the thermal treatment described in Chapter 5, model parameters can be compared to evaluate whether an additional pulse effect is present.

Comparison of the different moderate intensity PEF treatment conditions at E = 0.9 kV/cm and E = 2.7 kV/cm and τ = 1000 µs (Fig. 6.4, middle panel) to the thermal data (Fig. 6.4, right panel) showed that the thermal inactivation kinetics, in general, can not be linked to one of the two electric field conditions used. Statistical comparison of the temperature required to obtain 5 $\log_{10}$ reduction showed that conditions of E = 0.9 kV/cm and τ = 1000 µs were significantly different from a thermal equivalent process, for all tested micro-organisms. Likewise, conditions of E = 2.7 kV/cm and pulse duration of 1000 µs showed significant differences to the thermal process, except for *L. monocytogenes.* A lower maximum temperature was required for the PEF process than for the thermal reference process, suggesting that an additional non-thermal effect was present at an electric field strength of 2.7 kV/cm and pulse width of 1000 µs.

Comparison of the high intensity PEF treated data at E = 10 kV/cm and pulse duration of 2 µs (Fig. 6.4, left panel) to the thermal data (Fig. 6.4, right panel), showed that the E = 10 kV/cm inactivation curve completely overlapped with the thermal inactivation curve (*E. coli, L. plantarum, S.* Senftenberg). Interestingly, the E = 10 kV/cm inactivation curve of *S. cerevisiae* first overlaps with the inactivation curve of E = 20 kV/cm, but at temperatures above 60 °C it overlaps with the thermal inactivation curve. For *L. monocytogenes* we only measured inactivation at E = 10 kV/cm to a maximum temperature of 70 °C, the starting point for thermal inactivation of *L. monocytogenes.* It is unpredictable how the inactivation curve of E = 10 kV/cm would proceed at temperatures above 70 °C, and therefore no comparison to the thermal reference could be made.

Statistical analysis of the other four species (*E. coli, L. plantarum, S.* Senftenberg and *S. cerevisiae*) showed no significant difference between thermal treatment and PEF conditions at E = 10 kV/cm when comparing the temperature required to obtain 5 $\log_{10}$ reduction.

PEF inactivation data at E = 20 kV/cm clearly show a more efficient inactivation curve for all tested micro-organisms when compared to the thermal inactivation curve. This observation was confirmed for all species by statistical analysis of the temperature required for 5 $\log_{10}$ reduction. Also the electric field strength of 15 kV/cm, applied to *E. coli*, showed an additional electroporation effect, that could be significantly discriminated from thermal treatment and treatment at E = 20 kV/cm.

Moderate intensity PEF inactivation data at E = 2.7 kV/cm for τ = 100 and 15 µs (Fig. 6.6) were compared towards the thermal inactivation data (Fig. 6.4, right panel) as well. Inactivation curves for the PEF process and thermal process overlapped, and statistical analysis of the temperature required for 5 $\log_{10}$ reduction showed no significant difference between the PEF and thermal processes for *E. coli, L. monocytogenes* and *S. cerevisiae.*

6.3.4 Effect of different juice matrices

Physical and chemical characteristics of the product matrix, can strongly influence the effectivity of PEF on micro-organisms (Raso, Condón & Álvarez, 2014). To evaluate if the efficiency of the condition at electric field strength of 2.7 kV/cm and pulse width of 1000 µs would also be affected by the product matrix, experiments were carried out with matrices varying in pH and conductivity (Fig. 6.1). Inactivation kinetics of *E. coli* and *L. monocytogenes* in orange juice (pH = 3.8), coconut water (pH = 5.0) and watermelon juice (pH = 6.0) were carried out.

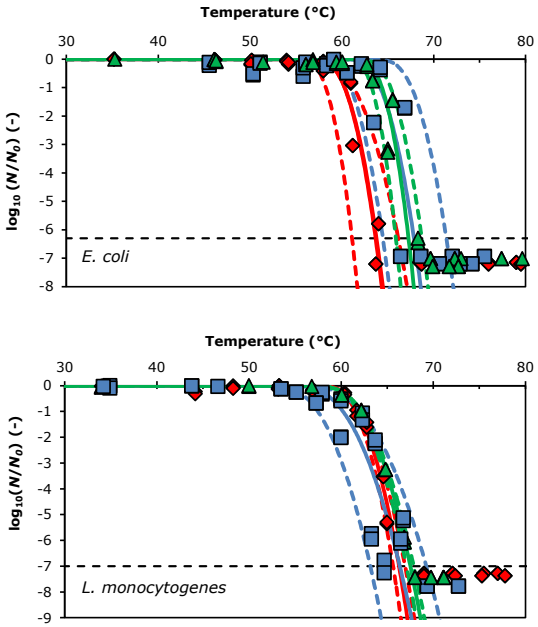


Figure 6.7. Reduction of viable counts of *E. coli* and *L. monocytogenes* preheated at 36 °C and exposed to a PEF treatment with bipolar pulses at E = 2.7 kV/cm, with a duration of τ = 1000 µs, suspended in orange juice (red diamonds), coconut water (green triangles) or watermelon juice (blue squares). Inactivation is measured as a function of maximum temperature (°C). Symbols represent measured data, continuous lines represent the mean value estimated with Gauss-Eyring model and dashed lines the 95% confidence interval. Horizontal dashed lines indicates the detection limit.

Inactivation data of *E. coli* and *L. monocytogenes* (Fig. 6.7, Table 6.3) showed that there was no significant difference in degree of inactivation between the matrices when bipolar pulses of τ = 1000 µs at E = 2.7 kV/cm were used.

Contradicting results about the effect of pH and conductivity on microbial inactivation were found in scientific literature. For example, several studies show that the pH of the matrix affects inactivation (Saldaña et al., 2010c; Wouters et al., 1999) whereas others concluded that pH was not influencing (Hülsheger, Potel & Niemann, 1981; Sale & Hamilton, 1967). Some studies show an influence of conductivity (Jayaram, Castle & Margaritis, 1993; Sensoy, Zhang & Sastry, 1997; Vega-Mercado et al., 1996; Wouters et al. 1999), while others report no effect (Álvarez et al., 2003a). The different observations and conclusions might be related to the set-up and equipment used, possibly leading to indirect effects, making it difficult to compare data. To overcome these differences, we compare the results in Fig. 6.7 with our previous study (Timmermans et al., 2014), where we used the same set-up as in this study. We observed that the efficiency of application of monopolar pulses of τ = 2 µs at an electric field strength of 20 kV/cm was clearly influenced by the pH of the product matrix, where more inactivation in high acid fruit juices was shown than in low acid fruit juices. As no difference in the degree on inactivation was observed for the PEF conditions at E = 2.7 kV/cm and τ = 1000 µs, these moderate intensity conditions might be interesting for a large group of liquid food products with neutral pH.

6.3.5 Concluding discussion

The aim of this study was to investigate if a combined process of moderate intensity PEF and heat could be used as alternative PEF condition to inactivate micro-organisms and serve as an alternative preservation process. A systematic comparison of the individual effects of moderate intensity electric field strength and pulse duration in combination with heat on microbial inactivation was carried out, and inactivation kinetics were compared to the high intensity PEF conditions and inactivation kinetics of heat only. PEF inactivation kinetics showed the importance of both higher electrical field strength and longer pulse width for the efficiency of the PEF process. Three of the seven PEF conditions tested exhibited an additional non-thermal effect next to the thermal element responsible for the inactivation: at E = 20 kV/cm and τ = 2 µs, at E = 15 kV/cm and τ = 2 µs, and at E = 2.7 kV/cm and τ = 1000 µs.

A remarkable difference between the commonly known high intensity PEF conditions at E = 20 kV/cm and the moderate intensity PEF conditions at E = 2.7 kV/cm, is their efficiency to inactivate micro-organisms in different products. Conditions at E = 20 kV/cm and pulse width of τ = 2 µs showed less microbial inactivation when the pH of the product matrix increased from acidic pH of 3.6 to a less acidic pH of 5.4 (Timmermans et al., 2014), while conditions at an electric field strength of E = 2.7 kV/cm and width of τ = 1000 µs were pH independent (Fig. 6.7). The underlying mechanism for this observation remains to be determined, but several findings reported previously triggered us to speculate about it.

Different pathways for the electroporation of biological cell membranes have been proposed, as mentioned in the introduction, where the most widely accepted theory involves the induction of an (external) electric field applied on the lipid domain of a

membrane of a micro-organism, leading to local instabilities and resulting in formation of pores in the membrane (electroporation). Due to the electroporation effect, the permeability of the cell membrane increases (electropermeabilization) and, depending on the applied electric field strength, leads to either cell death (irreversible, complete inactivation) or resealing of the cell membrane and recovery (reversible, sub-lethal or partial inactivation) (Saulis, 2010). It is assumed that at least an electric field strength of 5 – 10 kV/cm is required to cause electroporation of the microbial cell membrane irreversibly (Jaeger et al. 2016; Raso, 2016).

A study by Tsong (1990) point to an alternative mechanism targeting the protein channels, transport systems or transport pumps. The cell membrane contains many types of membrane transport systems, like pumps and specific channels for cations and anions, imbedded in the lipid bilayer, and many of these channels or pumps are voltage sensitive. The gating voltage to open a protein channel in *E. coli* is typically below 100 mV, which is considerably lower than the 1000 mV required for pore formation in the lipid double layer of the membrane (Schoenbach et al., 1997). Once a channel is forced to open by an excessive potential induced by the applied external field, it will experience an enormous local heating due to the high current density passing through the channel and as a consequence it may be thermally denatured (Tsong, 1990). Possibly the long pulses of τ = 1000 µs at an electric field strength of E = 2.7 kV/cm, denature the transport proteins, as relatively long pulses at a moderate intensity are given. Application of pulses with an intensity of E = 15 – 20 kV/cm and a duration of τ = 2 µs are likely responsible for pore formation in the lipid domain, as this is proven for red blood cells (Zimmerman, Pilwat & Riemann, 1994), model membranes (Neumann & Rosenheck, 1972) and bacteria (Hamilton & Sale, 1967).

However, it might be possible that the application of E = 2.7 kV/cm and τ = 1000 µs in this study both act on the lipid domain, and that different sized pores are formed, as pore formation is dependent on both the intensity of the pulses as well as the duration of a single pulse (Neumann et al., 1992; Tsong, 1990).

Finally, electrochemical reactions might be responsible for the inactivation at an electric field strength of E = 2.7 kV/cm using τ = 1000 µs pulses. Electrochemical reactions take place at the interface between the electrode and the liquid, and are dependent on electrode material and conditions used. Electrochemical reactions were avoided as much as possible by using titanium electrodes as they have low corrosion tendency compared to other metal materials. Furthermore, if reactions between the electrode material with the food products takes place, titanium dioxide is the most likely product to be formed, a component often used in the food and pharmaceutic industry. Electrochemical reactions in the area of the metal-liquid surface leading to surface etching are minimized by using bipolar pulses (Roodenburg, 2007), and combination of bipolar pulsed ohmic heating showed to minimize electrochemical reactions and the formation of gas bubbles compared to ohmic heating (Samaranayake, Sastry & Zhang, 2005). Samaranayake and co-workers concluded that pulsed ohmic heating at lower frequencies and longer pulse widths up towards τ = 100 µs are more effective in suppressing the electrochemical reactions of titanium electrodes. A minimum delay time of τ = 10 – 15 µs between the positive and negative pulses was found to be a critical factor to avoid hydrogen generation (Samaranayake, Sastry & Zhang, 2005).

In view of the industrial application of the PEF technology, conditions at E = 20 kV/cm and τ = 2 µs are suitable for the inactivation of micro-organisms in high-acid products, whereas conditions at E = 2.7 kV/cm and τ = 1000 µs can be applied to both high-acid and low-acid products. Furthermore, the moderate intensity conditions showed minor differences in degree of inactivation between the different microbial species tested, while high intensity conditions showed larger differences. In addition, it is expected that industrial application and scaling up of the moderate intensity electrical field strength might be easier than the application of higher electric field strength.

Taken together, the inactivation data at moderate intensity conditions E = 2.7 kV/cm and τ = 1000 µs can be used as an alternative (PEF) process for preservation of liquid food products. The lower maximum temperature required and shorter holding time when compared to a conventional thermal pasteurisation process are expected to result in a better product quality.

6.4 Appendix

An overview of the continuous-flow PEF system and liquid handling configuration is shown in Fig. S6.1. The temperature-time profile of the system and a breakdown into individual components of the liquid handling part is shown in Fig. S6.2. Heating spiral A was immersed in a water bath of 40 °C, and the two cooling spirals in section E were immersed in an ice-water bath of 0 °C. The temperature increase in the treatment chambers is a function of intensity and number of pulses applied given by Eq. 6.1 and 6.3. Thermocouples (Type T) were positioned before and after the treatment chambers and an additional NTC temperature sensor was mounted 2 mm from the exit from the grounded treatment chamber using thermal paste for a proper thermal contact (section C). A detailed description of section C (treatment chambers and electrodes) is shown in Fig. S6.3. Note that part C is variable for the 3 different configurations: I, II and III, as shown in Fig. S6.4.

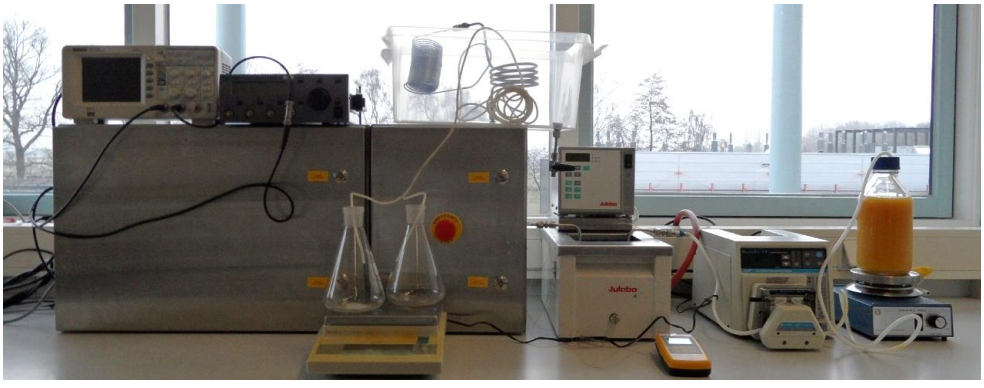


Figure S6.1. Overview of the continuous-flow PEF setup used in this thesis.

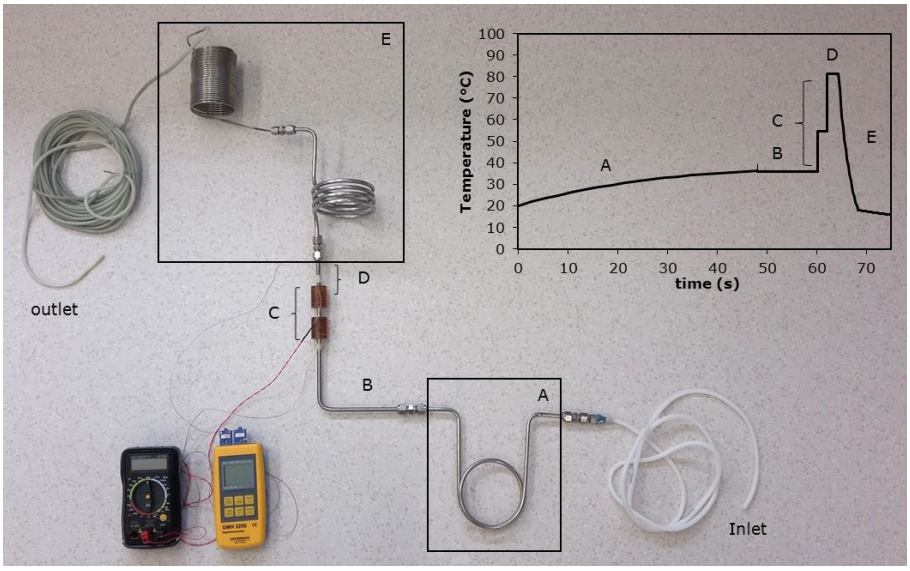


Figure S6.2. Overview of liquid handling part in continuous-flow PEF setup. Heating spiral (A) is immersed in a water bath set at 40 °C, cooling spirals (E) are immersed in ice-water of 0 °C. Note that the treatment chambers (part C) are variable for the three configurations, I, II and III. Configuration II is shown in this Figure. A 7.5 m long silicon hose with an inner diameter of 1 mm is mounted at the exit as a continuous pressure relief.

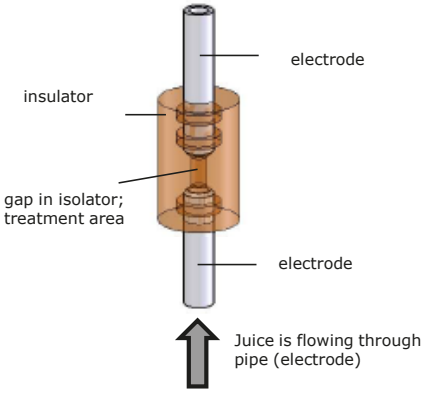


Figure S6.3. Drawing of insulator and electrode (metal pipe) in a co-linear PEF equipment. Electrodes can be positively or negatively charged or grounded. Treatment takes place in the gap of the insulator, where an electric field between the two electrodes is created.

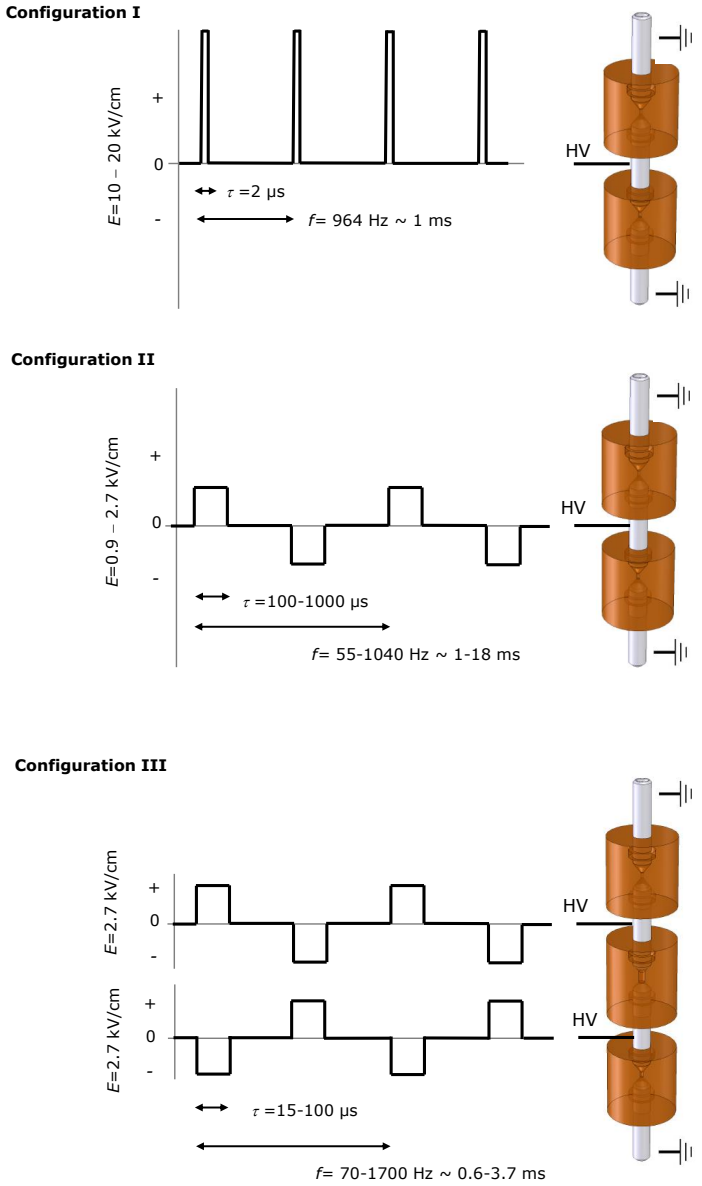


Figure S6.4. Overview and comparison of the three different PEF configurations discussed in this Chapter. High voltage (HV) and grounded electrodes are indicated in the drawing. Different voltage pulses varying in electric field strength (*E*), pulse width (*τ*), interval (frequency) and polarity (positive and/or negative) are applied to the HV electrodes during time.

In Fig. S6.5, a detailed view of the mounted components inside a stainless steel cabinet is shown. The set-up and connections to the electrodes for configuration III are displayed, the other two configurations showed similar connections. The treatment device is mounted in a supporting U-frame. Electrical connections are clipped on allowing

removal of the entire liquid handling part (Fig. S6.2) for sterilisation in an autoclave. The thermal conduction and thermal mass of the U-clips and U-frame are minimised using a poorly thermally conductive polymer for the U frame, and grounding the U clips with a wire. By using this construction more than 90% of the electrical energy is recovered as calometric heat, which provided the accuracy to allow comparison to a thermal process to discriminate between thermal and non-thermal effects responsible for inactivation. Furthermore, the time after leaving treatment chambers to cooling (section D) was made as short as possible, and yields 2.3 s at a flow rate of 13 mL/min.

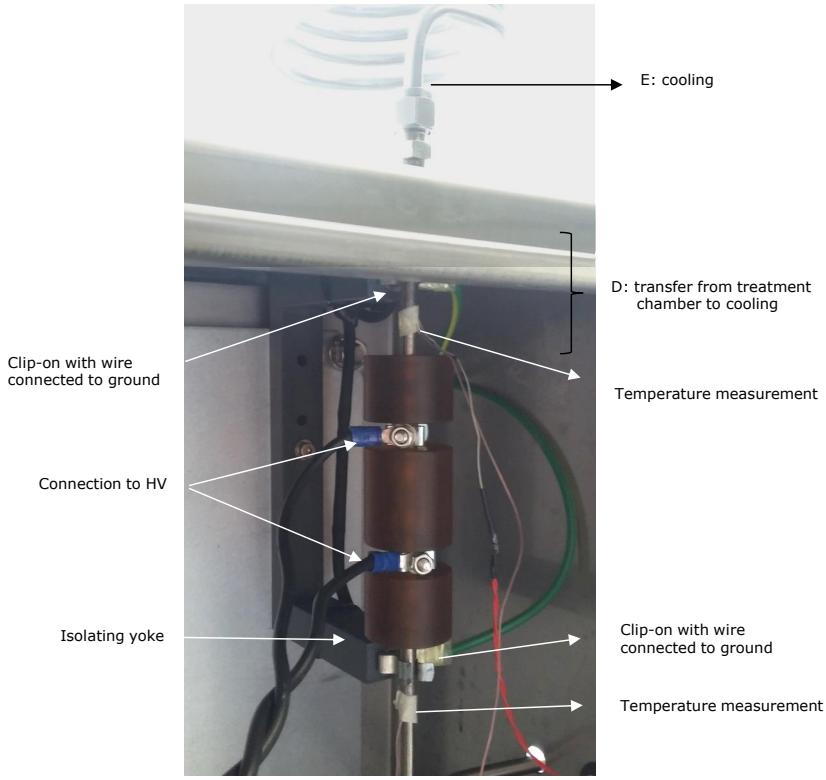


Figure S6.5. Example of lay-out and connections to electrodes, in configuration III.

Chapter 7

Effect of pasteurisation by Moderate Intensity Pulsed Electric Field treatment compared to heat treatment on quality attributes of fresh orange juice

Submitted as:

R.A.H. Timmermans, W.S.U. Roland, C. van Kekem, A.M. Matser & M.A.J.S. van Boekel. Effect of pasteurisation by Moderate Intensity Pulsed Electric Field treatment compared to heat treatment on quality attributes of fresh orange juice.

Abstract

This study described in this chapter evaluated the impact of pulsed electric field (PEF) processing at moderate electric field strength (E = 0.9 and 2.7 kV/cm) and long pulse width (τ = 1000 μs) at variable maximum temperatures on quality attributes of freshly squeezed orange juice, and compared the results to the impact of two conventional thermal processes using either mild or severe pasteurisation conditions. No differences for pH and soluble solids were found after application of any treatment, and only small differences were observed for color and vitamin C (ascorbic acid and dehydroascorbic acid) content after processing, mainly for conditions applied at higher temperature. Variations in the maximum temperatures of the PEF process and in the thermal processes led to differences in flavour compounds and the remaining activity of pectin methylesterase (PME) responsible for undesired cloud instability. Optimal PEF conditions were determined as an adequate alternative to thermal pasteurisation, including proper inactivation of pectinase enzymes, but with a better retention of quality.

7.1 Introduction

Thermal preservation is commonly applied to fruit juices to destroy spoilage and pathogenic micro-organisms as well as to stabilize the juice cloud by deactivating the pectinase enzymes naturally present in the juice. Cloud stability is an important quality parameter in orange juice, and can be affected by the impact of pectin degrading enzymes, particularly pectin methylesterase (PME) (Joslyn & Pilnik, 1961). PME de-esterifies the methyl groups on the galacturonic backbone of pectin, creates charged regions that can form complexes with Ca^{2+} and will precipitate, resulting in a clarified juice (Krop, 1974).

Conventional thermal processes for citric juice pasteurisation involve the use of temperatures of 65 – 99 °C and can be categorized as high-temperature short time (HTST) pasteurisation (T = 65 – 86 °C and holding time 1 – 43 s) and superpasteurization (T = 90 – 99 °C and holding times of 1 – 90 s) (Valero et al., 2010). Treatments with temperatures above 100 °C are considered as ultra-high-temperature (UHT) processing. The design of a thermal pasteurisation process of orange juice is dependent on the purpose of the treatment. Intense process conditions are required to inactivate PME, as PME is thermally more stable than vegetative micro-organisms (Chen & Wu, 1998). Furthermore, the type of juice to be processed plays a role in the required temperature-time combinations. Temperatures and holding times required for the pasteurisation of cloudy and pulpy juices are generally higher than those used for clear juice or juice reconstituted from concentrate before aseptic packaging (Cautela et al., 2010). Conditions at temperatures of 90 – 95 °C for 15 – 60 s are reported to be sufficient for microbial and enzymatic inactivation of single-strength juice (Chen, Shaw & Parish, 1992; Eagerman & Rouse, 1976). Braddock (1999a) reported that conditions at 90 – 99 °C for 15 – 30 s are industrially applied. When there is no need for complete PME inactivation, citrus industry usually applies moderate heat treatments of 72 °C for 10 – 30 s (Valero et al., 2010). No microbial growth was detected after heat treatment above 62 °C and holding time of 12 s (Hirsch et al., 2008).

When orange juice is heated, a complex series of chemical reactions are started involving sugars, amino acids, lipids, ascorbic acid, sulfur-containing components, phenolic compounds and peel oil components (Nagy, Rouseff & Lee, 1989). These reactions can result into a loss of fresh flavour (Perez-Cacho & Rouseff, 2008b; Tatum, Nagy & Berry, 1975), degradation of ascorbic acid (Chen, Shaw & Parish, 1992), and discolouration (Lee & Coates, 2003). To avoid quality degradation of orange juice by thermal processing, non-thermal preservation techniques such as high pressure processing and pulsed electric fields (PEF) have been studied during the last decades, as an alternative pasteurisation method aiming for a better product quality, whilst maintaining inactivation of vegetative micro-organisms.

PEF processing conditions required to inactivate pathogenic and spoilage micro-organisms in fruit juices are described in Chapter 6, using variations in the intensity of the pulse (electric field strength, E), duration of a single pulse (pulse width, τ) and maximum temperature. In chapter 6, pulse effects responsible for microbial

inactivation were distinguished from thermal effects, and two process windows were identified were pulse effects have an additional effect to the thermal effect responsible for microbial inactivation: 1) E = 15 - 20 kV/cm and τ = 2 µs, and 2) E = 2.7 kV/cm and τ = 1000 µs. The effect of the first pulse condition on quality aspects is known and described in previous work (Timmermans et al., 2011; Vervoort et al., 2011, Vervoort et al., 2012). These studies using electric field strength > 20 kV/cm and short pulses showed a good retention of colour, vitamin C content and flavour when compared to untreated juice, however no complete inactivation of PME was observed. These conditions are also applied in industry for preservation of juices (Buckow, Ng & Toepfl, 2013). The effect of the second pulse condition at E = 2.7 kV/cm, long pulse width of τ = 1000 µs and combination with heat on quality aspects is described in the present study, in order to evaluate whether these conditions can be a suitable alternative to conventional heat pasteurisation, including a complete inactivation of PME, but with better retention of quality.

The aim of the present study was to compare the impact of PEF processing at reduced electric field strength, long pulse duration and varying maximum temperatures between 45 – 90 °C to two conventional thermal processes, one at mild and another at more intense conditions in freshly squeezed orange juice by studying the quality attributes pH, brix, colour, ascorbic acid content, volatile flavour compounds and PME activity.

7.2 Material and methods

7.2.1 Juice preparation

Oranges of the variety Salustiana (grown in Spain) were delivered in boxes, stored at 4 – 6 °C and divided over two batches (juice A and juice B) before juice was squeezed on two different days using a commercial juice extractor (Speed pro self-service, Zumex, S.A., Spain). The juice was sieved (0.225 mm pore size) to remove the large pulp particles, mixed to make a homogeneous batch to avoid differences between individual oranges, divided and allocated to closed bottles and stored at 2 – 4 °C prior to processing on the same day.

7.2.2 PEF processing

A continuous-flow PEF process at lab-scale, described in Chapter 6 as configuration II, was used for electric field treatment. The juice was pumped at a flow rate of 13 ± 1 mL/min, and preheated to 45 ± 1 °C before PEF treatment to reduce the electric input required for the total process. Bipolar square wave pulses with a pulse width of τ = 1000 µs with variable pulse repetition (frequency) were given to heat up the product to a maximum temperature ranging between 45 and 90 °C, with intervals of 3 °C (see Table 7.1 for the frequency range used). The experiment was performed twice, once with an electric field strength of 2.7 kV/cm (juice A) and the second time at an electric field strength of 0.9 kV/cm (juice B). The voltage applied by the system was set at 540 V, and two co-linear treatment chambers made of polyetherimide (PEI, Ultem™ resin) with variable dimensions were used to obtain the desired electric field strength. Table

7.1 provides the dimensions of the treatment chambers and characteristic parameters of the PEF treatment. The electrodes were made of titanium.

Samples were collected in opposite orders: for juice A from the highest temperature possible (75 °C) to the lowest temperature (45 °C), and for juice B from the lowest temperature (45 °C) to the highest temperature (90 °C). Samples were collected after the cooling section and coded with the maximum temperature they received by the PEF-process. An example of a typical temperature-time profile of the PEF treatment is shown in Fig. 7.1-I. Juice with only preheating and no PEF treatment was used as a PEF-control (coded as '45 °C') and juice with no preheating nor PEF treatment was a general control (coded as 'untreated-start' when taken at begin of the day and coded as 'untreated-end' when taken at end of the day).

Pulse waveform, voltage and current traces were recorded to monitor the intensity in the treatment chambers using a digital oscilloscope (Rigol DS1102E). Temperatures at the inlet and outlet of the treatment chambers were measured with mini-hypodermic thermocouples (HYP-O-T-Type, Omega) and NTC thermistor monitor. The energy input and caloric power measured in the bulk were calculated according to equations given in Chapter 6. Specific electrical energy applied by the PEF treatment is given in Table 7.1, and energy balance between energy input and caloric power measured in the bulk were equal to each other within experimental error (< 5%), with absolute deviations of 0.5 – 3 °C based on maximum temperature.

Table 7.1. Characterizations of the pulse conditions and configurations used to apply a moderate intensity PEF treatment at a flow rate of 13 mL/min.

Electric Field Strength (kV/cm)	Pulse width (µs)	Dimensions of treatment chamber (diameter x length) (mm)	Residence time (ms)	Pulse repetition frequency (Hz)	Number of pulses applied	Specific electrical energy use (kJ/kg)	Tmax (°C)
0.9	1000	4.0 x 6.0	696	0 – 48	0 – 67	0 – 171	45 – 90
2.7	1000	1.0 x 2.0	14	0 – 112	0 - 3.2	0 - 114	45 – 75

7.2.3 Thermal processing

Thermal processing of the orange juices A and B was carried out at continuous-flow conditions at lab-scale. The orange juice was pumped at a flowrate of 22 ± 1 mL/min, preheated to the desired T_{max} of 72 °C or 95 °C in t = 43 s by heating through a heat spiral (SS-316, diameter 4 mm) that was immersed in a water bath at T = 75 °C or 97 °C, followed by a holding section at the desired temperature in a heat spiral (SS-316, diameter 1 mm) for 20 s, and directly cooled down in a cool spiral (SS-316, diameter 2 mm) immersed in a water bath at T = 20 °C for t = 23 s, followed by a cooling section in ice-water (Silicone tube, 1 mm) for t = 90 s, to obtain a product outlet temperature of about 10 °C. A heating profile of the thermal treatment is shown in Fig. 7.1-II. Treatment conditions were a mild thermal treatment (72 °C – 20 s) (LH) and a severe thermal treatment (95 °C – 20 s) (HH), based on conditions documented to be used in industrial practice (Braddock, 1999a; Tetra Pak, 2004; Valero et al., 2010).

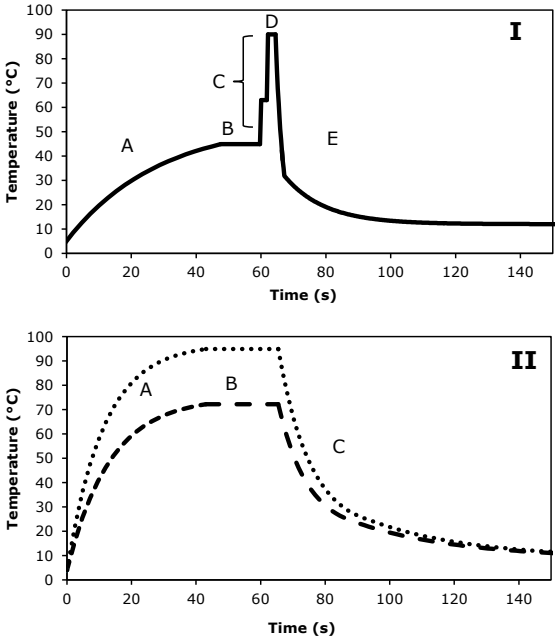


Figure 7.1. Example of temperature-time profile of the moderate intensity PEF treatment (I) and thermal treatment (II) of orange juice.
Panel I). Orange juice was preheated from T = 4 °C to 45 °C in 48s (A), maintained at T = 45 °C for t = 12 s (B) before entering the treatment chambers, heated up in the first treatment chamber, followed by a pause of t = 1.7 s, the time to go to the second treatment chamber, and heated up to a desired maximum temperature, i.e. T = 90 °C (C). Than orange juice is transferred to cooling section in t = 2.3 s (D) and cooled down for t = 122 s to temperatures < 10 °C (E). Panel II). Orange juice was preheated from T= 4 °C to 72 °C for low thermal treatment or to T = 95 °C for high thermal treatment in t = 43 s (A), maintained at the desired temperature for t = 20 s (B) and cooled down for t = 113 s to temperatures < 10 °C (C).

7.2.4 Sampling and storage of samples

For each treatment condition, five samples per quality attribute were collected in appropriate tubes, and immediately stored on ice after collection. All quality attributes were analysed in triplicate, by measuring a single analysis per collected sample tube, meaning that for each attribute 2 spare samples were stored. Samples for flavour analysis were directly prepared in vials, capped and stored at -80 °C. Samples for vitamin C content and enzyme analysis were stored directly in -80 °C as well. Other samples for colour, pH and brix were stored overnight at 4 °C and analysed the day after production.

7.2.5 Dissolved oxygen, conductivity, pH and soluble solids

The amount of dissolved oxygen in the orange juice was measured prior to processing, using a handmeter ExStik II DO600 (Extech instruments, USA) at a temperature of 7 °C. The electrical conductivity of the orange juice was measured with a conductivity meter (Greisinger GMH 3430). The pH of the orange juice (19.5 °C ± 1°C) was measured with a pH meter (827 pH lab, Metrohm) with a glass electrode (Metrohm) and calibrated

before every series of measurement at pH 4 and 7. The amount of soluble solids of the orange juice samples was measured by a digital hand-held refractometer (PR-1, Atago, Japan) and measured as °Brix at temperatures of 19.5 °C ± 1 °C.

7.2.6 Colour measurements

Colour measurements were performed using a Hunterlab ColorFlex spectrophotometer (Hunterlab, Reston, Virginia, U.S.A.). An illuminant of D_{65} and a 10° angle were used as observer. Standardization and measurement of the samples was similar to previous work (Timmermans et al., 2011). CIE *L*, a** and *b**-values were measured, representing *L** (lightness), ranging from 0 (black) to 100 (white), *a** quantifying greenness (negative) to redness (positive) and *b** quantifying blueness (negative) to yellowness (positive). Colour difference, ΔE, was calculated from *L**, *a**, and *b** parameters, using Hunter-Scotfield's equation (Eq. 7.1), where $a = a - a_0$, $b = b - b_0$ and $L = L - L_0$. The subscript '0' indicates initial colour of untreated-start.

$$\Delta E = \left(\Delta a^2 + \Delta b^2 + \Delta L^2\right)^{1/2} \quad \text{(Eq. 7.1)}$$

ΔE was calculated for each treatment condition, and dependent on the value of ΔE the sample was considered to have no noticeable difference (0 – 0.5), slightly noticeable (0.5 – 1.5), noticeable (1.5 – 3.0), well visible (3.0 – 6.0) or great (6.0 – 12.0) (Cserhalmi et al., 2006).

7.2.7 Vitamin C content

The extraction and analysis of the total vitamin C content was measured mainly according to the method described by Vervoort et al. (2011), and is the sum of ascorbic acid (AA) and its oxidized form, dehydroascorbic acid (DHAA). Some modifications to the method of Vervoort et al. (2011) were made and described below. In short, a dual detection system was used after HPLC separation, by which AA was directly detected by UV, and DHAA indirectly by fluorometric detection after a post-column on-line derivatization with o-phenylenediamine. AA and DHAA were extracted from juice by dilution of 2.5 mL in 1% (w/v) metaphosphoric acid with 0.5% (w/v) oxalic acid dihydrate, adjusted to pH 2.0 using 10 M NaOH-solution, to a volume of 2.5 mL. After homogenization, the samples were flushed with nitrogen and centrifuged for 10 min at 3,220 x *g* at 4 °C. The supernatant was filtered over a 0.2 µm syringe filter and diluted 1:1 with acetonitrile, to improve the stability of DHAA (Bognár & Daood, 2000). An aliquot of 5 µL was used for injection, and vials were stored in dark sample compartment at 4 °C before injection. Calibration curves were prepared from standard solutions, and composed of AA (10-250 µg/mL) and DHAA (1.25-15 µg/mL) in the extraction buffer, with a total volume of 10 mL. Stock solution of 1 mg/mL AA was made, and DHAA standard was prepared from the AA stock solution as described by Vervoort et al. (2011). Standard solutions were diluted 1:1 with acetonitrile. The analytical HPLC column used was a Waters symmetry C18 (250 x 4.6 mm, 5 µm particle size) with Spherisorb ODS2 guard cartridge (Waters). Mobile phase, post-column reagent and pump conditions were identical to Vervoort et al. (2011). AA was detected with a UV-detector set at λ_{max} = 263 nm, DHAA was detected by a fluorescence detector set

at excitation and emission wavelength 250 and 430 nm, respectively. Run time was 15 min for both analyses and AA was measured at retention time of 7.2 min and DHAA at retention time of 4.1 min.

Reproducibility of AA and DHAA in orange juice samples and standard solutions was evaluated and optimized beforehand. To equilibrate the system, 10 pre-injections of a standard, composed of 30 µg/mL AA and 5 µg/mL DHAA were carried out on a daily basis, followed by the calibration curve and a set of 10 orange juice samples. Measurements of the calibration curve and a set of 10 orange juice samples were repeated another three times and completed with the calibration curve (so in total 40 samples could be measured per day). The AA and DHAA content was quantified using bracketing calibration, based on peak area of the standards. Orange juice samples were measured over a couple of days in random order. Samples were thawed on the day of analysis to a maximum temperature of 10 °C.

7.2.8 PME activity

Pectin methylesterase (PME) activity was measured based on the method described by Redd, Hendrix & Hendrix (1986) by measuring the release of acid during pectin hydrolysis as a function of time at pH 7.0 and temperature of 35 °C. The reaction mixture consisted of 1 – 14 mL of orange juice sample (amount depended on estimated enzyme activity, with 1 mL for untreated and 14 mL for most intense thermal conditions) and 100 mL of pectin-salt solution (10 g/L citrus pectin (Alfa Aesar, J61021) and 12.35 g/L NaCl in demi water, stirred for 16 h). The pH of the juice-pectin-salt solution was rapidly brought to pH = 7.1 ± 0.05 using 0.5 M NaOH of 35 °C. During pectin hydrolysis, the pH was maintained constant at pH 7.0 by addition of 0.01 M NaOH using an automatic pH-stat titrator (Metrohm). Minimal measured time was 10 min and maximum measured time was 60 min, dependent of the slope.

Enzyme activity (PEu) was related directly to the amount of NaOH added per minute, as was calculated according to Eq. 7.2 (Redd, Hendrix & Hendrix, 1986).

$$\mathrm{PEu} = \frac{\text{ml NaOH x N of NaOH}}{\text{ml sample x time in min}} \qquad \text{(Eq.7.2)}$$

Orange juice samples were measured over a number of days in random order, and the samples to be analysed were thawed on the day of analysis, to a maximum temperature of 10 °C. Adequate measurements of the same untreated sample at the start of the day, after every three titrations and at the end of the day gave the possibility to correct for small deviations in PEu measured over time and between the two titrators. The measured PEu-value was multiplied by a scale factor; this factor was determined by division of the daily average of the untreated samples per titrator by the total average of the untreated samples.

7.2.9 Volatiles

The flavour compounds in the orange juice headspace were analysed by a combination of Solid Phase Micro Extraction (SPME) and gas chromatography mass spectrometry

(GC-MS), mainly according to the method described by Vervoort et al. (2012). Prior to the experiments, pre-tests were carried out to select an internal standard, that did not interact with orange juice, showed no overlap with other peaks in the chromatogram, was soluble in water and stayed stable over time in the -80 °C freezer. Menthone showed to meet these criteria. Menthone solution (Sigma Aldrich) was prepared (daily fresh) in water in concentration 1 µL/100 mL water. The fresh orange juice or just treated orange juice was immediately transferred in a glass vial of 10 mL, filled with 3 mL juice, 3 g NaCl and 1.5 mL (daily fresh) menthone solution (internal standard), and capped using crimp-seals with a PTFE/silicone septum. The vials were stored in the freezer at -80 °C until GC-analysis.

After controlled thawing, to a maximum temperature of 10 °C, vials were homogenized and placed in the cooling tray (7 °C) of the autosampler. The headspace analyses were conducted on a Trace GC (Thermo Finnigan) gas chromatograph (GC) coupled to a DSQ-II (Thermo Scientific) mass spectrometer (MS) which was equipped with a TriPlus (Thermo Scientific) autosampler. In a first step, each vial was equilibrated in the incubator at 40 °C for 20 min under agitation. Absorption and desorption conditions to a pre-conditioned solid phase micro-extraction (SPME) fibre with 85 µm carboxen/polydimethylsiloxane (CAR/PDMS) absorptive coating (StableFlex, Supelco) were identical to Vervoort et al. (2012). Thermal desorption and thermal cleaning of the fiber after each extraction was similar to the protocol of Vervoort et al., 2012, with modification that cleaning was 40 min at 300 °C in this study. The volatiles were injected in split mode (1/10) and subsequently separated on a ZB-5MS column (30 m x 0.25 mm id, 0.25 µm film thickness, Phenomenex, Torrance, CA, USA). Carrier gas, flow rate and column oven temperature and time were identical to Vervoort et al. (2012). Mass spectra were obtained by electron ionization at 70 eV, with a scanning range of 35 - 400 m/z. The MS ion source temperature was 230 °C.

The SPME fiber was checked for damage after every run and was replaced when necessary. Damages occurred due to the relatively aggressive nature of some orange juice volatiles. The GC-MS analyses were performed in triplicate, where three sample vials for each tested condition were taken, and the analyses of all samples were performed in random order. The GC-MS total ion chromatograms obtained were evaluated and peak areas were calculated using Xcalibur software (version 2.2). The peak areas were normalized by the peak areas of the internal standard menthone of the individual runs and corrected for the average internal standard peak areas of all juice A and all juice B runs. The reproducibility of the analyses was assessed by calculation of the standard deviation of the normalized peak areas of the respective compounds.

Identification of the peaks was performed by comparing the components' mass spectra with the reference mass spectra from the NIST Mass Spectral Library (NIST version 2.0). A match factor of > 800 was set as limit for identification. However, in some cases, comparison of the mass spectra was not sufficient to unambiguously assign a compound name to a peak. Therefore, measurements of series of alkanes (C_8-C_{20}, Sigma Aldrich) were performed to enable the calculation of a retention index (RI) for each compound (Kováts, 1958). The calculated RI was allowed to deviate maximally by 20 units from RIs found in literature published for the retention on the same column

material (Acree & Arn, 2004; Bylaite & Meyer, 2006; Dharmawan et al., 2007). External reference compounds 2-hexenal and octanal were purchased from Merck (Hohenbrunn, Germany), β-myrcene from Janssen Chimica (Beerse, Belgium), α-pinene and β-pinene from Fluka (Buchs, Switzerland), and sabinene, 3-carene, and ocimene from Sigma-Aldrich (Steinheim, Germany), and measured. The retention times and mass spectra of the respective peaks in the orange juice chromatograms were compared to those of the standard compounds to confirm peak identity.

7.3 Results and discussion

7.3.1 Dissolved oxygen and electrical conductivity

The amount of dissolved oxygen in orange juice was measured after extraction and sieving, before processing, and was 4.0 ± 1.0 ppm at 7 °C. The amount of dissolved oxygen was measured at intervals for five hours, and the value did not change over time. This amount of dissolved oxygen can be considered as low to normal when comparing the values to reported conditions for plant operations, where an amount of 6.5 ppm is considered as normal while an amount of 1.8 ppm represents a commercial deaerated orange juice (Trammel, Dalsis & Malone, 1986). As commercial degassing has a larger effect on volatile losses than a pasteurisation step (Jordán, Goodner & Laencina, 2003), no additional degassing step to reduce more oxygen was carried out in this study, as we wanted to examine the effect of different pasteurisation processes on flavour compounds. The electrical conductivity was 0.60 S/m at 45 °C for untreated juice A, and 0.53 S/m at 45 °C for untreated juice B.

7.3.2 pH and soluble solids

The quality of citrus juice is typically determined by the balance of sweetness and tartness, with sugars and acids as main contributors (Braddock, 1999b). The total soluble solids (expressed as °brix) and the acidity or pH of oranges varies for different species, stages in maturity and during the season (Harding, Winston & Fisher, 1940). The aim in this study was to evaluate if processing, either by moderate intensity PEF and long pulse duration or thermal treatment, changes these values compared to untreated juice. For both juices A and B, the values for soluble solids of PEF treated and thermal treated juice were not different from untreated juice, being 11.1 ± 0.1 °Brix for juices A and 11.6 ± 0.1 °Brix for juices B, which can be considered as a normal value (Bull et al., 2004; Hartyáni et al., 2011; Nagy & Smoot, 1977; Timmermans et al., 2011). Also the pH value for juice A and B did not change by thermal or PEF treatment, and values for juices A and B were pH = 3.51 ± 0.01 and pH = 2.99 ± 0.02, respectively. The pH of juice A was comparable to values often reported for orange juice, with a pH typically ranging from 3.4 to 4.3 (Bull et al., 2004; Hartyáni et al., 2011; Nagy & Smoot, 1977; Timmermans et al., 2011; Wibowo et al., 2015), so the pH of juice B can be considered as rather low.

The differences in soluble solids and pH between the batches A and B in untreated orange juice might be related to individual differences between oranges. A pre-test

carried out with oranges from the same delivery, showed large differences in soluble solids and pH of juice made from individual oranges, ranging between 9.4 – 11.6 °Brix and pH 3.08 – 3.40. It was therefore decided to make one large, pooled batch of orange juice per day to be able to compare only processing effects. Despite the intention of this approach, some differences in soluble solids and pH of the untreated batch A and batch B were obtained, making it difficult to compare the effect of a varying electrical field strength on quality attributes, as the initial quality of untreated juice was different and seemed to play and important role, as will be discussed in the next sections. Differences in pH and soluble solids initiated by processing were not expected, as samples were only analysed the day after production and not stored over a period of time, so the possibly surviving micro-organisms did not have the opportunity to start an organic acid fermentation and reduce the pH.

7.3.3 Colour

The colour of juice is one of the first quality factors that a consumer judges, and has a remarkable influence on its acceptance. Colour is also an indicator of the changes that naturally occur in a fresh food or of the changes during processing or storage. In the case of fresh orange juice, the typical colour is generally a mixture of pigments and carotenoids (Meléndez-Martínez, Vicario, & Heredia, 2007b). Colour was measured instrumentally, and Hunter L^*, a^* and b^*-values of the untreated and treated orange juice are shown in Fig. 7.2. Lightness (L^*-value) was different for the two batches of untreated orange juice, with values of 50.4 ± 0.2 and 48.8 ± 0.1 for batch A and B, respectively. Only preheating of the juice to 45 °C, before PEF treatment, already led to an increase of the L^*-value for about 1 point. Further temperature increment by the PEF or thermal process slightly increased the L^*-value for juice A, whereas this trend was not observed for juice B. These small changes are in agreement with results of Lee & Coates (2003) who observed an increase in L^*-value after pasteurisation of orange juice, which might be attributed to partial precipitation of unstable, suspended particles in the juice (Genovese, Elustondo & Lozano, 1997). This small difference in L^*-value might (also) be introduced by the pumping of the orange juice, as the difference in L^*-value was observed in the 45 °C samples as well. Redness (a^*-value) was different for the two batches of untreated orange juice, with values of 3.7 ± 0.2 for batch A and 2.5 ± 0.2 for batch B. Moderate intensity PEF and thermal processing of the juice did not change the a^*-value for juice A, while a slight decrease of this a^*-value (reduction of the redness) was visible for PEF and thermal treatment of juice B.

The values for yellowness (b^*-value) for the two batches were comparable for untreated juice, with values of 60.3 ± 0.3 for juice A and 59.9 ± 0.2 for juice B. However, the batches responded differently to the treatments applied. Preheating of juice A to a temperature of 45 °C increased the b^*-value with 1 point, but further temperature increase with the PEF treatment did not change the b^*-value. Thermal treatment led to a further increase of the b^*-value. Contrary, preheating to 45 °C led to a decrease in b^* value for juice B. An increase of the b^*-value of PEF treated samples was visible at maximum temperatures of 75 °C or higher, but this temperature effect was not visible in thermal treated juice. Results of the increase of the temperature dependent increase

of the *b**-value in juice A, are in agreement with results of Lee & Coates (2003) who observed an increase of the *b**-value after pasteurisation as well.

Although small deviations and trends for the juices in specific *L**, *a** and *b**-values were observed, it is rather difficult to draw conclusions on the overall impact of the processing on colour. It is more valuable to compare the total change in *L**, *a** and *b**-values, expressed in ΔE values, as consumers do not judge each particular attribute, but the combination of them (Cserhalmi et al., 2006). The ΔE values were calculated according to Eq. 7.1, and results are shown in Fig. 7.2. Preheating of orange juice to T = 45 °C gave a slightly noticeable change in colour for both juice A and juice B. Further temperature increment of the PEF treatment led to higher values of ΔE, which is mostly considered as a 'slightly noticeable' effect. Only maximum temperatures of PEF between 72 °C – 78 °C were characterized as 'noticeable' different. Further temperature increment as given by the thermal treatments showed a more 'noticeable' difference for juice A, where this was reduced for juice B. It can therefore be concluded that the application of the moderate intensity PEF treatment gave a (slightly) noticeable effect on colour compared to untreated juice.

7.3.4 Vitamin C

Vitamin C content is an important attribute to orange juice, as it has a large contribution to human nutrition. Although is it not the only fruit product containing vitamin C, it is certainly an important source, as the vitamin C content in orange juice is high, as is the consumption rate of orange juice by humans (Rampsersaud & Valim, 2017; Ting, 1980). Variations in vitamin C content can be found in different citrus products due to factors such as variety, maturity and cultural practices of the fruit (Harding, Winston & Fisher, 1940), but also due to processing practices and storage conditions of these products before they reach the consumer (Ting, 1980).

The vitamin C content was determined by measuring the concentration of ascorbic acid (AA) and dehydroascorbic acid (DHAA), together forming the vitamin C content, and values of untreated and processed orange juice for AA and DHAA are shown in Fig. 7.3. AA content for untreated juice was 511.5 ± 22.0 mg/L for juice A and 579.3 ± 20.9 mg/L for juice B, and DHAA content was 8.1 ± 0.7 mg/L for juice A and 6.6 ± 0.4 mg/L for juice B. According to various references, this orange juice can be considered as a rather rich source of vitamin C (Bull et al., 2004; Klimczak et al., 2007; Meléndez-Martínez et al., 2007a; Ting, 1980; Vervoort et al., 2011). Comparison of the amount of AA present in the samples before and after pasteurisation (either by the moderate intensity PEF process or thermal treatment) did not show any change introduced by the pasteurisation process applied (Fig. 7.3-I). Probably the low pH of the juice and low amount of dissolved oxygen were responsible for this AA retention, as these factors are known to be important factors to stabilize the AA (Combs & McClung, 2017).

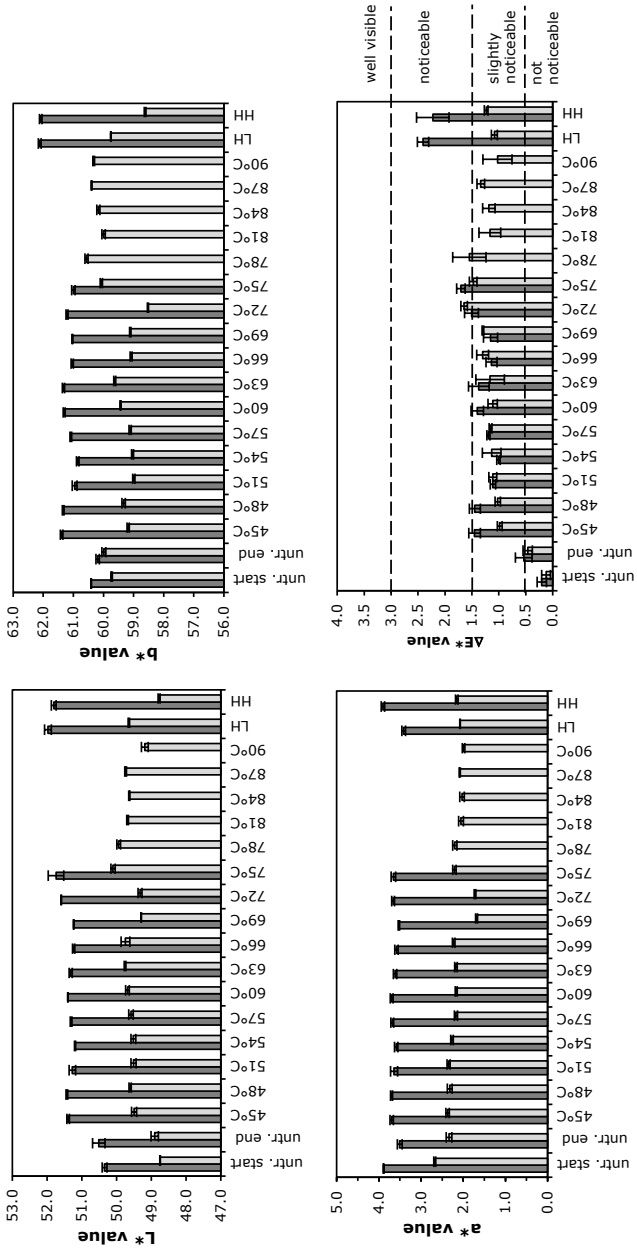


Figure 7.2. Effect of processing on colour of orange juice, according to Hunterlabs L^*, a^* and b^*-value and total colour difference (ΔE). Dark grey bars represent juice A, light grey bars represent juice B. Values of untreated juice at start and end of the day are given. Results of various moderate intensity PEF treatment are given according to the maximum temperatures applied, using an electric field strength of 2.7 kV/cm (dark grey bars) and 0.9 kV/cm (light grey bars). Thermal treatments are indicated as LH for low heat treatment (75 °C – 20s) and HH for high heat treatment (95 °C – 20s). Bars represent the average of three measurements with standard deviation.

Contrary, changes were detected for DHAA, which concentration increased when treatment temperature increased, either applied by the PEF process or by the thermal treatment (Fig. 7.3-II). DHAA is the first oxidation product of AA, and its levels can increase when exposed to high temperatures, light or oxygen, neutral pH, oxidases as well as to the presence of some traces of heavy metal ions. DHAA still exhibits vitamin C activity, since it can be reconverted into AA in the human body (Combs & McClung, 2017). However, this compound is unstable and most of it is lost as diketogulonic acid, which has no vitamin C activity (Combs & McClung, 2017).

The concentration of DHAA formed in our study was rather low compared to the concentration of AA. Reduction in the AA concentration was therefore not noticed within experimental error, as the standard deviations in AA measurements were larger than the concentrations of DHAA formed. The total amount of vitamin C, being the sum of AA and DHAA, did not change by any process or specific process condition used, either by moderate intensity PEF treatment or thermal treatment.

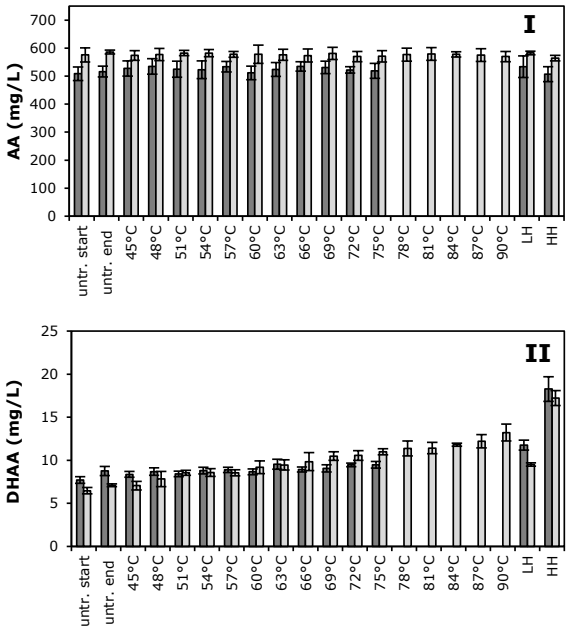


Figure 7.3. Effect of processing on ascorbic acid (AA) (panel I) and dehydroascorbic acid (DHAA) (panel II) concentration in orange juice. Dark grey bars represent juice A, light grey bars represent juice B. Values of untreated juice at start and end of the day are given. Results of various moderate intensity PEF treatment are given according to the maximum temperatures applied, using an electric field strength of 2.7 kV/cm (dark grey bars) and 0.9 kV/cm (light grey bars). Thermal treatments are indicated as LH for low heat treatment (75 °C – 20 s) and HH for high heat treatment (95 °C – 20 s). Bars represent the average of three measurements with standard deviation.

7.3.5 PME

Pectin methylesterase (PME) is generally responsible for cloud loss of orange juice by de-esterification of pectin (Krop, 1974), and it is therefore important to completely inactivate this enzyme to make shelf life stable products. The remaining enzyme activity of PME after the different treatments was determined, and results are shown in Fig. 7.4. The activity unit of PME (expressed as PEu) is defined as the amount of enzyme that releases 1 µmole of carboxylic acid groups in 1 mL of solution for one minute.

Processing clearly reduced enzyme activity, where higher maximum temperatures of the moderate intensity PEF or thermal process led to more reduction of the PME activity. A difference in PME activity between the two PEF processes and batches A and B was observed, mainly in the PEF treated samples with conditions up to a maximum temperature of 63 °C. PEF conditions applied with maximum temperature between 66 °C and 75 °C showed no difference between the processes and batches A and B, and no difference between the batches A and B were observed for the thermal treatments applied. The degree of inactivation and differences between batches A and B might be explained by different PME isoenzymes present. Orange juice comprises of multiple forms of pectin esterase, known as isoenzymes, with different kinetic properties (Versteeg, 1979), that can generally be classified into heat-labile PME and heat-stable PME. Typically, orange juice consists of 90 % of the heat-labile PME, which is destroyed at temperatures below or near 70 °C, and 10 % of the heat-stable PME that is destroyed around temperature of 90 °C or higher, dependent on holding times used (Eagerman and Rouse, 1976; Rotschild, Van Vliet & Karsenty, 1975; Snir et al., 1996). Based on the PME activity shown in Fig. 7.4, juices A and B contained the same degree of heat-stable PME fraction, being 11.1 % at maximum temperature of 69 °C for both juices. The thermo-labile fraction of juice B treated at 0.9 kV/cm was more temperature-sensitive than thermo-labile fraction of juice A treated at 2.7 kV/cm. The slight difference in pH content between juice A (pH = 3.5) and B (pH = 3.0) might play a role here, as pH plays an important role in the temperature requirements to inactivate PME. When the pH of juice was decreased, the susceptibility of the PME enzyme to heat inactivation increased (Eagerman & Rouse, 1976; Kew et al., 1957; Nath & Ranganna, 1977; Rotschild, Van Vliet & Karsenty, 1975; Van den Broeck et al., 1999), leading to more inactivation at equal maximum temperatures. Rouse & Atkins (1953) reported that by adjusting the juice to values below pH = 3.5, even temperatures as low as 50 °C resulted in 20 – 40% PME inactivation.

Juice is considered commercially stable when the remaining PME activity is smaller than 1×10^{-4} PEu/mL (Braddock, 1999a; Holland, Reeder & Pritchett, 1976; Rothschild, Van Vliet & Karsenty, 1975). This threshold value is indicated in Fig. 7.4 with a dashed line to facilitate an easier comparison. The results of this study showed that mild thermal treatment did not meet this specification, but intense thermal treatment did meet this criterion, as expected on forehand based on the temperature-time requirements reported for the thermal inactivation of the resistant PME fraction (Eagerman & Rouse, 1976). More interesting is that moderate intensity PEF treatments with long pulse duration at maximum temperatures of 78 °C or higher meets these criteria as well. Although this temperature of 78 °C is much lower than the intense thermal treatment applied at 95

°C, we cannot quantify if there is a beneficial 'pulse' effect of the moderate intensity PEF treatment compared to the conventional thermal treatment, as only two thermal treatment conditions were evaluated and one of them is below the desired threshold. PEF studies carried out using high intensity pulses claim that enzyme inactivation is predominantly caused by thermal effects (> 90 %), rather than by the voltage pulses themselves (Jaeger et al., 2010; Van Loey, Verachtert & Hendrickx, 2002), which seems to be confirmed in the present study using pulses with moderate intensity.

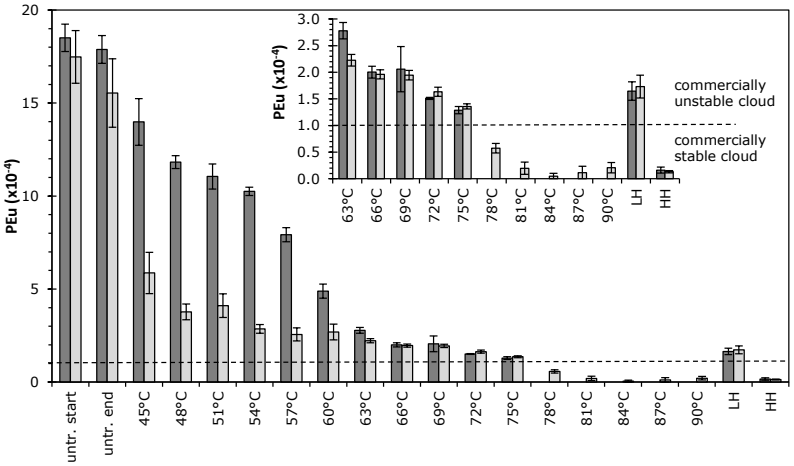


Figure 7.4. Effect of processing on remaining pectin methylesterase activity, expressed as pectin esterase units (PEu) in orange juice. Dark grey bars represent juice A, light grey bars represent juice B. Values of untreated juice at start and end of the day are given. Results of various moderate intensity PEF treatment are given according to the maximum temperatures applied, using an electric field strength of 2.7 kV/cm (dark grey bars) and 0.9 kV/cm (light grey bars). Thermal treatments are indicated as LH for low heat treatment (75 °C – 20 s) and HH for high heat treatment (95 °C – 20 s). Bars represent the average of three measurements with standard deviation. Juice cloud is considered commercially stable when PEu values are below 1.0 x 10^{-4}.

7.3.6 Volatiles

Flavour is one of the most important attributes of orange juice, and processing is known to irreversibly change the 'fresh-like' attributes of the juice in a negative way (Braddock, 1999a). Early research showed that the unique flavour of orange juice could be ascribed to a couple of specific aldehydes, but extensive analytical studies during the past decades showed that a mixture of several compounds in the proper proportions and concentrations is necessary for a good flavour (Shaw & Wilson, 1980; Perez-Cacho & Rouseff, 2008a). The perceived flavour is a combination of volatile aroma compounds and non-volatile taste compounds that can interact with each other and with the matrix. Processing of orange juice will affect the volatile aroma compounds much more than the non-volatile taste compounds. Therefore the focus of the current study lies in the investigation of the relative amounts of volatile compounds and their changes upon processing.

The volatile compounds from the headspace of the orange juice were extracted by solid-phase micro extraction (SPME) and subsequently separated with gas chromatography (GC) and identified by mass spectrometry (MS). A total of 34 compounds was analysed in this study. A typical chromatogram is shown in Fig. 7.5. A relatively low number of esters, aldehydes and alcohols was detected in juice A and juice B, no ketones, ethers and acids were detected at all, whereas a relative high number of terpenes was detected in the juice when compared to literature (Perez-Cacho & Rouseff, 2008a).

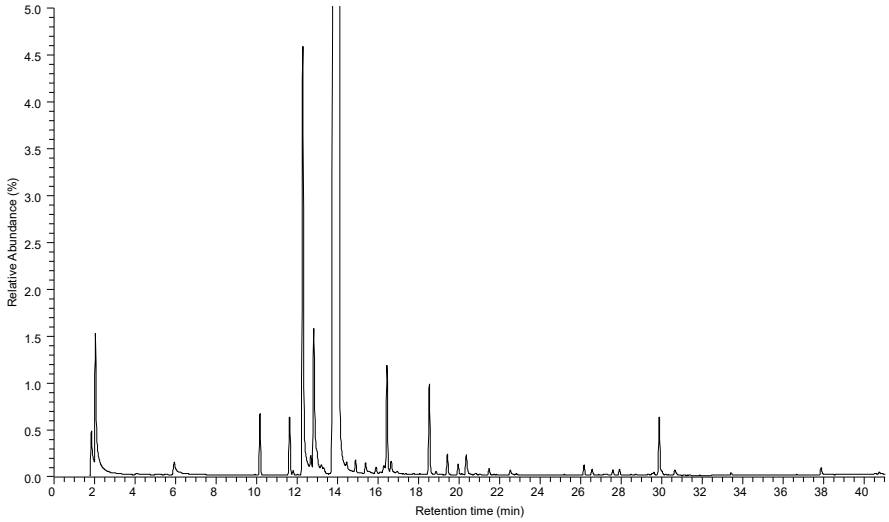


Figure 7.5. Typical GC-MS chromatogram of orange juice flavour compounds.

Retention index values of all compounds were determined and are given in Table 7.2. 32 compounds were identified according to the criteria mentioned above. Two peaks could not be identified, and one compound has been tentatively identified as β-phellandrene, as the match factor of the MS fragmentation spectra and the calculated RI deviated slightly from the criteria described above. For all compounds, the areas under the peaks were calculated and normalized according to the internal standard. For each compound, the areas under the peaks were compared for all processing conditions.

In general, relatively small changes in the peak area for the different compounds were introduced by processing, and therefore only trends could be described. Six trends in relative flavour concentrations were observed when peak areas of untreated, moderate intensity PEF treated and thermal treated juices were compared. Examples to illustrate the six trends are shown in Fig. 7.6. Fig. 7.6A illustrates a trend where the compound ethanol in juice B seemed to be unaffected by processing (untreated = PEF = heat), Fig. 7.6B illustrates the trend with higher concentrations of β-linalool in untreated and PEF treated than in heat treated juice A (untreated = PEF > heat), Fig. 7.6C illustrates the trend with higher concentrations of limonene in untreated than in PEF and heat treated juice B (untreated > PEF = heat), Fig. 7.6D illustrates the trend with lower concentrations of octanal in PEF treated juice A than in untreated juice A,

and even lower concentrations after thermal treatment (untreated > PEF > heat), Fig. 7.6E illustrates lower concentrations of 4-terpineol in untreated juice B than in PEF and thermal treated (untreated < PEF < heat), and Fig. 7.6F illustrates lower concentrations of 4-terpineol in untreated and PEF treated juice A than in thermally treated (untreated = PEF < heat).

Due to analytical inconsistencies, the GC-MS analyses for A-HL, B-60, B-63, B-75, and B-87 had to be excluded. However, an adequate number of GC-MS analyses remained in order to compare untreated orange juice to orange juice treated at various temperatures with moderate intensity PEF and with conventional heat treatment.

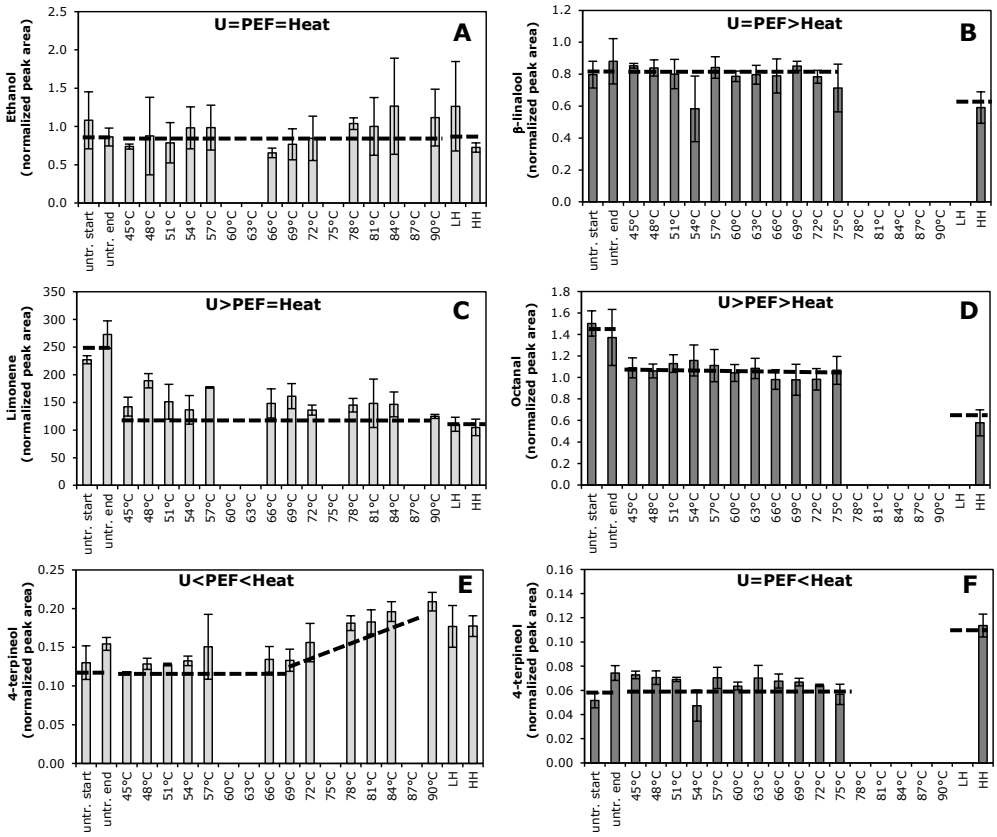


Figure 7.6. Trends illustrating the effect of processing on the detected volatiles present in orange juice, discriminating untreated juice (U), moderate intensity PEF treated juice (PEF) and thermal treated juice (heat). Six trends were identified, one trend showed no processing effect: U = PEF = heat (shown in Fig. 7.6A for ethanol), three trends showed a decrease of the compound after PEF and/or thermal processing compared to untreated, being U = PEF > heat (shown in Fig. 7.6B for β-linalool), U > PEF = heat (shown in Fig. 7.6C for limonene), and U > PEF > heat (shown in Fig. 7.6D for octanal); and two trends showing an increase of the compound after PEF and/or thermal processing compared to untreated: U < PEF < heat (shown in Fig. 7.6E for 4-terpineol) and U = PEF < heat (shown in Fig. 7.6F for 4-terpineol). Dark grey bars represent juice A, light grey bars represent juice B. Bars represent the average of three measurements with standard deviation. Trends for each specific compound are shown in the supplementary data, and summarized in Table 7.2.

The trends for each compound are given in Table 7.2, and figures including normalized peak areas per compound and treatment are provided in the supplementary material. The aroma compounds that have been reported in literature to contribute directly or indirectly to the overall orange juice flavour are indicated in Table 7.2, and the impact of processing on (only) these compounds will now be discussed.

No impact of moderate intensity PEF or thermal processing was observed for the measured peak areas of the esters ethyl acetate and ethyl butyrate, and the alcohol ethanol, as no effect or no trend of processing could be seen in the peak areas when compared to untreated juice.

Processing of the juice by either a moderate intensity PEF treatment or thermal treatment reduced the peak area of the aldehydes nonanal (juice A), decanal (juice A), dodecanal (juice B), terpene hydrocarbons limonene, α-pinene (juice B), β-myrcene (juice B) and terpene aldehydes neral and geranial, compared to untreated juice.

However, for this study, it was more interesting to evaluate whether a difference between the moderate intensity PEF treatment and thermal treatment was observed. Thermal treatment reduced the relative concentration of the positive flavour contributors 1-octanol and β-linalool, whereas the areas of the moderate intensity PEF treatment for these compounds did not change compared to those of untreated juice.

A beneficial effect for the moderate intensity PEF treatment compared to the thermal treatment was also observed for terpene hydrocarbons β–pinene, α-pinene (juice A), β-myrcene (juice A) and aldehydes octanal, nonanal (juice B), and decanal (juice B), although the areas for these moderate intensity PEF-treated compounds were lower compared to values in untreated juice. For some of the last mentioned compounds, a PEF temperature dependent decrease might be found within the group 'PEF treated', although differences in the peak areas were small and it might be debatable if a tendency to decline is present or not, as analyses for temperatures at 75 °C and 87 °C were missing and the peak area of 78 °C was often lower. Table 7.2 indicates that the relative amount of β–pinene, nonanal, and decanal decreased when the maximum temperature of the moderate intensity PEF treatment exceeded the indicated temperatures, estimated at 84 °C, 81 °C, and 81 °C, respectively. Although the reduction of some compounds in the PEF-treated juice might be temperature dependent, the remaining concentration measured at the most intense PEF treatment at 90 °C was still higher than in the conventional thermal treatment.

PEF and thermal treatment could also lead to the formation of components, such as α-terpinene, 4-terpineol and α-terpineol. These latter two compounds are typical reaction products of acid-catalysed degradation of limonene and β-linalool, formed at high temperatures and may be recognized as compounds causing off-flavour when present in high concentrations (Bazemore, Goodner & Rouseff 1999; Mastello et al., 2015; Perez-Cacho & Rouseff, 2008b). A temperature dependent increase of both compounds was found in PEF treated juice, when the maximum temperatures of the treatment exceeded 69 °C (juice B). Interestingly, no effects in juice A were observed, although this juice was measured up to maximum temperatures of 75 °C. Thermal processing led to an increase of these off-flavour compounds, to a similar extent as the most intense PEF treatments tested.

Table 7.2. Volatile compounds in treated and untreated orange juice.

No.	RI	Compound name	Class	Identification[a]
1	<800	Ethanol	Alcohol	MS
2	<800	Ethyl acetate	Ester	MS
3	802	Ethyl butyrate	Ester	MS, RI
4	863	2-Hexenal	Aldehyde	MS, RI, St.
5	932	α-Pinene	Monoterpene	MS, RI, St.
6	972	Sabinene	Monoterpene	MS, RI, St.
7	977	β-Pinene	Monoterpene	MS, RI, St.
8	990	β-Myrcene	Monoterpene	MS, RI, St.
9	1002	β-Phellandrene	Monoterpene	tentative
10	1005	Octanal	Aldehyde	MS, RI, St.
11	1008	3-carene	Monoterpene	MS, RI, St.
12	1014	*Unidentified*		-
13	1017	α-Terpinene	Monoterpene	MS, RI
14	1036	Limonene	Monoterpene	MS, RI
15	1048	Ocimene	Monoterpene	MS, RI, St.
16	1059	γ-Terpinene	Monoterpene	MS, RI
17	1073	1-Octanol	Alcohol	MS, RI
18	1086	Terpinolene	Monoterpene	MS, RI
19	1099	β-Linalool	Monoterpene alcohol	MS, RI
20	1105	Nonanal	Aldehyde	MS, RI
21	1182	4-Terpineol	Monoterpene alcohol	MS, RI
22	1196	α-Terpineol	Monoterpene alcohol	MS, RI
23	1207	Decanal	Aldehyde	MS, RI
24	1239	Neral	Monoterpene aldehyde	MS, RI
25	1267	Geranial	Monoterpene aldehyde	MS, RI
26	1377	α-Copaene	Sesquiterpene	MS, RI
27	1389	β-Cubebene	Sesquiterpene	MS, RI
28	1408	Dodecanal	Aldehyde	MS, RI
29	1420	β-Caryophyllene	Sesquiterpene	MS, RI
30	1431	*Unidentified*		-
31	1456	α-Caryophyllene	Sesquiterpene	MS, RI
32	1484	γ-Selinene	Sesquiterpene	MS, RI
33	1493	Valencene	Sesquiterpene	MS, RI
34	1522	δ-Cadinene	Sesquiterpene	MS, RI

a RI: Identification based on retention indices found in literature. MS: Identification based on MS fragmentation spectra in NIST compound library.

St: Identification based on retention time and MS spectra of an external reference compound.

b Trends between untreated, PEF treated and heat treated orange juice as indicated in Fig. 7.6. Between the different PEF treatments of juice B, tendencies of increase or decrease of the component is observed at indicated temperatures.

c Impact on fresh orange juice flavour reported in the review of Perez-Cacho & Rouseff 2008a, including only compounds that have been shown to be aroma active in GC-Olfactometry studies and that have been identified as relevant for orange juice flavour by two or more independent research groups.

d Impact on processed orange juice, from GC-Olfactometry studies, according to Perez-Cacho & Rouseff 2008b and Bazemore, Goodner and Rouseff 1999.

e Impact on processed orange juice, from GC-Olfactometry studies, according to Perez-Cacho & Rouseff 2008b and Mastello et al. 2015.

f reported by Shaw 1991, not reported from GC-Olfactometry study.

n.d. not detected

Trends in juice A[b]	Trends and Tendencies in juice B[b]		Impact on orange juice flavour
U=PEF=Heat	U=PEF=Heat		Possibly lifts aroma[f]
U=PEF=Heat	U=PEF=Heat		Background aroma, contributing to naturalness[c]
U=PEF=Heat	U=PEF=Heat		Key odorant in fresh orange juice[c]
U=PEF=Heat	n.d.		
U>PEF>Heat	U>PEF=Heat		Background aroma, contributing to naturalness[c]
U>PEF>Heat	U>PEF>Heat	PEF>84°C decrease	
U>PEF>Heat	U>PEF>Heat	PEF>84°C decrease	Background aroma, contributing to naturalness[c]
U>PEF>Heat	U>PEF=Heat		Background aroma, contributing to naturalness[c]
U=PEF=Heat	U=PEF=Heat		
U>PEF>Heat	U>PEF>Heat		Key odorant in fresh orange juice[c]
U>PEF>Heat	U>PEF=Heat		
U=PEF>Heat	U=PEF=Heat		
U=PEF<Heat	U=PEF<Heat		Background aroma, contributing to naturalness[c]
U>PEF=Heat	U>PEF=Heat		Necessary for aroma, function uncertain[c]
U>PEF=Heat	U>PEF>Heat	PEF>84°C decrease	
U=PEF<Heat	U=PEF=Heat		
U=PEF>Heat	U=PEF>Heat		Background aroma, contributing to naturalness[c]
U>PEF=Heat	U>PEF=Heat		
U=PEF>Heat	U=PEF>Heat		Key odorant in fresh orange juice[c]
U>PEF=Heat	U>PEF>Heat	PEF >81°C decrease	Key odorant in fresh orange juice[c]
U=PEF<Heat	U<PEF<Heat	PEF ≥78°C increase	Background aroma, contributing to naturalness[c], Marker for heat-abuse, off-flavour at high conc.[d]
U=PEF<Heat	U<PEF<Heat	PEF >78°C increase	Marker for heat-abuse, off-flavour at high conc.[e]
U>PEF=Heat	U>PEF>Heat	PEF >81°C decrease	Key odorant in fresh orange juice[c]
U>PEF=Heat	U>PEF=Heat		Key odorant in fresh orange juice[c]
U>PEF=Heat	U>PEF=Heat		Key odorant in fresh orange juice[c]
U>PEF>Heat	U>PEF>Heat		
U>PEF>Heat	U>PEF>Heat	PEF >84°C decrease	
n.d.	U>PEF=Heat		Background aroma, contributing to naturalness[c]
U>PEF>Heat	U>PEF>Heat		
U>PEF>Heat	U>PEF>Heat		
U=PEF=Heat	U>PEF=Heat		
U=PEF=Heat	U=PEF>Heat		
U>PEF>Heat	U>PEF>Heat	-	
U>PEF=Heat	U=PEF>Heat		

Our results using moderate intensity PEF are similar to those shown by Leizerson & Shimoni (2005a) who found a higher concentration of pinene, myrcene, octanal, limonene and decanal in ohmic heat treated than in conventional heated orange juice. Also the study of Min and co-workers (2003) observed a better retention of concentration of α-pinene, myrcene, octanal, limonene and decanal after high intensity PEF treatment at 40 kV/cm than after a conventional heat treatment. Both studies described in literature, as well as our study, had a lower heat load for the PEF or ohmic treatment compared to the conventional heat treatment, probably leading to the initiation of fewer chemical reactions, and therefore resulting in more retention of flavour compounds.

Overall, it can be said that the impact of the thermal treatments on the volatile flavour compounds was very moderate, and up to PEF processing temperatures of 78 °C minimal changes were observed. Some deviations between moderate intensity PEF treated and thermally processed orange juice were found for individual compounds, where moderate intensity PEF treatment resulted in a better retention of flavour compounds. No strong off-flavour compounds such as *p*-cymene and carvone (Perez-Cacho & Rouseff, 2008b) were detected in the juices studied, and off-flavour compounds such as 4-vinylguaiacol and furaneol (Walsh, Rouseff & Naim, 1997) could not be detected as they need a solvent extraction. The increase of α-terpinene, 4-terpineol, and α-terpineol was minimal at the most intense PEF and thermal treatments studied, when compared to the concentrations in untreated juice.

7.4 Conclusion

In this chapter we compared the effect of PEF processing at reduced electric field strength (E = 0.9 and 2.7 kV/cm) and long pulse width (τ = 1000μs) in combination with varying maximum temperature to two conventional thermal pasteurisation processes on several quality aspects in orange juice.

Variations in the initial quality of the two (untreated) juices A and B made it hard to evaluate if there was an effect on quality aspects when a different electric field strength was applied. Furthermore, the juice had a relatively low pH, so some chemical reactions that could influence the quality attributes ascorbic acid content, colour and some flavour compounds, might be lower than expected beforehand and could be more pronounced in other products with higher pH. Larger differences in quality between moderate intensity PEF processing and thermal processing could also be expected when experiments would be performed on larger pilot- or industrial scale. The heat transfer for PEF treatment is scale independent due to volumetric heating, but for the conventional thermal process a lower heat transfer will be expected at larger scale than on the lab-scale unit, leading to longer process times, which may affect product quality negatively.

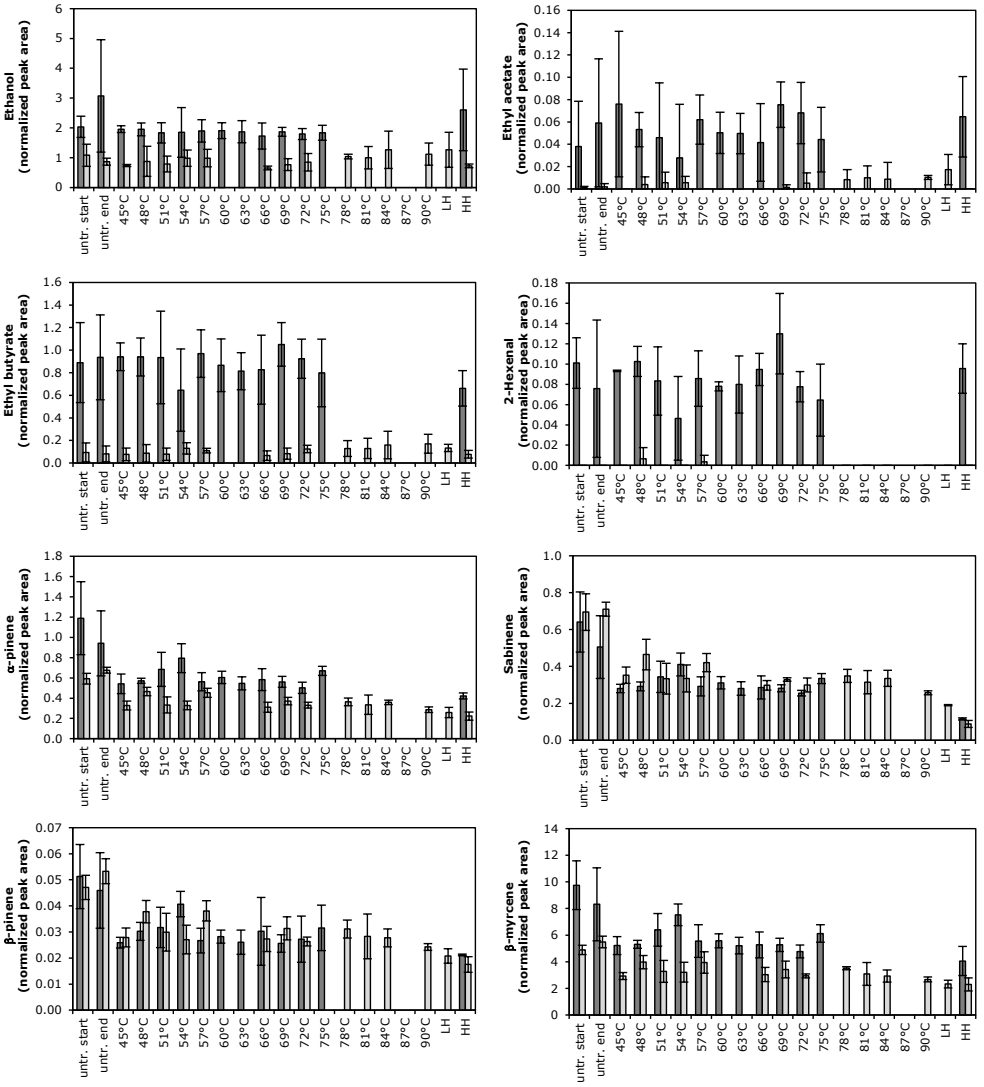
However, the impact of the varying maximum temperature by the moderate intensity PEF and conventional thermal process did show an effect on some quality attributes. The pH and soluble solids did not show a difference after any treatment either by moderate intensity PEF or conventional heat. Only small differences were observed

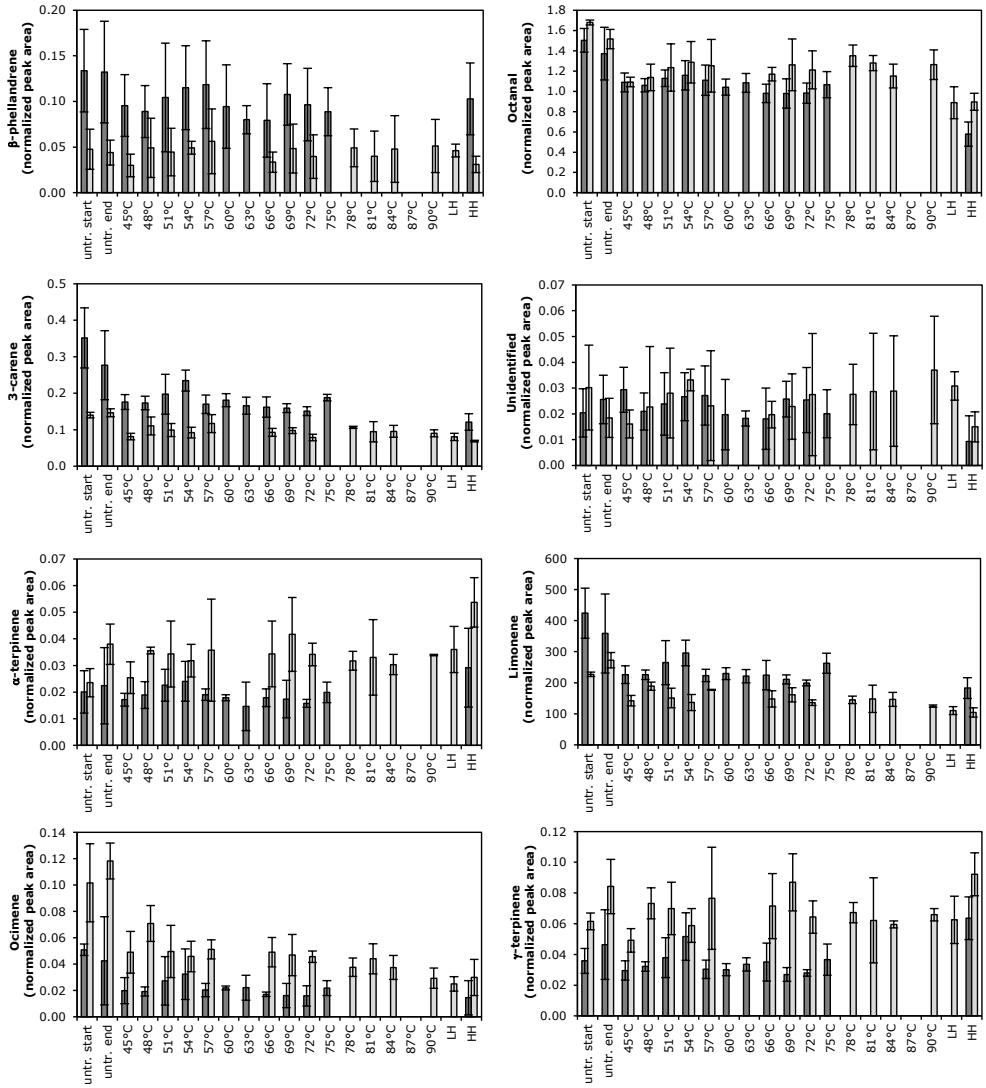
for colour and vitamin C content, where an increased temperature impact led to slight deviations, mainly for the most intense thermal treatment. A real impact of processing was measured in the enzymatic activity of PME, which reduced when the temperature was increased. Reduction of the remaining PME activity to levels below 1.0×10^{-4} PEu were reached with moderate intensity PEF processing at maximum temperatures of 78 °C or higher. According to literature, this level is sufficient to obtain a shelf stable orange juice. The impact of processing on the flavour components showed that the beneficial contributors to the flavour were better retained after moderate intensity PEF treatment than after the conventional thermal treatment, that the concentration of compounds with a negative flavour impact was similar at most intense PEF conditions of 90 °C to the intense conventional heating, and that no compounds causing strong off-flavours were detected.

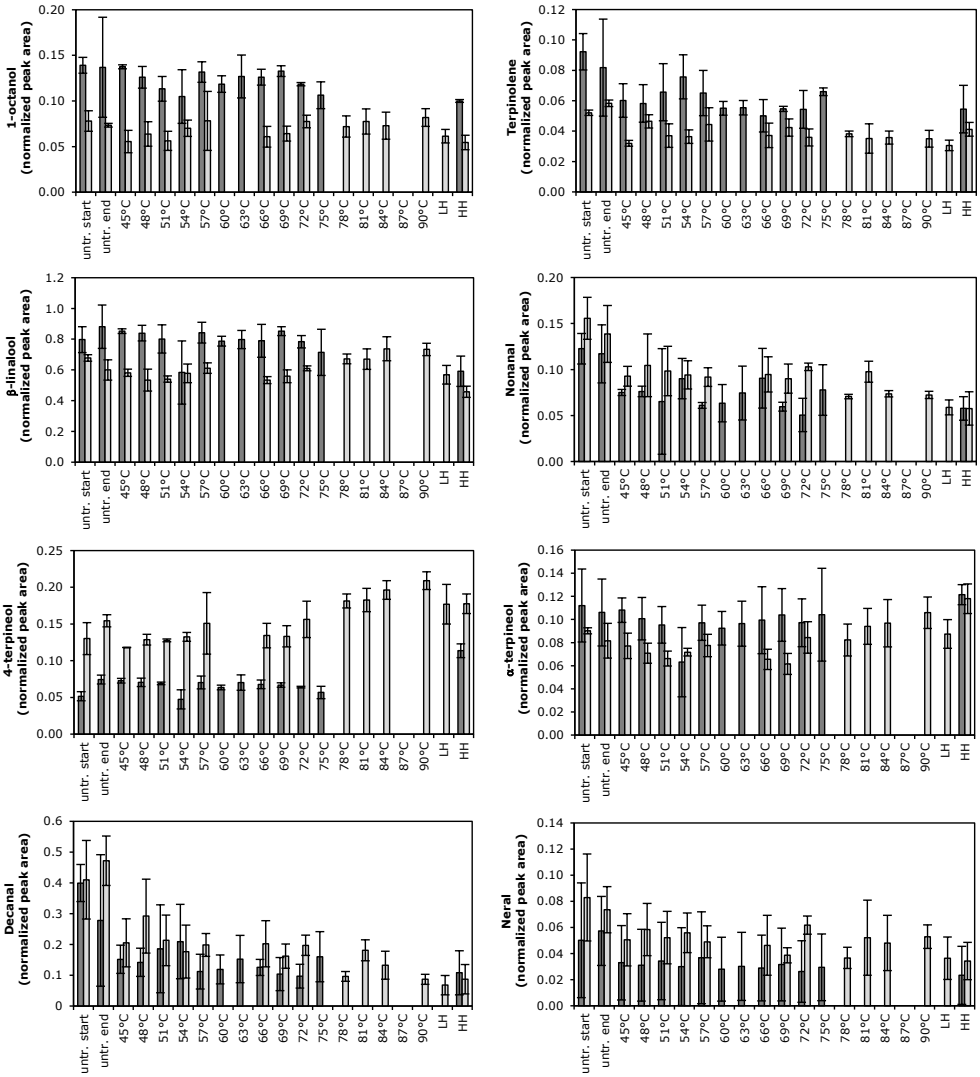
Comparison of the effect of the two electric field strengths used in this study (E = 0.9 or 2.7 kV/cm with pulse width of τ = 1000 µs) did not show remarkable differences on quality attributes, while identical pulse conditions tested did show a significant higher inactivation of micro-organisms at an electric field strength of 2.7 kV/cm compared to 0.9 kV/cm (Chapter 6). PEF processing conditions at moderate intensity (E = 2.7 kV/cm), long pulse duration (τ = 1000 µs) and combination with heat up to maximum temperatures of 65 – 90 °C led to more microbial and enzymatic inactivation than high intensity PEF conditions at 20 kV/cm, short pulse duration (τ = 2 µs) and lower maximum temperatures (T < 60 °C) (Chapter 6; Timmermans et al., 2011; Timmermans et al., 2014; Vervoort et al., 2011; Vervoort et al., 2012), and therefore a longer shelf life using the moderate intensity PEF processing conditions could be expected, although this remains to be determined.

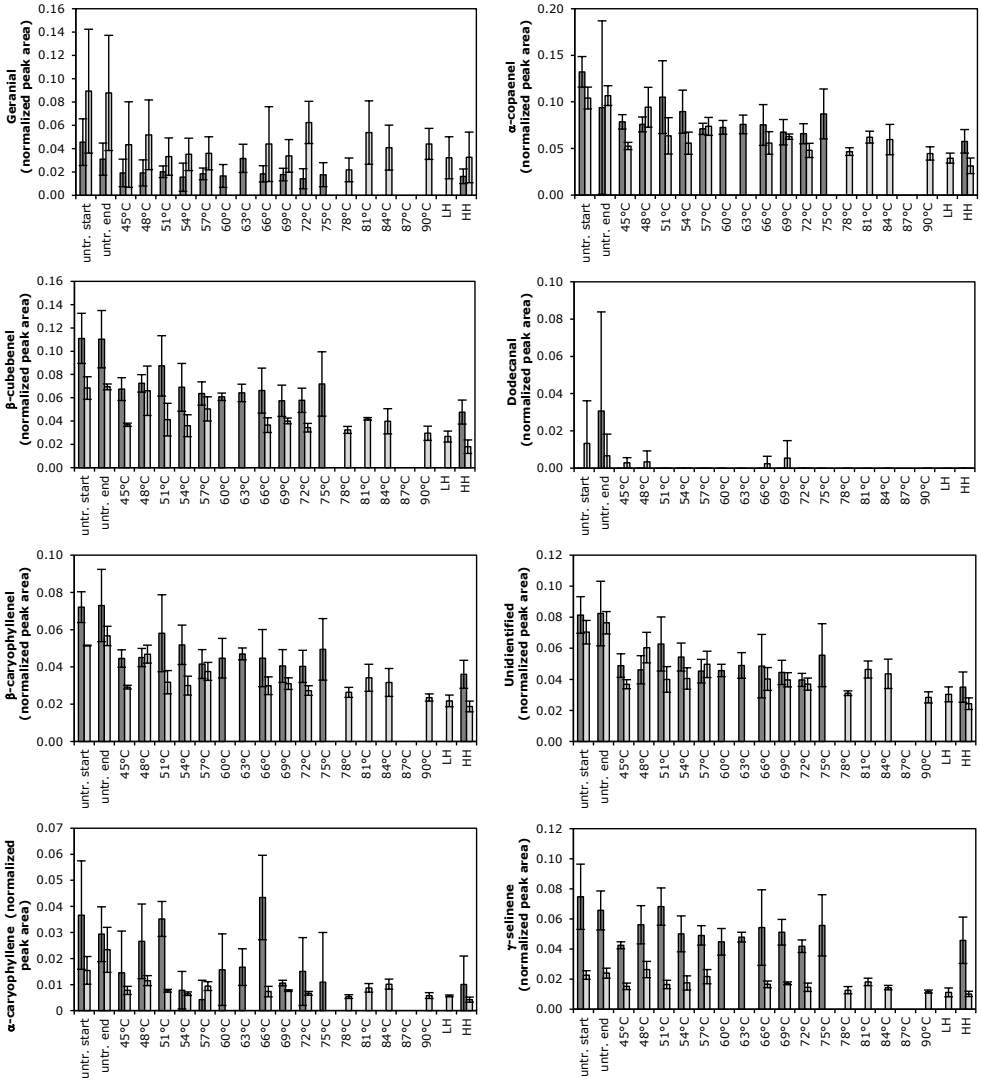
Based on the results of this study, PEF processing at moderate electric field strength, long pulse duration at a maximum temperature of 78 °C would be an optimal process condition, which is suitable as alternative pasteurisation process to thermal treatment for low pH orange juice, as the PME level is reduced to the desired number, microbial inactivation criteria could be met (shown in Chapter 6) and quality aspects colour, vitamin C content and flavours are better retained than in thermal treated and still very close to untreated juice.

7.5 Appendix


Ethanol (normalized peak area)
Ethyl acetate (normalized peak area)
Ethyl butyrate (normalized peak area)
2-Hexenal (normalized peak area)
α-pinene (normalized peak area)
Sabinene (normalized peak area)
β-pinene (normalized peak area)
β-myrcene (normalized peak area)
untr. start
untr. end
45°C
48°C
51°C
54°C
57°C
60°C
63°C
66°C
69°C
72°C
75°C
78°C
81°C
84°C
87°C
90°C
LH
HH


β-phellandrene (normalized peak area)
Octanal (normalized peak area)
3-carene (normalized peak area)
Unidentified (normalized peak area)
α-terpinene (normalized peak area)
Limonene (normalized peak area)
Ocimene (normalized peak area)
γ-terpinene (normalized peak area)
untr. start
untr. end
45°C
48°C
51°C
54°C
57°C
60°C
63°C
66°C
69°C
72°C
75°C
78°C
81°C
84°C
87°C
90°C
LH
HH


1-octanol (normalized peak area)
Terpinolene (normalized peak area)
β-linalool (normalized peak area)
Nonanal (normalized peak area)
4-terpineol (normalized peak area)
α-terpineol (normalized peak area)
Decanal (normalized peak area)
Neral (normalized peak area)
untr. start
untr. end
45°C
48°C
51°C
54°C
57°C
60°C
63°C
66°C
69°C
72°C
75°C
78°C
81°C
84°C
87°C
90°C
LH
HH


Geranial (normalized peak area)
α-copaenel (normalized peak area)
β-cubebenel (normalized peak area)
Dodecanal (normalized peak area)
β-caryophyllenel (normalized peak area)
Unidientified (normalized peak area)
α-caryophyllene (normalized peak area)
γ-selinene (normalized peak area)
untr. start
untr. end
45°C
48°C
51°C
54°C
57°C
60°C
63°C
66°C
69°C
72°C
75°C
78°C
81°C
84°C
87°C
90°C
LH
HH

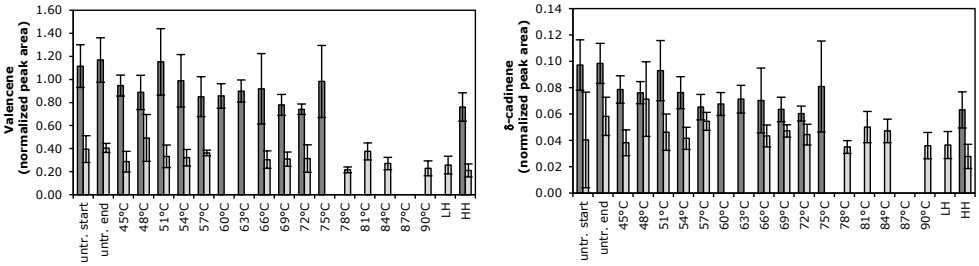


Figure S7.1. Effect of processing on each detected flavour compound in orange juice. Dark grey bars represent juice A, light grey bars represent juice B. Values of untreated juice at start and end of the day are given. Results of various moderate intensity PEF treatment are given according to the maximum temperatures applied, using an electric field strength of 2.7 kV/cm (dark grey bars) and 0.9 kV/cm (light grey bars). Thermal treatments are indicated as LH for low heat treatment (75 °C – 20s) and HH for high heat treatment (95 °C – 20s). Bars represent the average of three measurements with standard deviation. Trends for each compound are given in Table 7.2, according to the 6 possibilities shown in Fig. 7.6.

Chapter 8

General Discussion

8.1 Introduction

Pulsed Electric Field (PEF) processing holds promises as alternative preservation technology applicable to liquid food products, such as fruit juices, aiming for pasteurisation at milder temperatures to guarantee a safe food product, while retaining fresh characteristics. Microbial inactivation by PEF is affected by multiple parameters including processing conditions, microbial characteristics and culture conditions, and treatment medium (Chapter 1), but conditions used vary largely between studies, making it difficult, if not impossible, to compare data. Most studies on PEF processing as alternative, mild pasteurisation technology use high intensity PEF, with pulses at electric field strength (E) > 20 kV/cm and pulse width in the range of (τ) of 2 − 20 µs, for a short period of time to minimize the heat load. The use of moderate electric field strength, typically in the range of E = 0.5 − 5.0 kV/cm has been extensively studied as a pre-step in mass transfer processes in food- and biotechnology to disintegrate plant cells, and in the field of biology to cause electroporation of micro-organisms, but has not been exploited to achieve inactivation of micro-organisms as alternative processing technology. One of the research questions raised in Chapter 1 was whether or not a combination of moderate electric field strength and heat could be used as alternative pasteurisation process.

The work described in this thesis aimed at providing insight in how moderate and high intensity PEF processing conditions, (liquid) product matrix and characteristics of targeted species affect microbial inactivation, shelf life, enzyme activity and quality of fruit juices. In this thesis, moderate intensity PEF is defined as field strength less than 5 kV/cm and high intensity PEF at field strength larger than 5 kV/cm, independent of the duration of a single pulse.

Inactivation kinetics of different PEF conditions and equipment configurations were studied using various species of spoilage and pathogenic micro-organisms, and evaluated in different juice matrices varying in pH and conductivity (Chapter 2 and 6). A systematic evaluation on the impact of PEF parameters: electric field strength, pulse width and maximum temperature, was carried out. Special attention was paid to distinguish non-thermal from thermal effects by selection of a suitable experimental set-up. By design, continuous flow PEF systems have a typical temperature holding time of a few seconds, which is too short to compare accurately to experimental measurements of a thermal treatment in capillairies that are routinely applied. Therefore, predictive modelling was employed as a tool to predict inactivation at such short holding times. A primary thermodynamic model based on Gaussian distribution and Eyrings rate constant for the transition state has been developed to describe the inactivation kinetics of enzymes and micro-organisms exposed to heat and chemical (acid) treatment (Chapter 4). It was found that the Lumry-Eyring model for unfolding proteins could be used and that the model parameters standard activation enthalpy and entropy are rationally linked to the reference temperature and Z-value commonly used in food microbiology. By exploiting these associations, a well-found extrapolation for thermal treatment at short holding times could be made (Chapter 5). A systematic evaluation of the combined effect of electric field strength, pulse duration and heat to an equivalent thermal reference process with same holding time was made, to study if

the PEF conditions applied comprise an additional non-thermal pulse effect next to the thermal element responsible for microbial inactivation (Chapter 6). Inactivation was not only studied in a laboratory setting, the effects of high intensity PEF processing conditions and storage temperature were also studied for yeast and moulds naturally present in a fresh fruit smoothie during shelf life under conditions applied by industry (Chapter 3). Finally, moderate intensity PEF conditions were subject to a case study on freshly squeezed orange juice to evaluate the effect on enzyme inactivation and food quality aspects (Chapter 7). The assumptions used in the experimental set-up in this study will now be discussed, followed by a discussion on the microbial inactivation by PEF and the impact of PEF on food quality, including suggestions for future research and the potential for food industry.

8.2 Experimental set-up of the PEF treatment

A systematic evaluation of PEF process conditions that are currently feasible at industrial scale, using real food matrices was conducted to better understand the effect of process parameters on shelf life and quality. For this, inactivation kinetics was studied in a continuous flow system, using square wave pulses and a maximum electric field strength of 23 kV/cm at laminar flow conditions. Inactivation kinetics was determined by selecting two single pulse characteristics (electric field strength and pulse width) while a varying number of pulses was applied. Flow conditions and the inlet- or preheating temperature were kept constant, and no cooling between the treatment chambers was applied, so the electrical energy applied was proportional to the temperature increment and maximum temperature reached during processing.

In chapter 2, it was shown that change of the initial preheating temperature of 20 °C to 36 °C enhanced the inactivation of *Salmonella* Panama and *Saccharomyces cerevisiae*. It was therefore decided to keep this preheating temperature of 36 °C for all further experiments, to allow comparison of the effect of pulse conditions, although more preheating would be desirable for an industrial scale application, to reduce the amount of electric energy required.

Moderate and high intensity PEF with selected pulse width and variable frequency were evaluated to assess the influence of electric field strength, pulse width and temperature on microbial inactivation. Monopolar pulses at high intensity/short pulse duration were compared to bipolar pulses at moderate intensity pulses. According to a study of Qin et al. (1994) bipolar pulses are more effective than monopolar pulses in microbial inactivation, but this effect could not be incorporated in our study, due to technical lay-out of the system.

Minimal effects of electrochemical reactions at the electrodes were expected on the microbial inactivation, as electrode material with low corrosion tendency was selected (titanium). Furthermore, at high intensity only short pulses were applied using a pulse transformed to avoid leak currents that are the main source of electrochemical reactions (Morren, Roodenburg & De Haan, 2003). For longer pulse width, bipolar pulses were applied to minimise electrochemical reactions at the metal-liquid surface (Roodenburg, 2007; Samaranayake, Sastry & Zhang, 2005).

Homogeneous treatment could be assumed by an adequate design of the treatment chamber using a high ratio between electrode gap and area (Van den Bosch, 1997). Different treatment chambers were designed to vary in electric field strength, with ratio's between 1.4 and 2.0.

The above mentioned set-up with a fixed flow to standardise preheating and cooling time resulted in a small deviation in the residence time for the different treatment chambers, with differences in the millisecond time range. Despite these efforts to control preheating and cooling, the differences in residence time and the necessary difference in pulse frequency to select a target temperature resulted in a different idle time, and possibly effect the electroporation efficiency and electropermeabilization of the cell. This aspect should be considered more carefully in future research.

Special attention was paid to the lay-out of the PEF system by including a grounded electrical connection to ensure operational safety, but constructed such that the heat flow by conduction was carried by the processing fluid and was not lost by unintentional cooling. Temperature measurements were taken under stationary conditions in continuous flow, before and directly after the treatment chambers, so the energy balance of the electrical energy input and caloric power measured in the bulk was accurate within experimental error (< 5%), with an absolute deviation of 0.5 – 3 °C. This accurate measurement allowed to make a comparison to a thermal reference process with similar holding time, to discriminate non-thermal effects from the thermal effects on a quantitative basis.

Microbial culture conditions were standardised across the different experiments. To mimic the effect of juice during cultivation, 1% glucose was added during the last overnight cultivation, which resulted in a reduction of the pH (Table 5.1). Recovery of sub lethal damaged micro-organisms was enhanced by addition of sodium pyruvate to the medium to avoid overestimation of the PEF process. (Standard) neutral pH medium was used for plating, although a more acidic pH of the medium might better reflect the effect of microbial inactivation in acidic juices, and is therefore recommended for future research.

Due to the variable lay-out of different PEF systems and process conditions used by different research groups, it is essential that experimental data and exposure conditions are well documented. Lack of essential data and information on temperature-time exposure and energy use in many papers published in the past, make it difficult to interpret and evaluate experiments and data. Recent guidelines presented on reporting the key information on PEF processing is a positive development (Mastwijk et al., 2007; Raso et al., 2016) and should be incorporated in the peer review system.

8.3 Microbial inactivation

8.3.1 Individual effects of electric field strength and pulse duration

The individual effect of electric field strength and pulse duration in combination with heat on the inactivation of different microbial species was evaluated (Chapter 6). Typically, PEF inactivation kinetics is expressed as a function of maximum outlet temperature or electric energy applied (Toepfl, Heinz & Knorr, 2007b; Wouters et al., 1999). The expression of a PEF process as function of maximum outlet temperature allows comparison to inactivation kinetics of a thermal reference process and thereby determine if additional pulse effects contribute to inactivation.

Other approaches used to express the impact of PEF processing on microbial inactivation, include kinetics as a function of either total pulse time (Álvarez et al., 2000; Grahl & Märkl, 1996; Hülsheger, Potel, & Niemann, 1981; Sale & Hamilton, 1967), electric field strength (Grahl & Märkl, 1996; Hülsheger, Potel & Niemann, 1981; Sale & Hamilton, 1967) or number of pulses applied (Vega-Mercado et al., 1996); this will be discussed below.

The inactivation of *E. coli, L. monocytogenes* and *S. cerevisiae* in orange juice presented in Chapter 6 (Fig. 6.4) was fitted as a function of total treatment time (Fig. 8.1, left panel) and number of pulses applied (Fig. 8.1, right panel). As expected, the $\log_{10}$ reduction increased with the total treatment time for a specific set of pulse conditions (Fig. 8.1-left panel) (Álvarez et al., 2000; Grahl & Märkl, 1996; Sale & Hamilton, 1967). Inactivation curves are mainly dependent on the electric field strength, with the highest electric field strength corresponding to the shortest total treatment time, and the lowest electric field strength corresponding to the longest total treatment time (Fig. 8.1-left panel). Variation in pulse width at the same electric field strength appears to be of minor importance when inactivation is expressed as a function of total treatment time, as inactivation curves at E = 2.7 kV/cm showed inactivation at the same order of magnitude for variable pulse width of τ = 1000 µs, τ = 100 µs and τ = 15 µs (Fig. 8.1, left panels).

Inactivation data can also be described as function of treatment time using linear approximation, resulting in threshold values indicating the critical treatment time to start inactivation (t_c) for a specific electric field strength applied (Hülsheger, Potel & Niemann, 1981). Linear approximation was used to determine t_c-values for the *E. coli* and *S. cerevisiae* data shown in Fig. 8.1 by estimating the x-axis intercept (Fig. 8.2). The t_c-value appears to be mainly dependent on electric field strength, as only minor differences for pulse duration were observed for *E. coli* (2.7 kV/cm), and no differences for *S. cerevisiae* were detected. Results of Grahl & Märkl (1996) and Hülsheger, Potel & Niemann (1981) showed some effect of electric field strength on t_c-value, though our study shows a more pronounced dependence of t_c on E than the reported studies. Differences in matrix composition, microbial strains and PEF settings used in the various studies are a possible explanation for the differences between the studies.

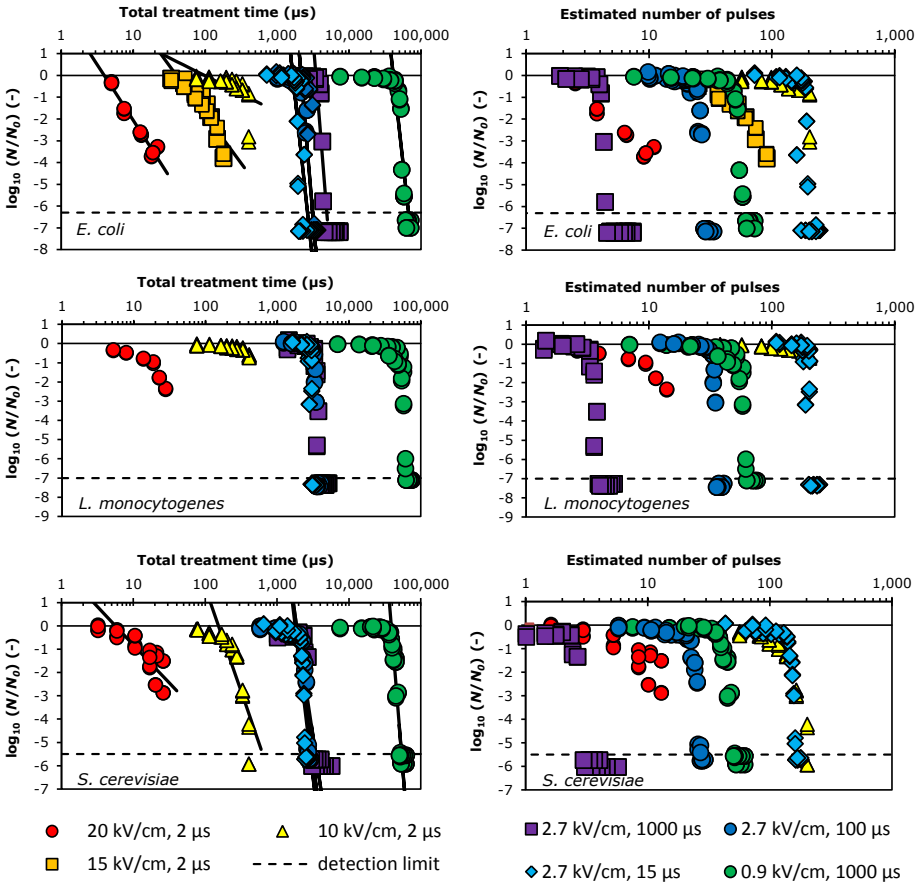


Figure 8.1. Reduction of viable counts $\log_{10}(N/N_0)$ of *E. coli, L. monocytogenes* and *S. cerevisiae* in orange juice after PEF treatment. Symbols represent measured inactivation data at variable electric field strength and pulse width, and lines the linear approximation. Inactivation data is identical to data shown in Chapter 6 (Fig. 6.4), but expressed as a function of total treatment time (left panel) or number of pulses applied, calculated according to Eq. 1.1 (right panel). The dashed lines represents the detection limit.

El Zakhem and co-workers (2006b) measured the characteristic damage time as a function of electric field strength in *S. cerevisiae*, where cell damage was measured as difference in electrical conductivity measured in the suspension and not by enumeration of survivors by plate counting as used in our study. The characteristic damage time is defined as the time required to reach half of the saturation conductivity, but is shown in Fig. 8.2 at the same scale as the threshold time to start damage/inactivation. Interestingly, the trend observed for critical treatment time as a function of electric field strength (our data) is similar to the decrease of damage time shown by El Zakhem et al. (2006b). Furthermore, the study of El Zakhem et al. (2006b) was carried out at controlled temperature, and showed that increase of the temperature reduced the damage time. In our study, the temperature was not controlled at a specific temperature, but started at a set temperature of 36 °C and increased as a result of the energy applied. Nevertheless, the temperature at the critical time could be calculated,

based on Eq. 1.2b (Chapter 1) assuming σ = 0.6 S/m, and reached approximately 39.5 °C (20 kV/cm), 59.5 °C (10 kV/cm), 57.0 °C (2.7 kV/cm) and 82.5 °C (0.9 kV/cm) for *S. cerevisiae.* Even though this conductivity of σ = 0.6 S/m is an underestimation of the actual conductivity, and actual temperatures would be even higher, these critical temperatures were compared to the inactivation temperatures in Fig. 6.4 (Chapter 6). The calculated temperature using the estimated t_c-value corresponds to the measured inactivation temperature at E = 20 kV/cm, however the predicted temperature for all other PEF conditions was higher temperatures than actually measured. Therefore it is proposed that linear approximation cannot be used to make an accurate prediction of t_c, as the line shape of the inactivation curve shows a synergistic effect of temperature and pulses on inactivation.

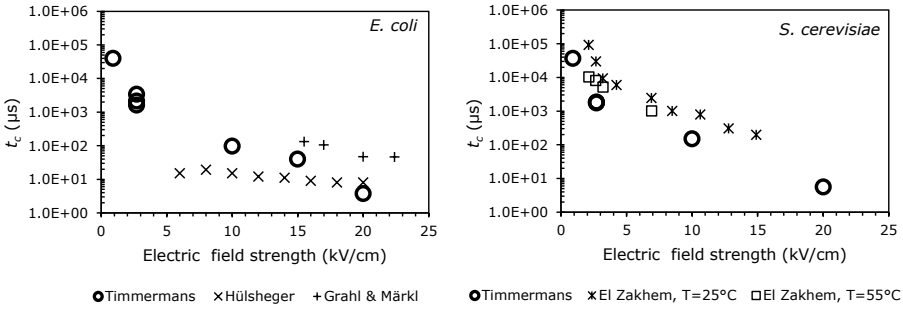


Figure 8.2. Critical treatment time (t_c) as a function of electric field strength for PEF treatment of *E. coli* and *S. cerevisiae.* Different symbols represent the origin of the data of different studies (El Zakhem et al., 2006b; Grahl & Märkl, 1996; Hülsheger, Potel & Niemann, 1981; Timmermans et al., Chapter 6).

The effect of number of pulses applied on inactivation was calculated according to Eq. 1.1. and is shown in Fig. 8.1 (right panels). As laminar flow conditions were used, it can be anticipated that the fastest particle traverses the treatment zone with twice the speed of an average-speed particle. As at least two pulses were given to an average-speed particle (Fig. 8.1, right panel), this means that the fastest particle has received at least one pulse.

Generally, it has been reported that an increase in number of pulses will increase the degree of inactivation (Hülsheger, Potel & Niemann, 1981; Vega-Mercado et al. 1996). This is also observed for the specific pulse conditions, represented by different symbols in Fig. 8.1. However, comparison of the specific pulse conditions showed a difference in the number of pulses required. This difference could be attributed to the different energy density per pulse (u, J/m^3), as calculated according to Eq. 8.1 and shown in Table 8.1.

$$u = \sigma \cdot E^2 \cdot \tau \qquad \text{(Eq. 8.1)}$$

In general, the energy density per pulse is inversely proportional to the number of pulses required, with a PEF condition of E = 2.7 kV/cm, τ = 1000 µs being highest in energy density requiring the least number of pulses for inactivation. In addition to the energy density, also the electric field strength plays a role, as conditions with similar energy input of 0.44 – 0.49 J/m^3 followed the order 20 kV/cm > 2.7 kV/cm > 0.9 kV/cm, suggesting that additional non-thermal pulse effects are present for some conditions, as discussed in Chapter 6.

Table 8.1. Energy density per pulse (J/m³) calculated according to Eq. 8.1 for electric field strength, pulse width and conductivity of 0.6 S/m, typical the energy of 1 pulse at orange juice of 40 °C, as used in inactivation studies described in Chapter 6.

E (kV/cm)	τ (µs)	u (J/m³)
20	2	0.48
15	2	0.27
10	2	0.12
2.7	1000	4.37
2.7	100	0.44
2.7	15	0.07
0.9	1000	0.49

A third approach to express the impact of PEF processing on microbial inactivation is as function of electric field strength (Fig. 8.3), categorised for individual pulse width tested. In general, an increase in electric field strength increases the amount of inactivation, when the electric field strength exceeds the critical threshold value E_c (Heinz et al., 2002; Hülsheger, Potel & Niemann, 1981; Sale & Hamilton, 1967). This is observed in our study as well, when only data points below maximum outlet temperatures of 50 °C are shown (Fig. 8.3, left panel). More inactivation was observed when the number of pulses, amount of energy or maximum temperature increased. When all data points, including the ones with temperatures above 50 °C are included in a plot, also inactivation at reduced electric field strength was observed (Fig. 8.3, right panel). However, for these data points the thermal and non-thermal effects responsible for inactivation are mixed. A discrimination of these effects will be discussed in section 8.3.3.

Different expressions to visualise microbial inactivation as a function of PEF process parameters are shown in Fig. 8.1-8.3. Due to the set-up used in our experiments, that allows a combined, synergistic effects of temperature and pulses, it is impossible to interpret expressions as function of electrical field strength only (Fig. 8.3). Furthermore, linear approximation to determine a relationship between electric field strength and critical treatment time shows only indications on t_c, as predictions do not hold when compared to measured data. Based on this expression as a function of total treatment time, no effect of pulse width at constant electric field strength was observed for *S. cerevisiae,* indicating that the same t_c-value gives similar inactivation at any pulse width, while measured inactivation data shown in Fig. 6.6 as function of maximum temperature clearly show more inactivation at longer pulse width. In conclusion, these discussed approaches to visualise inactivation might be useful to study individual effects and relations when experiments were carried out at constant temperatures, but not in a combined process of temperature and pulses as used in this thesis.

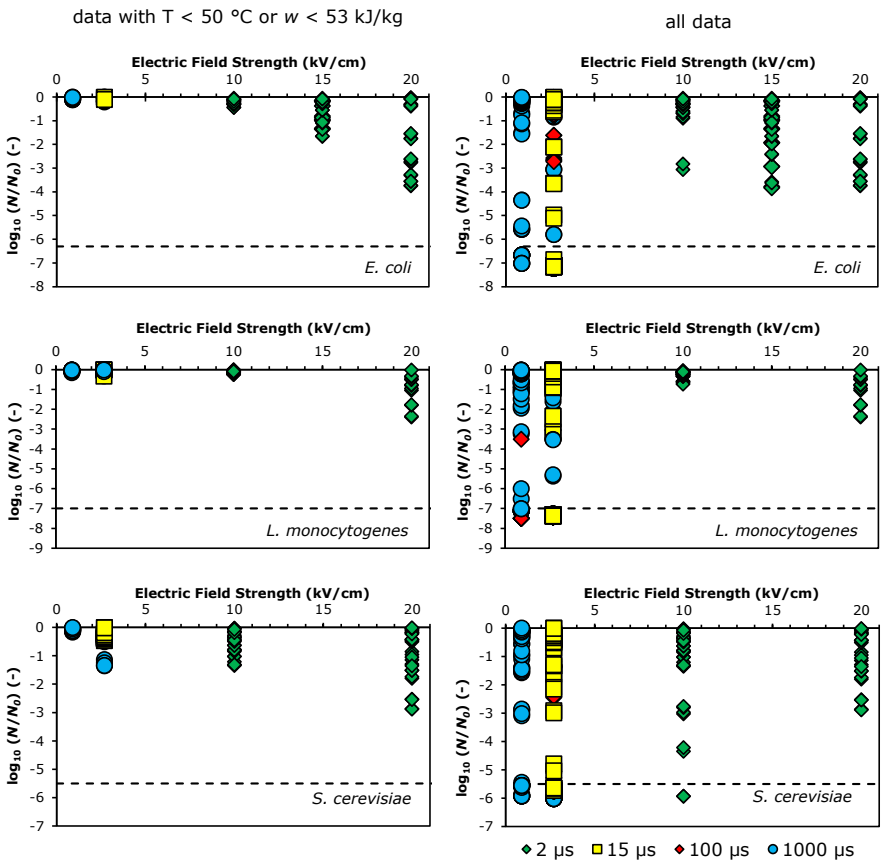


Figure 8.3. Reduction of viable counts $\log_{10}(N/N_0)$ of *E. coli, L. monocytogenes* and *S. cerevisiae* in orange juice after PEF treatment as a function of electric field strength applied. Symbols represent inactivation data at variable pulse duration. Data points in the left panel, taken from Fig. 6.4, show inactivation up to maximum temperatures of 50 °C, data points in the right panel show all inactivation data displayed in Fig. 6.4.

8.3.2 Relationship between PEF conditions and matrix properties

Microbial inactivation using high intensity PEF conditions at E = 20 kV/cm, τ = 2 µs, with maximum electrical energy input of about 80 kJ/kg and maximum temperatures of 58 °C, was dependent on the pH of the (liquid) matrix, as shown in Chapter 2, and Fig. 8.4. PEF processing effectively inactivated target micro-organisms in apple and orange juices at high acid conditions (pH 3.5 - 3.7), but lost its efficiency when the pH of the matrix increased to low acidic values in watermelon juice (pH 5.3). Acidification of the low acidic watermelon juice to a pH of 3.7 showed an increased PEF efficiency again (Chapter 2).

Contrary, moderate intensity PEF conditions at E = 2.7 kV/cm, τ = 1000 µs, with maximum electrical energy input of 120 – 130 kJ/kg and maximum temperatures of 68 °C, did not show any effect of the pH of the matrix on the microbial inactivation

(Chapter 6 and Fig. 8.4), as no significant differences in microbial inactivation were observed in orange juice (pH = 3.8), coconut water (pH = 5.0) and watermelon juice (pH = 6.0).

A significant difference between the two PEF combinations of high field strength/ short duration or moderate field strengths/long duration was the curvature of the inactivation kinetics plot using the Gauss-Eyring model (Fig. 8.4). At E = 20 kV/cm inactivation increased gradually with increasing energy input, while at E = 2.7 kV/cm the inactivation increased progressively when a threshold value was exceeded.

Although the conditions applied at moderate intensity PEF showed complete inactivation of *E. coli* and *L. monocytogenes* and high intensity PEF conditions did not (Fig. 8.4), we cannot conclude that these moderate intensity PEF conditions are more effective, as the high intensity PEF conditions were only evaluated up to a maximum temperature of about 58 °C. At this temperature of 58 °C, no inactivation at moderate intensity PEF was observed. To draw a conclusion about effectivity of moderate and high intensity PEF in products at variable pH, the high intensity PEF conditions should be measured in these products with maximum temperatures up to 70 °C.

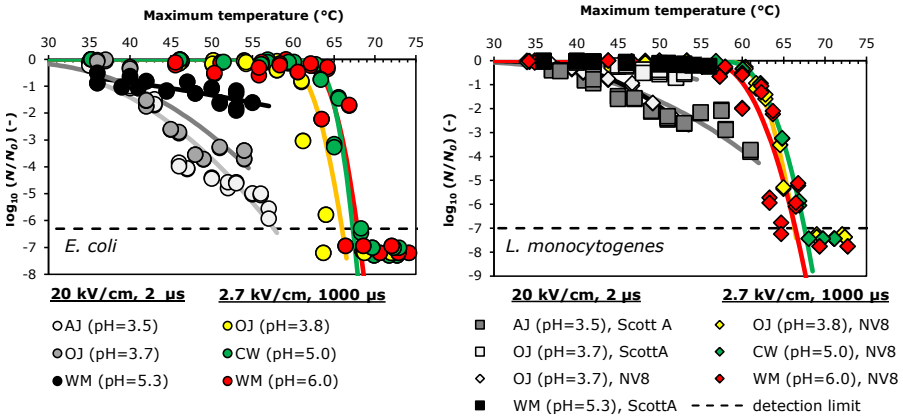


Figure 8.4. Reduction of viable counts $\log_{10}$ (N/N_0) of *E. coli* and *L. monocytogenes* in apple juice (AJ), orange juice (OJ), watermelon juice (WM) and coconut water (CW) after PEF treatment at high intensity pulses of E = 20 kV/cm with τ = 2 µs or reduced intensity pulses at E = 2.7 kV/cm with τ = 1000 µs. Symbols represent inactivation data at various conditions and matrices varying in pH (taken from in Fig. 2.2 and 6.4). Different strains of *L. monocytogenes* (Scott A and NV8) were used, while *E. coli* strain was the same in all experiments.

Studies on the effect of microbial inactivation using high intensity PEF on juices with neutral pH are limited. Reported studies showed about 3.5 $\log_{10}$ inactivation of *E. coli* in carrot juice, pH = 6.0 (Zhong et al., 2005) and also 3.5 $\log_{10}$ inactivation of *L. monocytogenes* in melon and watermelon juice at pH 5.8 and 5.5, respectively (Mosqueda-Melgar, Raybaudi-Massilia & Martín-Belloso, 2007), although energy input in these studies was calculated to be 1.8 – 90 times higher than used in our study. For dairy applications, electrical energy inputs of 120 – 240 kJ/kg and intermediate cooling are reported necessary for the inactivation of pathogenic micro-organisms (Buckow et al., 2014). This minimal requirement of 120 kJ/kg is in the range of that used at

moderate electric field strength of E = 2.7 kJ/kg used in our study, so future research could focus on the suitability of these moderate intensity PEF conditions for dairy applications.

For both high intensity as well as moderate intensity PEF conditions, the same *E. coli* strain (ATCC 35218) was used, but for studies with *L. monocytogenes,* two different strains were used. Initial experiments at E = 20 kV/cm were performed with *L. monocytogenes* Scott A, and *L. monocytogenes* NV8 was selected to evaluate conditions at moderate intensity PEF combined with heat, as the latter was reported to be the most heat-resistant strain (Van der Veen et al., 2009). Later in this thesis, PEF resistance at E = 20 kV/cm was evaluated for *L. monocytogenes* NV8, and this strain showed higher sensitivity to this PEF treatment than *L. monocytogenes* Scott A, with a reduction of 2.1 $\log_{10}$ vs. 0.7 $\log_{10}$ at T_{max} = 52 °C, respectively (Fig. 8.4).

More *L. monocytogenes* strains were evaluated at E = 20 kV/cm and this showed a large diversity in sensitivity towards the PEF treatment; varying in 0.5 $\log_{10}$ inactivation for the Scott A variant up to nearly 3 $\log_{10}$ inactivation for variants ATCC15313 and LO28 at temperature of 48 °C. Further analysis of the various strains, including *L. monocytogenes* Scott A, at E = 2.7 kV/cm will give more insights in the effectivity of this moderate intensity condition. Additional measurements on PEF and/or heat resistance of particular strains, variants or mutants might give more insight in the mechanism responsible for inactivation.

8.3.3 Comparison of PEF process to an equivalent thermal process

In Chapter 4, a rational thermodynamic model (Gauss-Eyring model) based on Gaussian distribution and Eyrings rate constant has been developed that can accurately account for non-linear inactivation kinetics of micro-organisms and enzymes exposed to a thermal and chemical (acid) treatment. The primary line shape of the Gauss-Eyring model is a bivariate log-normal distribution with temperature and time as independent variables. Remarkably, it was found necessary to treat model parameters as stochastic variables, as a result of the underlying physics of unfolding proteins (Chapter 4), which also described inactivation of micro-organisms very well, suggesting that protein unfolding is a determining factor for microbial inactivation when applying heat and chemical treatment in the same way as enzymes. The mathematical framework was provided by the Lumry-Eyring model for unfolding of proteins using transition state theory. The model parameters are standard activation enthalpy and entropy that are directly related to the reference temperature *Tr* and *Z*-value, commonly used for kinetic analysis in food microbiology. The model performance was evaluated using published data on enzyme inactivation and microbial inactivation over a broad range of temperatures and pHs (Chapter 4). The Gauss-Eyring model was evaluated for our own data in Chapter 5, and was a satisfactory model for both isothermal as well as isotime inactivation data for micro-organisms in this study. In addition, the aimed extrapolation to inactivation at short treatment times as used in PEF treatments could be made. Interestingly, the model fitted very well to the inactivation data taken from the same individual overnight cultures with very narrow confidence intervals for parameter estimates, but the goodness-of-fit assuming a single sub-population

decreased dramatically when introducing variability of inactivation data obtained from different overnight cultures, albeit that preculturing conditions were standardised for these cultures. This observation is in line with published results of Aryani and co-workers, who observed higher biological variability than experimental variability in the thermal inactivation of *L. monocytogenes* (Aryani et al., 2015) and *L. plantarum* (Aryani, Den Besten & Zwietering, 2016).

Explanations for this biological variability may include heterogeneity of the stock culture, introducing different single colonies to start the different cell cultivations, but could also be the result of spontaneous mutations during cultivation (Nahku et al., 2011). Each single overnight culture of *E. coli* was examined for the presence of sub poplations, to evaluate if this could explain the variability. The presence of two sub populations in each culture has been assumed, similar to the approach used for the examples described in Chapter 4. Evaluation of goodness-of-fit for the model with two subpopulations was done by evaluation of the estimated values for the model parameters. If acceptable fit parameters were found, model performance for each single overnight culture was evaluated by comparing the BIC values for models using one or two subpopulations. A model based on a single population was preferred for cultivates Ec-6 and Ec-7 (as coded to individual cultures as described in Table 5.2), while a model based on two populations was preferred for the other cultivates.

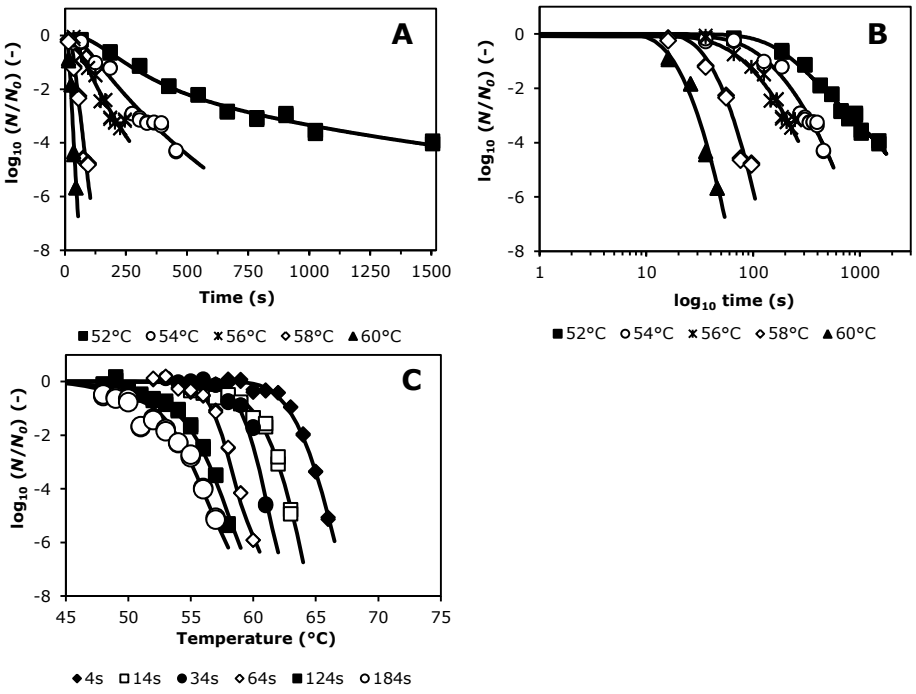


Figure 8.5. Thermal inactivation of *E.coli* in orange juice at isothermal (A, B) and isotime (C) conditions. Data points represent part of the experimental values obtained from individual overnight cultures from the same frozen stock, lines are the result of optimised fit of the Gauss-Eyring model fitted to (sub) populations.

In a next step, parameter reduction was carried out by sharing *Tr* and *Z* values for the different cultures, if possible. The reduction of number of parameters was optimised using the BIC criterion, balancing between the variance (indicated by SSr) and the remaining number of parameters.

Among the eight different grown cultures, 3 dominant sub populations can be resolved with distinct *Tr* and *Z* value (Table 8.2), but specific σ-value for each cultivation. By allowing two sub-populations being present in an overnight culture, the (remaining) variability in the model fit was substantially reduced, which could be observed by comparing the model fit (Fig. 8.5 and Fig. 5.3) and 95% CI of the parameter estimates (Table 8.2 and Table 5.2). Also the BIC value of this model fit (40 parameters, SSr = 19.3) with subpopulations was significantly lower (BIC = -631.2) than the BIC value obtained for the average of the individual fits (24 parameters, SSr=43.0) (BIC = -462.2). So, the above described approach and Gauss-Eyring model indicate that the observed heterogeneity towards heat treatment between the different overnight cultures could be explained by the presence of sub populations in the culture.

Table 8.2. Parameter estimations of the dominant (sub) populations in thermal inactivation of *E. coli* in orange juice (Chapter 5), using the Gauss-Eyring model. Values between brackets show the 95% confidence interval estimated for the model parameters. In the last column, the presence of the subpopulation in the different cultivations is indicated and corresponding σ-value for the (sub) population is given.

Subpopulation	*Tr* (*t*=1s) (°C)	*Z* (°C)	σ (°C)	Present in cultivation
1	62.4 [62.3-62.5]	5.3 [5.3-5.3]	1.73 [1.71-1.75]	Ec-2
			1.16 [1.14-1.18]	Ec-5
			1.14 [1.09-1.19]	Ec-6
2	66.8 [66.7-66.8]	6.2 [6.2-6.2]	0.96 [0.94-0.99]	Ec-1
			0.75 [0.71-0.78]	Ec-4
			0.78 [0.77-0.79]	Ec-7
3	71.2 [71.2-71.3]	8.5 [8.5-8.6]	0.78 [0.77-0.79]	Ec-2
			1.18 [1.14-1.21]	Ec-3
			1.90 [1.87-1.92]	Ec-8

In this study, thermal inactivation data was collected in capillary tubes subjected to different temperature-time combinations using a water bath to obtain a series of inactivation kinetics (Hauben et al., 1997; Mackey & Derrick, 1986; Michalski et al., 1999). Although this is a generally accepted method for microbial inactivation studies resulting in high reproducibility, the disadvantage of this method for our comparison is that the small liquid sample in the capillary requires conductive heating, whereas heating in a continuous flow PEF treatment is instantaneous and uniform by Joule heating. Advanced in-house small-scale continuous flow heaters with short holding times have been described for other studies (Berendsen et al., 2015; Van der Veen et al., 2009) that do not have this limitation. The study of Berendsen et al. (2015) showed that inactivation kinetics of *B. subtilis* spores in a batch heating system generally led to a significant lower *Z*-value than in a continuous-flow heating system. In this scenario, extrapolation and prediction of the inactivation curve at t = 1 s as shown in Chapter 5, a change from batch system to continuous-flow system is expected to have a larger impact on the *Z*-value than on the *Tc(t)* value (based on data of Berendsen et al., 2015). According to the Gauss-Eyring model an increase of the *Z*-value would lead to a shift of the inactivation curve (Fig. 5.2), meaning that a higher maximum temperature

would be required for the t = 2.3 s curve than is shown in Fig. 6.4. Thus, the presented comparison of the PEF data to the thermal inactivation data (Chapter 6), is possibly an underestimation of the non-thermal 'electroporation' effect.

The comparison of the PEF inactivation data to the thermal inactivation data as discussed in Chapter 6, indicated that three PEF processing conditions have an additional non-thermal pulse effect responsible for the inactivation: E = 20 kV/cm, τ = 2 µs, E = 15 kV/cm, τ = 2 µs and E = 2.7 kV/cm, τ = 1000 µs. To further evaluate this observation, the thermal inactivation data is plotted *versus* the PEF inactivation data for the various PEF treatment conditions using the same microbial strains in orange juice (Fig. 8.6). The data points of the indicated PEF conditions with an additional non-thermal pulse effect are located to the left of the thermal inactivation line, clearly showing this additional non-thermal pulse effect. Data points belonging to E = 0.9 kV/cm, τ = 1000 µs were less effective than the thermal treatment, conceivably caused by difference between batch and continuous-flow experiments as mentioned before. The conditions at E = 0.9 kV/cm have no electroporation effect and therefore was considered as a thermal reference process at continuous flow, and will be used for comparison. Data points belonging to PEF conditions at E = 10 kV/cm and at E = 2.7 kV/cm with τ = 100 and τ = 15 µs are generally displayed within the 95% CI interval of the batch thermal treatment, and statistical comparison performed in Chapter 6 showed that these conditions were not significantly different from the (batch) thermal reference process when compared for temperatures required for 5 $\log_{10}$ reduction. The same comparison to a thermal reference process at continuous flow could be made. Comparison of the PEF treated data at E = 10 kV/cm to E = 0.9 kV/cm showed no significant differences, suggesting that this condition did not comprise an additional non-thermal pulse effect. Again, the data of *L. monocytogenes* was not used for this comparison, as virtually no inactivation at this condition was observed at maximum temperatures. Comparison of the PEF treated data at E = 2.7 kV/cm, τ = 15 µs and τ = 100 µs showed a significant difference for all tested micro-organism when compared to condition E = 0.9 kV/cm, except for *E. coli* at τ = 100 µs.

In conclusion, additional non-thermal pulse effects were established for high intensity pulses at E = 15 -20 kV/cm and short pulse width, but also for moderate intensity pulses at E = 2.7 kV/cm at long pulse width. Dependent on the chosen thermal reference, additional non-thermal pulse effects may be assigned to shorter pulse widths at E = 2.7 kV/cm as well.

In addition to the aforementioned paragraph where we focussed on the question 'is there a non-thermal pulse effect', it is also relevant to quantify the temperature reduction caused by a moderate or high intensity PEF treatment compared to a thermal process. Therefore, the temperature required to obtain a 5 $\log_{10}$ reduction for a specific PEF treatment was compared to the temperature required for a thermal reference (shown in Table 6.3), being either a batch treatment (thermal reference at t = 2.3 s) or continuous flow process (E = 0.9 kV/cm), and temperature differences are shown in Fig. 8.7. Note that the trends for the thermal batch and continuous flow treatment are similar, and differences can only be seen in absolute values.

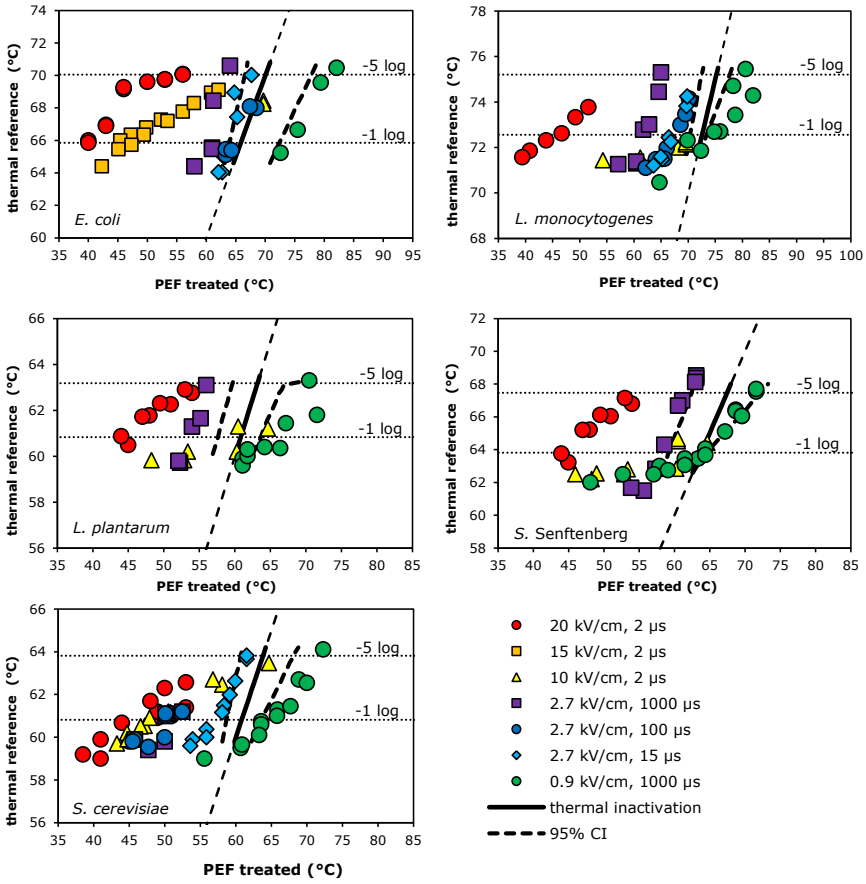


Figure 8.6. Reduction of viable counts $\log_{10}(N/N_0)$ of *E. coli, L. monocytogenes, L. plantarum, S.* Senftenberg and *S. cerevisiae* in orange juice as a function of temperature applied by the PEF treatment and thermal reference obtained using a batch process. Data of thermal reference and 95% CI is similar to data shown in Fig. 6.4, and PEF data has been shown in Fig. 6.4 and 6.6.

Although the tested micro-organisms responded differently to the PEF and thermal treatment, the largest temperature reduction was observed at high intensity PEF condition E = 20 kV/cm, τ = 2 µs, followed by moderate intensity PEF condition E = 2.7 kV/cm, τ = 1000 µs. Interestingly, the absolute differences between the various species are smaller at the mentioned moderate intensity PEF condition than at high intensity PEF, suggesting for another underlying mechanism responsible for electroporation. Evaluation of the effect of this temperature reduction on quality will be discussed in section 8.4.

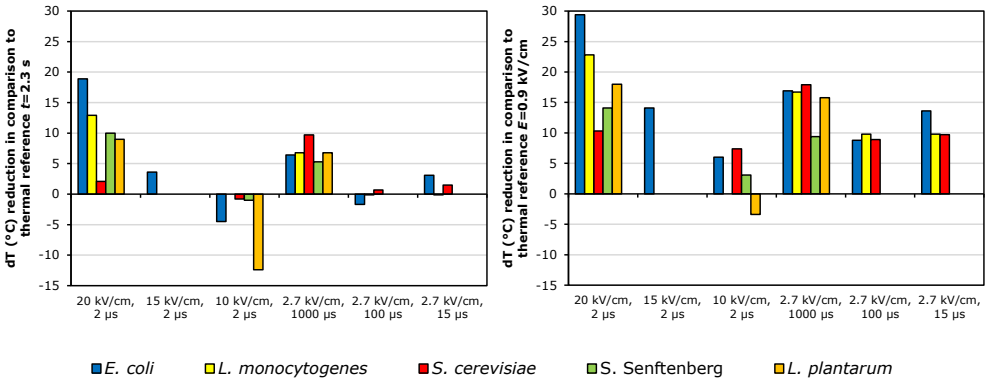


Figure 8.7. Temperature reduction between specific PEF conditions and thermal reference process to obtain a 5 log reduction of tested micro-organisms, based on Table 6.3. Comparison to a thermal reference at t = 2.3 s (batch) is shown left and comparison to E = 0.9 kV/cm (continuous-flow) is shown right.

8.3.4 Proposed mechanism for electroporation

The work described in this thesis clearly shows that additional non-thermal pulse effects can be observed for two different process windows: at high intensity pulse conditions, but also at moderate intensity pulse conditions. Microbial inactivation using these two process windows resulted in different responses to the variations in microbial morphology and matrix composition. Different underlying mechanisms might explain the different responses for inactivation, as briefly mentioned in Chapter 6.

The generally accepted explanation for electroporation is the generation of pores in the lipid domain of a cell membrane by the application of the external electric field, as discussed in Chapter 1. Once a critical transmembrane potential is induced, a time dependent transition occurs as long as the field is maintained above a critical value, typically milliseconds. As soon as the field intensity drops below the critical value, a stabilization process starts, which brings the membrane to the permeabilised state (ms). In a next step, resealing occurs slowly, and takes seconds to minutes. Finally, some changes in the membrane properties remain present for a longer time scale (up to hours), before cell behaviour is back to normal (Teissié, Golzio & Rols, 2005; Tsong, 1990).

The size and shape of a cell determine the transmembrane voltage threshold, where smaller sized cells have an increased transmembrane voltage threshold, and therefore require larger electric field strengths to induce pore formation. Dependent on the pulse conditions applied (electric field strength and amount of energy) and environmental conditions, the cell can either recover from the stress or will be inactivated. The transmembrane potential or transmembrane pH gradient was not measured in this thesis project, but based on the data obtained we can speculate about the damage caused by the different PEF conditions used.

PEF conditions at E = 15 - 20 kV/cm and τ = 2 µs showed an additional non-thermal pulse effect in orange juice (pH = 3.8). Application of the same PEF condition of E = 20 kV/cm led to a reduced inactivation in low acidic pH (pH = 5.3), and it is suggested that application of these PEF conditions led to reversible electroporation, and that inactivation

in acidic conditions is the result of additional stress delivered by the matrix pH. This observation is in line with the observation of Aronsson & Rönner (2001), who observed more inactivation when more stress hurdles, including pH stress, were combined in a PEF process. PEF conditions applied at E = 2.7 kV/cm and τ = 1000 µs, shown in this thesis, resulted in an additional non-thermal pulse effect next to the thermal element responsible for microbial inactivation in orange juice. The pH of the matrix showed no influence on the microbial inactivation, and it is therefore hypothesised that this PEF condition results in irreversible electroporation, when a specific threshold was exceeded.

This 'irreversible electroporation' at conditions E = 2.7 kV/cm and τ = 1000 µs might be explained by the higher energy density per pulse used for this condition compared to the other evaluated conditions (Table 8.1). Higher electric field intensity and long pulse width lead to the formation of larger sized pores as described in a previous study (Tsong, 1990), and therefore more electropermeabilization for a longer time across the membrane could occur, conceivably resulting in more damage. Only remark to this theory is that the applied electric field strength should exceed the critical transmembrane potential of the specific micro-organism, and the E = 2.7 kV/cm used in our work might be too low.

As mentioned in section 1.3.4, different theories of electroporation have been investigated by various research groups, including electroporation occurring in the protein channels or transport systems (Tsong, 1990). The cell membrane contains different types of membrane transport systems that are imbedded in the lipid bilayer, and several of these pumps and specific channels for cations and anions are voltage sensitive. Voltage-dependent ion channels are a class of transmembrane proteins that are activated by changes in the electrical membrane potential (Graham & Craigen, 2004; Ulmschneider et al., 2013), and act as gated pores to allow the passage of ions across the membrane. In Fig. 8.8, the three different states of a voltage-gated ion channel are shown; closed (resting state), open (activated state) and inactivated.

The difference in transmembrane potential ($\Delta\varphi_m$) to open an ion channel in *E. coli* is estimated to be below 100 mV (Schoenbach et al., 1997), which is considerably lower than the 1000 mV required for pore formation in the lipid bilayer of the membrane (Hülsheger, Potel & Niemann, 1981). Similar $\Delta\varphi_m$ values and ratio between the values are mentioned by Tsong (1991), who estimated that 50 mV is needed to open ion channels, and 150 – 500 mV to form a pore in the lipid bilayer of mammalian cells. Based on the above, it could be expected that when an external field strength is applied, voltage sensitive channel proteins will open before the critical transmembrane potential for electroporation of the lipid bilayer is reached and electroporation in the lipid domain starts. Single channel measurements indicate that the transit time for the channel opening and closing is typically in the microsecond time range or faster (Berg, Tymoczko & Stryer, 2002). When protein channels are opened by an external field, they may experience an extreme local current density, leading to irreversible denaturation due to Joule heating or electromodification of their functional groups (Tsong, 1991). When the field is turned off, a denatured membrane protein may either renature slowly or is unable to restore its initial conformational state. If the duration of an electric pulse is prolonged to ms or longer, these heat denatured pores will expand even further due to the mechanical effect of the hydrodynamic flow induced by the electro-osmosis of ions (Dimitrov & Sowers, 1990).

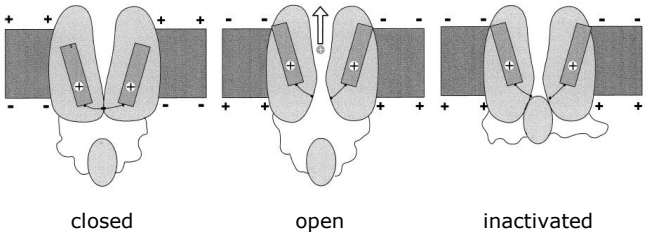


Figure 8.8. Different stages of voltage-gated Na^+ channel, resting state (ion channel closed), activated state (ion channel open) and inactivated state (Bezanilla, 2000).

PEF conditions at E = 2.7 kV/cm and τ = 1000 µs showed to be more effective than at τ = 100 µs or τ = 15 µs. Based on Tsongs theory, a pulse duration of τ = 1000 µs might be long enough to denaturate transport proteins. Because the inactivation data at E = 2.7 kV/cm (for any pulse width) was more effective than the inactivation at E = 0.9 kV/cm, τ = 1000 µs (Chapter 6), we expect that both the electric field strength and pulse width effect the inactivation of transport proteins. A schematic representation how these parameters might affect electroporation is shown in Fig. 8.9, indicating that high threshold voltages are required for electroporation in lipid domain and lower threshold voltage combined with a long pulse duration are required for electroporation in the transport proteins.

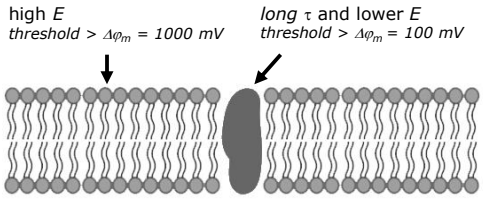


Figure 8.9. Hypothesised schematic representation of electroporation in the lipid domain and transport protein channel, with their threshold values for *E. coli* (Schoenbach et al., 1997).

As an example, the transmembrane voltage (V_m) for the micro-organisms examined in this study was approximated as a function of electric field strength (Fig. 8.10), assuming several simplifying restrictions, as given in Eq. 1.4. The shape factor f (A) of the rod shaped bacteria could be calculated according to Eq. 1.5b, but can be approximated by assuming a cylinder with two hemispheres on each end (Hülsheger, Potel & Niemann, 1983). The shape factor (f (A)) for such particles is given by Eq. 8.2, with l as length, d as diameter.

Note that for spherical shaped cells the form factor is 1.5. As the actual cell size was not measured in this thesis, characteristic dimensions were taken from literature (Bergey, 1986) as shown in Table 8.3.

$$f(A) = l / (l - 0.33 \cdot d) \qquad \text{(Eq. 8.2)}$$

Table 8.3. Cell diameter (*d*), length (*l*) and shape factor (*f*(A)) for micro-organisms tested in this study. Data taken from Bergey (1986).

	***d* (µm)**	***l* (µm)**	***f* (A)**
E. coli	1.3	4.0	1.12
L. plantarum	1.1	5.5	1.07
L. monocytogenes	0.5	1.5	1.12
S. cerevisiae	4.0	10.0	1.15
S. Senftenberg	1.1	3.5	1.12

As a second simplification, the factor $f(\lambda)$ describing the electrical conductivities of the suspending medium, plasma medium, cell membrane, ratio of the membrane thickness and the cell radius, can be approximated by 1 (Neumann, 1989). The angle Θ indicates the directions of the radius at the point considered on the cell and the field, and coincides with the direction of the electric field when $\Theta = 0$ (and cos (Θ) = 1). Hence, the highest membrane potential difference is assumed to occur at the two poles of the cell in field direction. For calculations Θ was assumed to be 0.

The transmembrane voltage is given by the approximation $V_m \approx \Delta\varphi_m$ (Neumann, 1989). The membrane potential $\Delta\varphi_m$ is defined as the difference between inside and outside the cell. The threshold values of this transmembrane potential to open the protein channel and to induce pore formation in the lipid domain, as indicated by Schoenbach et al. (1997) for *E. coli,* are indicated by dotted lines in Fig. 8.10. A reduced electric field strength of about ~3 kV/cm showed to be sufficient to open the voltage-gated protein channels of all five micro-organisms (Fig. 8.10). Higher electric field strengths are required to induce the formation of pores in the lipid domain. Interestingly, in this theory this difference in cell size of the different micro-organisms plays a smaller role when opening the protein channel than when inducing a pore in the lipid bilayer.

In general, the experimental data shown in Fig. 6.4 (Chapter 6) are in agreement with this example visualized in Fig. 8.10, showing smaller differences between the different micro-organisms when applying an electric field strength of E = 2.7 kV/cm than when a higher electric field strength of E = 10 or 20 kV/cm is applied. Fig. 8.10, clearly shows that the calculations for most sensitive (*S. cerevisiae*) and the most resistant (*L. monocytogenes*) micro-organism are in line with the experimental data. For the other micro-organisms, some differences between experimentally determined data were observed possibly caused by assumptions of the estimated cell size. Furthermore, the additional non-thermal effects responsible for electroporation in the lipid domain are theoretically found above field strengths of 10 kV/cm for the bacteria (Fig. 8.10), which could be confirmed with the experimental data showing this effect for *E. coli* at E > 15 kV/cm.

In conclusion, based on the above discussed points, it seems plausible that the electroporation effect on the cell membrane, can occur both by pore formation in the lipid domain and specifically on the protein channels in the lipid domain, and that electric field strength and pulse width determine which mechanism dominated. Further work is required to investigate this proposed mechanism.

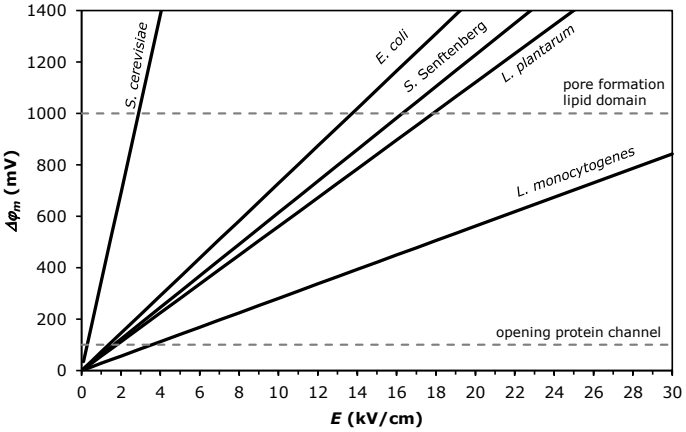


Figure 8.10. Example of calculated transmembrane voltage or transmembrane potential $\Delta\phi$ as a function of electric field strength, for the examined micro-organisms in this study, according to Eq. 8.2 and with characteristic dimension taken from Bergey (1986) (Table 8.3) and $\Theta = 0$. Critical transmembrane potential to open protein channels (< 100 mV) and form pores in lipid domain cells (1000 mV) (Schoenbach et al, 1997) are indicated.

8.4 Food quality attributes, enzymes and shelf life

8.4.1 Food quality attributes and enzyme inactivation

The impact of PEF processing at moderate electric field strength (E = 0.9 and 2.7 kV/cm) and pulse width of τ = 1000 µs at variable maximum temperatures on quality attributes of freshly squeezed orange juice were studied and compared to untreated juice and the impact of two conventional thermal processes using either mild or severe pasteurisation conditions (Chapter 7). No differences for pH and soluble solids were found after application of any treatment, and only small differences were observed for colour and vitamin C (ascorbic acid and dehydroascorbic acid) content after processing, mainly for conditions applied at higher temperature. Also the impact of processing on volatile flavour components was moderate, with minimal changes in PEF processed juice up to temperatures of 78 °C. Some deviations between moderate intensity PEF treated and thermally processed orange juice were found for individual compounds, where moderate intensity PEF treated resulted in a better retention of the flavour compounds. A minimal amount of 'heat-marker' compounds α-terpinene and 4-terpineol were formed at the most intense conditions, indicating that the thermal load was minimal even at the most intense PEF treatment. The only real impact of processing was measured in the enzyme activity of pectin methylesterase (PME) responsible for undesired cloud instability. Dependent on the maximum temperature applied by the PEF process, a reduction of the remaining enzyme activity was measured, up to levels below 1.0 x 10^{-4} PEu at temperatures of 78 °C or higher, mentioned as critical value to obtain shelf stable juices (Braddock, 1999a). So, dependent on the intended shelf life and desired cloud stability, adequate PEF processing conditions could be selected.

Due to the relatively low pH of the orange juice (pH = 3.5 and pH = 3.0), less chemical reactions induced by processing occurred than was expected beforehand, that could result in loss of ascorbic acid content, colour and flavour compounds. However, it should be mentioned that most studies showed quality loss during shelf life and not directly after processing (Leizerson & Shimoni, 2005b; Min et al., 2003; Vervoort et al., 2011; Vervoort et al., 2012; Yeom et al., 2002).

Surprisingly, the moderate intensity PEF treatment showed more inactivation of PME than was expected beforehand, based on the thermal-time requirements reported in literature. Probably the low pH of the juice played a role, reducing the temperature-time requirements to obtain the desired inactivation (Eagerman & Rouse, 1976; Rouse & Atkins, 1953; Van den Broeck et al., 1999). It cannot be concluded whether or not the moderate intensity PEF process has an additional non-thermal effect compared to a thermal process for PME inactivation, as only two thermal reference conditions were measured, which is insufficient for a kinetic comparison with a thermo-labile and thermo-stable fraction. Future work should incorporate sufficient thermal references with variable temperature-time combinations, so the Gauss-Eyring model can be used to predict the thermal inactivation of both thermo-labile and thermo-stable PME fractions at short holding times, similar to the procedures described in Chapter 3 and 4 for microbial inactivation. Comparison of this thermal inactivation curve as well as the effect of the moderate intensity PEF treatment on PME activity should be taken from the same batch, similar to the procedure in Chapter 6.

Research of Schilling et al. (2008) reported a synergistic effect of high intensity PEF (E = 25 kV/cm) and heat at inlet temperatures of 60 °C to inactivate POD and PPO enzymes. However, it could not be excluded that local hot spots in the PEF treatment chamber might be responsible for a higher enzyme inactivation in the combined thermal-PEF treatment as suggested by Van Loey, Verachtert & Hendrickx (2002). This issue could also occur in our situation, while the effect could not be incorporated in the thermal inactivation model that acts as a reference (Jaeger et al., 2010). Another factor that might contribute to (additional) enzyme inactivation is by electrochemical reactions at the electrode surface. Although pulsed modulation and proper electrode material were used to reduce these reactions (Morren, Roodenburg & de Haan, 2003), it could not be excluded that no electrochemical reactions, including a local pH shift, occur at pulse duration of 1000 µs (Chapter 7), possibly resulting in a reduced enzyme activity.

8.4.2 Shelf life

Besides microbial inactivation and minimal impact of processing on food nutrients and vitamins, the stability of the product during storage is important. The shelf life of a product is defined as the period in which the product is still acceptable for human consumption, and is determined by the quality of the raw material, product formulation, processing, packaging and storage conditions (Jaeger, Meneses & Knorr, 2014). The application of high intensity PEF pulses to extend the shelf life of a fresh fruit smoothie has been compared to untreated smoothie by examination of microbial spoilage during shelf life (Chapter 3). Variations were made in the settings of the most important

process parameter, electric field strength (E = 13.5 – 24.0 kV/cm), leaving other PEF process parameters constant.

The case study on apple-strawberry-banana smoothie shows that spoilage of untreated smoothie is dominated by the outgrowth of yeasts, while initial number of moulds present in the smoothie declined during the storage of untreated smoothie. PEF inactivated most of the yeasts present in the fresh smoothie, thereby providing outgrowth opportunities for the moulds, which were visually observed after 14 days (7 °C) or 18 days (4 °C). The intensity of the electric field strength both affected the log reduction of yeast and the lag-time, the time the cell or spore requires to grow out. A similar effect of electric field strength on the degree of inactivation has been observed for moulds, although field strength did not influence the time to visual mould growth. This result suggests that outgrowth of moulds during storage could be ascribed to mould spores surviving the PEF process, or that a contamination with mould spores occurred after the processing or during bottling.

The current data does not allow discrimination between outgrowth of vegetative mycelium and mould spores. For future work it is recommended to include an additional heat treatment of the sample and plating step to inactivate the vegetative mycelium, to determine the number of spores present. Future research could also focus on the effect of both moderate as well as high intensity PEF with variable maximum temperatures to inactivate vegetative hyphae and mould spores in different matrices, including viscous products, or inoculate with spores to test this hypothesis.

The shelf-life extension of the PEF treated fruit smoothie was significantly shorter than when similar PEF conditions were applied on fruit juices. A study on orange juice with similar PEF conditions (E = 23 kV/cm, τ = 2 µs, total treatment time = 36 µs, T_{in} = 38 °C, T_{out} = 58 °C, w = 76 kJ/kg) showed a shelf life extension up to 2 months at refrigerated (4 °C) storage, as yeast and mould counts were below detection limit at t = 58 d, while untreated orange juice was spoiled between 9 and 20 days (Timmermans et al., 2011). Similar observations of high intensity PEF treated orange juice stored at 4 °C have been reported by others, with shelf life extension up to 22 days (E = 20 kV/cm, w = 120 kJ/kg) (Toepfl, 2011), 112 days (E = 35 kV/cm, total treatment time = 59 µs, T_{in} = 25 °C, T_{out} = 59 °C) (Yeom et al., 2002) and 196 days (E = 40 kV/cm, total treatment time = 97 µs, T_{in} = 45 °C, T_{out} = 65 °C) (Min et al. 2003), while untreated juice was spoiled by yeasts and moulds after 4 – 8 days, 8 – 14 days and 14 – 21 days, respectively. The relatively short shelf life extension for the reported fruit smoothies might be explained by the matrix composition. Studies on the effect of PEF lethality on fruit smoothie products are limited, but a study of Walkling-Ribeiro et al. (2008b) showed a reduced PEF effectivity in the inactivation of *E. coli* in tropical fruit smoothie when compared to juice matrices. Possibly, the higher viscosity and presence of seeds influences the flow behaviour and residence time distribution in the treatment chamber, resulting in treatment time variations or inhomogeneous distribution of the electric field, leading to a non-uniform treatment of the smoothie. Whether this can explain the observed effect remains to be determined.

8.5 Conclusions and future perspectives

This thesis provided a systematic evaluation of individual PEF process parameters electric field strength, pulse width and temperature. This knowledge can be used to optimize PEF pasteurisation processes for fruit juices. In short, the conclusions for each research question will be stated below:

- Combination of heat and moderate intensity PEF at E = 2.7 kV/cm and τ = 1000 µs can be used as an effective PEF process, as alternative to the currently used high intensity PEF conditions of E = 15 − 20 kV/cm, used to preserve fruit juices.
- Besides electric field strength, also pulse width was identified as critical process parameters to achieve microbial inactivation. In addition, temperature played was an important factor in PEF efficiency, both the inlet temperature as well as maximum temperature, related to the required electrical energy for inactivation. The specific values of these parameters required to achieve inactivation are dependent on the microbial characteristics and properties of the product matrix, where high field strength/short pulse width showed more variability for the different micro-organisms tested than moderate field strength/long pulse duration. Furthermore, moderate field strength/long pulse width was not influenced by the pH of the matrix, while high field strength/short pulse width showed to be less effective at low acidic pH.
- Comparison of the PEF process to an equivalent thermal process with same holding time showed that PEF conditions at E = 15 − 20 kV/cm, τ = 2 µs and at E = 2.7 kV/cm, τ = 1000 µs comprised a non-thermal pulse effect responsible for microbial inactivation.
- The intensity of the electric field strength affected the $\log_{10}$ reduction of yeasts and moulds for a high intensity PEF treatment. The PEF treatment results in a shift on spoilage population: in untreated smoothie yeasts cause spoilage after 8 days (stored at 4 or 7 °C), whereas in PEF treated smoothie yeasts were (partly) inactivated which provided outgrowth opportunities for moulds, and led to spoilage by moulds after 14 days (stored at 7 °C) or 18 days (stored at 4 °C).
- A case study on the effect of these moderate field strength/long pulse width conditions in fresh orange juice showed minimal impact on vitamin C content, colour and volatile flavour profile when compared to untreated juice, while the required inactivation of PME enzyme, relevant for fruit juice industry, was obtained.

The work described in this thesis shows that PEF at moderate field strength/long pulse duration could be used as an effective pasteurisation process with minimal impact on nutritional aspects. Future research has to be carried out to determine the effect of these process conditions during refrigerated shelf life, including analysis of microbial outgrowth and enzyme stability, as well as impact on quality attributes and sensorial aspects. Potentially, moderate intensity PEF could also be used for processing of ambient stored juice. Before evaluation of these moderate field strength/long pulse width conditions at ambient storage conditions will be carried out, it is advised to evaluate if *Alicyclobacillus* spores could be inactivated using these PEF conditions, as

these spores can germinate and grow out in high acid juices when stored at ambient temperature or higher and spoil the juice by production of off-flavours.

Future research could also focus on the effect of the moderate field strength/long pulse width process on enzyme inactivation, to evaluate if these PEF conditions comprise a non-thermal effect responsible for enzyme inactivation. The Gauss-Eyring model could be used to model this effect. In addition, the potential of this model could be further explored by evaluation of other data-sets with expected variability or heterogeneity.

Before the optimal PEF processing conditions could be established, multiple tests of the product could be evaluated to include possible effects of biological variation, in for instance enzyme activity. In addition, the effect of microbial inactivation of a cocktail of strains or species with reduced inoculation level should be evaluated, as in the current study only single strains with a high inoculation level were evaluated.

Another point of interest for future studies is to evaluate the impact of moderate field strength/long pulse width on microbial inactivation in more complex matrices, including viscous products, or products that contain proteins, fats and fibres. It is expected that the influence of particles has the same disturbing effect on the electric field distribution at moderate and high intensity PEF. However, the absolute effect of a disturbance and change in electric field strength is larger for high intensity PEF than for moderate intensity electric field strength, expecting to result in a more homogeneous field distribution and higher effectivity for moderate intensity PEF than high intensity PEF. As the microbial inactivation with reduced electric field strength conditions were pH-independent, dairy products are an interesting class of products to be treated with these new conditions, possibly requiring a lower PEF energy use than the reported 120 – 240 kJ/kg for high intensity PEF (Buckow et al., 2014; Mathys et al., 2011).

In addition, future work could focus on the minimal requirements of the PEF process to have an effective and economical PEF process for pasteurisation. In the research described in this thesis, the preheating temperature was set at 36 °C in all experiments evaluating microbial inactivation, resulting in a large amount of PEF energy for the moderate intensity PEF conditions. Furthermore, heated product was immediately cooled down to keep the best quality as possible. Additional experiments have to be carried out to determine the minimal ΔT requirements of the moderate intensisty PEF process to obtain microbial and enzyme inactivation, as well as the effect of a higher heat load on the quality of the treated product. Furthermore, the research in this thesis was carried out at laminar flow conditions, and future research needs to demonstrate the effect under turbulent flow conditions to predict the effectivity at industrial scale.

Finally, the proposed future work will give indirectly more insight in the possible mechanism responsible for inactivation, though performance of diagnostic measurements is recommended to unravel the critical, detailed mechanism and minimal process requirements responsible for inactivation.

In conclusion, this thesis provided novel insight in the use of pulsed electric field processing as a mild pasteurisation process with improved quality as alternative to thermal pasteurisation of fruit juice. The work described in this thesis resulted in more insight in the individual effects of electric field strength and pulse duration, and presented an effective combination of long pulse duration and moderate intensity

electric field strength as alternative PEF condition to the currently used high intensity electric field strength conditions for preservation. In addition, these moderate intensity/ long pulse duration conditions are promising for industrial application, as they showed to be less sensitive for differences in the characteristics of the micro-organisms than high intensity PEF conditions and they are effective in both high-acid as well as low-acid products.

References

Abee, T., Koomen, J., Metselaar, K.I., Zwietering, M.H. & den Besten H.M.W (2016). Impact of Pathogen Population Heterogeneity and Stress-Resistant Variants on Food Safety. *Annual Reviews Food Science and Technology, 7*, 439–45.

Abram, F., Smelt, J.P.P.M., Bos, R. & Wouters, P.C. (2003). Modelling and optimization of inactivation of *Lactobacillus plantarum* by pulsed electric field treatment. *Journal of Applied Microbiology, 94,* 571-579.

Acree, T. & Arn, H. (2004). Flavornet and human odor space. http://www.flavornet.org. Last accessed on 15th of May 2017.

Ahern, T.J. & Klibanov, A.M. (1985). The Mechanism of Irreversible Enzyme Inactivation at 100 °C, *Science*, *228*, 1280-1284.

AIJN (2010). Liquid Fruit Market Report 2010. *AIJN European Fruit Juice Association,* Brussels.

AIJN (2016). Liquid Fruit Market Report 2016. *AIJN European Fruit Juice Association,* Brussels.

AIJN (2017). Liquid Fruit Market Report 2017. *AIJN European Fruit Juice Association,* Brussels.

Álvarez, I., Raso, J., Palop, A. & Sala, F.J. (2000). Influence of different factors on the inactivation of *Salmonella senftenberg* by pulsed electric fields. *International Journal of Food Microbiology, 55,* 143-146.

Álvarez, I., Pagán, R., Raso, J. & Condón, S. (2002). Environmental factors influencing the inactivation of *Listeria monocytogenes* by pulsed electric fields. *Letters in Applied Microbiology, 35,* 489-493.

Álvarez, I., Pagán, R., Condón S. & Raso, J. (2003a). The influence of process parameters for the inactivation of *Listeria monocytogenes* by pulsed electric fields. *International Journal of Food Microbiology, 87,* 87-95.

Álvarez, I., Raso, J., Sala, F.J. & Condón, S. (2003b). Inactivation of *Yersinia enterocolitica* by pulsed electric fields. *Food Microbiology, 20,* 691-700.

Álvarez, I., Virto, R., Raso, J. & Condón, S. (2003c). Comparing predicting models for *Escherichia coli* inactivation by pulsed electric fields. *Innovative Food Science and Emerging Technologies, 4,* 195-202.

Álvarez, I., Mañas, P., Condón, S. & Raso, J. (2003d). Resistance variation of *Salmonella enterica* serovars to pulsed electric fields treatment. *Journal of Food Science, 68,* 2316-2320.

Álvarez, I., Condón, S. & Raso, J. (2006). Microbial inactivation by pulsed electric fields. In: Raso, J. & Heinz, V. (Eds.). *Pulsed Electric Fields Technology for the Food Industry. Fundamentals and application.* Springer, New York.

Anderson, W.A., McClure, P.J., Baird-Parker, A.C. & Cole, M.B. (1996). The application of a log-logistic model to describe the thermal inactivation of *Clostridium botulinum* 213B at temperatures below 121.1°C. *Journal of Applied Bacteriology, 80,* 283-290.

Aragao, G.M.F., Corradini, M.G., Normand, M.D .& Peleg, M. (2007). Evaluation of the Weibull and log normal distribution functions as survival models of *Escherichia coli* under isothermal and non isothermal conditions. *International Journal of Food Microbiology, 119*, 243–257.

Aronsson, K. & Rönner, U. (2001). Influence of pH, water activity and temperature on the inactivation of *Escherichia coli* and *Saccharomyces cerevisiae* by pulsed electric fields. *Innovative Food Science & Emerging Technologies, 2,* 105-112.

Aronsson, K., Lindgren, M., Johansson, B.R. & Rönner, U. (2001). Inactivation of microorganisms using pulsed electric fields: the influence of process parameters on *Escherichia coli, Listeria innocua, Leuconostoc mesenteroides* and *Saccharomyces cerevisiae. Innovative Food Science and Emerging Technologies, 2,* 41-54.

Aronsson, K., Rönner, U. & Borch. E. (2005). Inactivation of *Escherichia coli, Listeria innocua* and *Saccharomyces cerevisiae* in relation to membrane permeabilization and subsequent leakage of intracellular compounds due to pulsed electric field processing. *International Journal of Food Microbiology, 99,* 19-32.

Aryani, D.C., Den Besten, H.M.W., Hazeleger, W.C. & Zwietering, M.H. (2015). Quantifying variability on thermal resistance of *Listeria monocytogenes. International Journal of Food Microbiology, 193,* 130-138.

Aryani, D.C., Den Besten, H.M.W. & Zwietering, M.H. (2016). Quantifying variability in growth and thermal inactivation kinetics of *Lactobacillus plantarum. Applied and Environmental Microbiology, 82,* 4896-4908.

Ashurst, P. (2016). The stability and shelf life of fruit juices and soft drinks. In: Subramaniam, P. (Ed.). *The stability and shelf life of Food.* Woodhead publishing.

Athena Visual Studio (2016). Athena Visual Studio. Software for Modeling, Estimation and Optimization, version 14.2. http://www.athenavisual.com

Baldwin, D.E. (2012). Sous vide cooking: a review. *International Journal of Gastronomy and Food Science, 1,* 15-30.

Barba, F.J., Parniakov, O., Pereira, S.A., Wiktor, A., Grimi, N., Boussetta, N., Saraiva, J.A., Raso, J., Martin-Belloso, O., Witrowa-Rajchert, D., Lebovka, N. & Vorobiev, E. (2015). Current applications and new opportunities for the use of pulsed electric fields in food science and industry. *Food Research International, 77,* 773-798.

Barbosa-Cánovas, G.V., Góngora-Nieto, M.M., Pothakamury, U.R. & Swanson, B.G. (1999). Fundamentals of High-Intensity Pulsed Electric Fields (PEF). In: Barbosa-Cánovas, G.V., Góngora-Nieto, M.M., Pothakamury, U.R., & Swanson, B.G. (Eds.). *Preservation of Foods with Pulsed Electric Fields.* Academic Press.

Battcock, M. & Azam-Ali, S. (1998). Fermented fruits and vegetables, a global perspective. FAO Agricultural Service Bulletin, 134, FAO, Rome.

Bazemore, R., Goodner, K. & Rouseff, R. (1999). Volatiles from unpasteurized and excessively heated orange juice analysed with solid phase microextraction and GC-Olfactometry. *Food Chemistry and Toxicology, 64,* 800-803.

Bendicho, S., Barbosa-Cánovas, G.V. & Martín, O. (2002). Reduction of protease activity in simulated milk ultrafiltrate by high intensity pulsed electric fields. *Institute of Food Technologists Annual Meeting.* Anaheim, California, USA. Extended abstracts, 91E-19.

Bendicho, S., Estela, C., Giner, J., Barbosa-Cánovas, G.V. & Martín, O. (2002). Effects of high intensity pulsed electric field and thermal treatments on lipase from *Pseudomonas fluorescens. Journal of Dairy Science, 85,* 19-27.

Bendicho, S., Barbosa-Cánovas, G.V. & Martín, O. (2003). Reduction of protease activity in milk by continuous flow high-intensity pulsed electric field treatments. *Journal of Dairy Science, 86,* 697-703.

Berendsen, E.M., Zwietering, M.H., Kuipers, O.P. & Wells-Bennik, M.H.J. (2015). Two distinct groups within the *Bacillus subtilis* group display significantly different spore heat resistance properties. *Food Microbiology, 45,* 18-25.

Berg, J.M., Tymoczko, J.L. & Stryer, L. (2002). Membrane channels and pumps. In: Berg, J.M., Tymoczko, J.L. & Stryer, L. (Eds.). *Biochemistry*. Fifth Edition, Freeman and Company, New York.

Bergey, D.H. (1986). Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore.

Berk, Z. (2016a). Production of single-strength citrus juices. In: Berk, Z. (Ed.) *Citrus juice processing*. Academic Press, Elsevier.

Berk, Z. (2016b). Production of citrus juice concentrates. In: Berk, Z. (Ed.) *Citrus juice processing*. Academic Press, Elsevier.

Betts, R. (2010). Microbial update yeasts and moulds. *International Food Hygiene, 21,* 11-13.

Bevilacqua, A., Speranza, B., Sinigaglia, M. & Corbo, M.R. (2015). A focus on the death kinetics in predictive microbiology: benefits and limits on the most important models and some tools dealing with their application in foods. *Foods*, *4,* 565-580.

Bezanilla, F. (2000). The voltage sensor in voltage-dependent ion channels. *Physiological Reviews, 80,* 555-592.

Bigelow, W.D. (1921). The logarithmic nature of thermal death time curves. *The Journal of Infectious Diseases, 29,* 528-536.

Bognár, A. & Daood, H.G. (2000). Simple in-line postcolumn oxidation and derivatization for the simultaneous analysis of ascorbic and dehydroascorbic acids in foods. *Journal of Chromatographic Science,* 38, 162-168.

Boseley, S. (2013). Smoothies and fruit juices are a new risk to health, US scientists warn. *The Guardian.* 7th September 2013.

Braddock, R.J. (1999a). Single strength orange juice and concentrate. In: Braddock, R.J. (Ed). *Handbook of citrus by-products and processing technology.* Wiley, New York, 53-83.

Braddock, R.J. (1999b). Composition, properties and evaluation of fruit components. In: Braddock, R.J. (Ed). *Handbook of citrus by-products and processing technology.* Wiley, New York, 53-83.

Buckow, R., Semrau, J., Sui, Q., Wan, J. & Knoerzer, K. (2012). Numerical evaluation of lactoperoxidase inactivation during continuous pulsed electric field processing. *Biotechnology Progress, 28,* 1363-1375.

Buckow, R., Ng, S. & Toepfl, S. (2013). Pulsed Electric Field Processing of orange juice: a review on microbial, enzymatic, nutritional, and sensory quality and stability. *Comprehensive Reviews in Food Science and Food Safety, 12,* 455-467.

Buckow, R., Chandry, P.S., Ng, S.Y., McAuley, C.M. & Swanson, B.G. (2014). Opportunities and challenges in pulsed electric field processing of dairy products. *International Dairy Journal, 34,* 199-212.

Bull, M.K., Zerdin, K., Howe, E., Goicoechea, D., Paramanandhan, P., Stockman, R., Sellahewa, J., Szabo, A.E., Johnson, R.L. & Stewart, C.M. (2004). The effect of high pressure processing on the microbial, physical and chemical properties of Valencia and Navel orange juice. *Innovative Food Science and Emerging Technologies, 5,* 135-149.

Bylaite, E. & Meyer, A.S. (2006). Characterisation of volatile aroma compounds of orange juices by three dynamic and static headspace gas chromatography techniques. *European Food Research and Technology, 222*, 176-184.

Campos, F.P. & Cristianini, M. (2007). Inactivation of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* in orange juice using ultra high-pressure pasteurisation. *Innovative food science and emerging technologies, 8*, 226-229.

Castillo, S., Pérez-Alfonso, C.O., Martínez-Romero, D., Guillén, F., Serrano, M. & Valero, D. (2014). The essential oils thymol and carvacrol applied in the packing lines avoid lemon spoilage and maintain quality during storage. *Food Control, 35*, 132-136.

Castro, A.J., Swanson, B.G., Barbosa-Cánovas, G.V. & Dunker, A.K. (2001a). Pulsed electric field denaturation of bovine alkaline phosphatase. In: Barbosa-Cánovas, G.V., & Zhang, Q.H. (Eds.). *Pulsed Electric Fields in Food Processing.* Technomic Publishing, Lancaster.

Castro, A.J., Swanson, B.G., Barbosa-Cánovas, G.V. & Zhang, Q.H. (2001b). Pulsed electric field modification of milk alkaline phosphatase activity. In: Barbosa-Cánovas, G.V., & Zhang, Q.H. (Eds.). *Pulsed Electric Fields in Food Processing.* Technomic Publishing, Lancaster.

Cautela, D., Castaldo, D., Servillo, L. & Giovane, A. (2010). Enzymes in citrus processing. In: Bayindirli, A. (Ed.). *Enzymes in fruit and vegetable processing. Chemistry and Engineering Applications.* CRC Press, Taylor & Francis Group.

Cebrián, G., Sagarzazu, N. Pagán, R., Condón, S. & Mañas, P. (2008). Resistance of *Escherichia coli* grown at different temperatures to various environmental stresses. *Journal of Applied Microbiology, 105,* 271-278.

Cebrián, G., Sagarzazu, N., Aertsen, A., Pagán, R., Condón, S. & Mañas, P. (2009). Role of the alternative sigma factor σ^B on *Staphylococcus aureus* resistance to stresses of relevance to food preservation. *Journal of Applied Microbiology, 107,* 187-196.

Cebrián, G., Raso, J., Condón, S. & Mañas, P. (2012). Acquisition of pulsed electric fields resistance in *Staphylococcus aureus* after exposure to heat and alkaline shocks. *Food Control, 25,* 407-414.

Cebrián, G., Condón, S. & Mañas, P. (2016). Influence of growth and treatment temperature on *Staphylococcus aureus* resistance to pulsed electric fields: relationship with membrane fluidity. *Innovative Food Science and Emerging Technologies, 37,* 161-169.

Cerf, O. (1977). Tailing of survival curves of bacterial spores. *Journal of Applied Microbiology, 42*, 1-19.

Chen, C.S. & Wu, M.C. (1998). Kinetic models for thermal inactivation of multiple pectinesterases in citrus juice. *Journal of Food Science, 63,* 747–750.

Chen, C.S., Shaw, P.E. & Parish, M.E. (1992). Orange and tangerine juices. In: Nagy, S., Chen, C.S. (Ed.). *Fruit juice processing technology.* AG Science Inc. Auburndale.

Codex Stan 247-2005. General Standard for fruit juices and nectars.

Combs, G.F. & McClung, J.P. (2017). Vitamin C. In: Combs, G.F. & McClung, J.P. (Eds.) *The vitamins*. (5th edition). Academic Press, chapter 10.

Corlet, D.A. & Brown, M.H. (1980). pH and acidity. In: Silliker, J.H., Baird-Parker, A.C., Brylan, F.L., Christian, J.H.B., Clark, D.S., Olson, J.C., Roberts, T.A. (Ed.). *Microbial Ecology of Foods*. Academia Press, New York, 92-111.

Corradini, M.G. & Peleg, M. (2009). Dynamic Model of Heat Inactivation Kinetics for Bacterial Adaptation. *Applied and Environmental Microbiology, 75,* 2590–2597.

Cortés, S., Torregrosa, F., Esteve, M.J. & Fígola, A. (2006). Carotenoid profile modification during refrigerated storage in untreated and pasteurized orange juice and orange juice treated with high-intensity pulsed electric fields. *Journal of Agricultural and Food Chemistry, 54,* 6247-6254.

Coster, H.G.L. & Zimmermann, U. (1975). The mechanism of electrical breakdown in the membranes of *Valonia utricularis*. *Journal of Membrane Biology, 22*, 73–90.

Cotter, P.D. & Hill, C. (2003). Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews, 67,* 429-453.

Creighton, T.E. (1988). Towards a better understanding of protein folding pathways, *Proceedings of the National Academy of Sciences of the United States of America, 85*, 5082-5086.

Cserhalmi, Zs., Sass-Kiss, Á., Tóth-Markus, M. & Lechner, N. (2006). Study of pulsed electric field treated citrus juices. *Innovative Food Science and Emerging Technologies, 7,* 49-54.

Dantigny, P., Guilmart, A. & Bensoussan, M. (2005). Basis of predictive mycology. *International Journal of Food Microbiology, 100,* 187-196.

Danyluk, M.D., Goodrich-Schneider, R.M., Schneider, K.R., Harris, L.J. & Worobo, R.W. (2012). Outbreaks of foodborne disease associated with fruit and vegetable juices, 1922-2010. EDIS Publication FSHN12-04. Available from http://ucfoodsafety.ucdavis.edu/files/223883.pdf.

Da Silva, F.V.M. & Gibbs, P.A. (2009). Principles of thermal processing. In: Simpson, R. (Eds.). *Engineering Aspects of thermal Food Processing.* CRC Press.

Den Besten, H.M.W., Mataragas, M., Moezelaar, R., Abee, T. & Zwietering, M.H. (2006). Quantification of the effects of salt stress and physiological state on thermotolerance of *Bacillus cereus* ATCC 10987 and ATCC 14579. *Applied and Environmental Microbiology, 72*, 5884-5894.

Dharmawan, J., Kasapis, S., Curran, P. & Johnson, J.R. (2007). Characterization of volatile compounds in selected citrus fruits from Asia. Part I: freshly-squeezed juice. *Flavour and Fragrance Journal, 22,* 228-232.

Dill, K.A., Ghosh, K. & Schmit, J.D. (2011). Physical limits of cells and proteomes. *Proceedings of the National Academy of Sciences, 108,* 17876–17882.

Dimitrov, D.S. & Sowers, A.E. (1990). Membrane electroporation – fast molecular exchange by electroosmosis. *Biochimica et Biophysica Acta, 1022,* 381-392.

Doevenspeck, H. (1960). Verfahren und Vorrichtung zur Gewinning der einzelnen Phasen aus dispersen Systemen, Germany, German Patent DE 2907887 A1.

Donsì, F., Ferrari, G. & Pataro, G. (2010). Applications of Pulsed Electric Field Treatments for the enhancement of Mass Transfer from Vegetable Tissue. *Food Engineering Reviews,* 2, 109-130.

Doyle, M.E. & Mazzotta, A.S. (2000). Review studies on the thermal resistance of *Salmonella. Journal of Food Protection, 63,* 779-795.

Driessen, F. M. (1983). Lipases and Proteinases in Milk: Occurrence, Heat Inactivation, and Their Importance for the Keeping Quality of Milk Products. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.

Dunn, J. (2001). Pulsed Electric Field Processing: an overview. In: Barbosa-Cánovas, G.V. & Zhang, Q.H. (Eds.). *Pulsed Electric Fields in Food Processing. Fundamental Aspects and Applications.* Technomic Publishing Company.

Dutreux, N., Notermans, S., Wijtzes, T., Góngora-Nieto, M.M., Barbosa-Cánovas, G.V. & Swanson, B.G. (2000). Pulsed electric fields inactivation of attached and free-living *Escherichia coli* and *Listeria innocua* under several conditions. *International Journal of Food Microbiology, 54,* 91-98.

Eagerman, B.A. & Rouse, A.H. (1976). Heat inactivation temperature-time relationships for pectinesterase inactivation in citrus juice. *Journal of Food Science, 41,* 1396-1397.

Elez-Martínez, P., Escolà-Hernández, J., Soliva-Fortuny, R.C. & Martín-Belloso, O. (2004a). Inactivation of *Saccharomyces cerevisiae* suspended in orange juice using high-intensity pulsed electric fields. *Journal of Food Protection, 67*, 2596-2602.

Elez-Martínez, P., Escolà-Hernández, J., Soliva-Fortuny, R.C. & Martín-Belloso, O. (2004b). Inactivation of *Lactobacillus brevis* in orange juice by high-intensity pulsed electric fields. *Food Microbiology*, *22,* 311-319.

Elez-Martínez, P., Aguiló-Aguayo, I. & Martín-Belloso, O. (2006). Inactivation of orange juice peroxidase by high-intensity pulsed electric fields as influenced by process parameters. *Journal of the Science of Food and Agriculture, 86,* 71-81.

Elez-Martínez, P. & Martín-Belloso, O. (2007). Effects of high intensity pulsed electric field processing conditions on vitamin C and antioxidant capacity of orange juice and gazpacho, a cold vegetable soup. *Food Chemistry, 102,* 201-209.

Elez-Martínez, P., Suárez-Recio, M. & Martín-Belloso, O. (2007). Modeling the reduction of pectin methyl esterase activity in orange juice by high intensity pulsed electric fields. *Journal of Food Engineering, 78,* 184-193.

Elez-Martínez, P., Martín-Belloso, O., Rodrigo, D. & Sampedro, F. (2007). Impact of pulsed electric fields on food enzymes and shelf-life. In: Lelieveld, H.L.M., Notermans, S. & de Haan, S.W.H. (Eds.). *Food preservation by pulsed electric fields. From research to application.* Woodhead publishing.

El Ghaouth, A.E., Wilson, C.L. & Wisniewski, M. (2003). Control of postharvest decay of apple fruit with *Candida saitoana* and induction of defence responses. *Phytopathology, 93*, 344-348.

El Zakhem, H., Lanoisellé, J.-L., Lebovaka, N.I., Nonus, M. & Vorobiev, E. (2006a). Behaviour of yeast cells in aqueous suspension affected by pulsed electric field. *Journal of Colloid and Interface Science, 300*, 553-563.

El Zakhem, H., Lanoisellé, J.L., Lebovka, N.I., Nonus, M. & Vorobiev, E. (2006b). The early stages of *Saccharomyces cerevisiae* yeast suspensions damage in moderate pulsed electric fields. *Colloids and surfaces B: Biointerfaces, 47,* 189-197.

El Zakhem, H., Lanoisellé, J.-L., Lebovaka, N.I., Nonus, M. & Vorobiev, E. (2007). Influence of temperature and surfactant on *Escherichia coli* inactivation in aqueous suspensions treated by moderate pulsed electric fields. *International Journal of Food Microbiology, 120,* 259-265.

Espachs-Barroso, A., Barbosa-Cánovas, G.V. & Martín-Belloso, O. (2003). Microbial and enzymatic changes in fruit juice induced by high-intensity pulsed electric fields. *Food Reviews International, 19,* 253-273.

Evrendilek, G.A., Zhang & Richter, E.R. (1999). Inactivation of *Escherichia coli* O157:H7 and *Escherichia coli* 8739 in apple juice by pulsed electric fields. *Journal of Food Protection, 7,* 793-796.

Evrendilek, G.A., Jin, Z.T., Ruhlman, K.T., Qiu, X., Zhang, Q.H. & Richter, E.R. (2000). Microbial safety and shelf-life of apple juice and cider processed by bench and pilot scale PEF systems. *Innovative Food Science and Emerging Technologies, 1,* 77-86.

Evrendilek, G.A., Tok, F.M., Soylu, E.M. & Soylu, S. (2008). Inactivation of *Penicillium expansum* in sour cherry juice, peach and abricot nectars by pulsed electric fields. *Food Microbiology, 25,* 662-667.

Eynard, N., Rodriguez, F., Trotard, J. & Teissié, J. (1998). Electrooptics studies of *Escherichia coli* electropulsation: orientation, permeabilization and gene transfer. *Biophysical Journal, 75,* 2587-2596.

Eyring, H. (1935). The Activated Complex in Chemical Reactions. *The Journal of Chemical Physics, 3,* 107–115.

Fachin, D., Van Loey, A., Indrawati, I., Ludikhuyze, L.& Hendrickx, M. (2006). Thermal and High-Pressure Inactivation of Tomato Polygalacturonase: A Kinetic Study. *Journal of Food Science, 67,* 1610-1615.

FAO, Food and Agriculture Organization (2016). Citrus Fruit statistics 2015. *Market and Policy Analyses of Raw Materials, Horticulture and Tropical Products Team.*

Fiala, A., Wouters, P.C. Van den Bosch, H.F.M. & Creyghton, Y.L.M. (2001). Coupled Electrical-fluid model of Pulsed Electric Field Treatment in a model food system. *Innovative Food Science and Emerging Technologies, 2,* 229-238.

Fleet, G.H. (2003). Yeasts in fruit and fruit products. In: Boekhout, T., Roberts, R. (Eds.). *Yeast and Food. Beneficial and Detrimental Aspects.* Behr's Verlag, Hamburg.

Fleming, D.W., Cochi, S.L., MacDonald, K.L., Brondum, J., Hayes, P.S., Plikaytis, B.D., Holmes, M.B., Audurier, A., Broome, C.V. & Reingold, A.L. (1985). Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *The New England Journal of Medicine, 312*, 404-407.

Fontan, R.C.I., Santos, L.S., Bonomo, R.C.F., Lemos, L.S., Ribeiro, R.P. & Veloso, C.M. (2009). Thermophysical properties of coconut water affected by temperature. *Journal of Food Process Engineering, 32,* 382-397.

García, D., Gómez, N., Mañas, P., Condón, S., Raso, J. & Pagán, R. (2005a). Occurrence of sublethal injury after pulsed electric fields depending on the micro-organism, the treatment medium pH and the intensity of the treatment investigated. *Journal of Applied Microbiology, 99,* 94-104.

García, D., Gómez, N., Raso, J. & Pagán, R. (2005b). Bacterial resistance after pulsed electric fields depending on the treatment medium pH. *Innovative Food Science and Emerging Technologies, 6*, 388-395.

Geeraerd, A.H., Herremans, C.H. & Van Impe, J.F. (2000). Structural model requirements to describe microbial inactivation during a mild heat treatment. *International Journal of Food Microbiology, 59,* 185-209.

Genovese, D.B., Elustondo, M.P. & Lozano, J.E. (1997). Colour and cloud stabilization in cloudy apple juice by steam heating during crushing. *Journal of Food Science, 62*, 1171-1175.

Gerlach, D., Alleborn, N., Baars, A., Delgado, A., Moritz, J. & Knorr, D. (2008). Numerical simulations of pulsed electric fields for food preservation: a review. *Innovative Food Science and Emerging Technologies, 9,* 408-417.

Geveke, D.J. & Kozempel, M.F. (2003). Pulsed electric field effects on bacteria and yeast cells. *Journal of Food Processing Preservation, 27,* 65-72.

Gil, M.M., Miller, F.A., Brandão, T.R.S. & Silva, C.L.M. (2016). Combined effects of temperature, pH and water activity on predictive ability of microbial kinetic inactivation model. *Procedia Food Science, 7,* 67-70.

Giner, J., Gimeno, V., Espachs, A., Elez, P., Barbosa-Cánovas, G.V. & Martín, O. (2000). Inhibition of tomato (*Licopersicon esculentum* Mill.) pectin methylesterase by pulsed electric fields. *Innovative Food Science and Emerging Technologies, 1,* 57-67.

Giner, J., Gimeno, V., Barbosa-Cánovas, G.V. & Martín, O. (2001). Effects of pulsed electric field processing on apple and pear polyphenoloxidases. *Food Science and Technology International, 7,* 339-345.

Giner, J. Ortega, M., Mesegué, M., Gimeno, V., Barbosa-Cánovas, G.V. & Martín, O. (2002). Inactivation of peach polyphenoloxidase by exposure to pulsed electric fields. *Food Engineering and Physical Properties, 67,* 1467-1472.

Giner, J., Gimeno, V., Palomes, M., Barbosa-Cánovas, G.V. & Martín, O. (2003). Lessening polygalacturonase activity in a commercial enzyme preparation by exposure to pulsed electric fields. *European Food Research and Technology, 217,* 43-48.

Giner, J., Grouberman, P., Gimeno, V. & Martín, O. (2005). Reduction of pectinmethylesterase activity in a commercial enzyme preparation by pulsed electric fields: comparison of inactivation kinetic models. *Journal of Science of Food and Agriculture, 85,* 1613-1621.

Goettsch, C. (2014). Revolutie in voedselbereiding. *Voedingsmiddelenindustrie, 1,* 8-9.

Gomes Neto, N.J., Magnani, M., Chueca, B., García-Gonzalo, D, Pagán, R. & de Souza, E.L. (2015). Influence of general stress-response alternative sigma factors σ^S (RpoS) and σ^B (SigB) on bacterial tolerance to the essential oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. and pulsed electric fields. *International Journal of Food Microbiology, 211,* 32-37.

Gómez, N., García, D., Álvarez, I., Condón, S. & Raso, J. (2005a). Modelling inactivation of *Listeria monocytogenes* by pulsed electric fields in media of different pH. *International Journal of Food Microbiology, 103,* 199-206.

Gómez, N., García, D., Álvarez, I., Raso, J. & Condón, S. (2005b). A model describing the kinetics of inactivation of *Lactobacillus plantarum* in a buffer system of different pH and in orange an apple juice. *Journal of Food Engineering, 70*, 7-14.

Góngora-Nieto, M.M., Pedrow, P.D., Swanson, B. & Barbosa-Cánovas, G.V. (2003). Impact of air bubbles in a dielectric liquid when subjected to high field strengths. *Innovative Food Science & Emerging Technologies, 4,* 57-67.

Graham, B.H. & Craigen, W.J. (2004). Genetic approaches to analyzing mitochondrial outer membrane permeability. *Current topics in developmental biology, 59,* 87-118.

Grahl, T. & Märkl, H. (1996). Killing of microorganisms by pulsed electric fields. *Applied Microbiology and Biotechnology, 45,* 148-157.

Graumlich, T.R., Marcy, J.E. & Adams, J.P. (1986). Aseptically packaged orange juice and concentrate: a review of the influence of processing and packaging conditions on quality. *Journal of Agricultural and Food Chemistry, 34,* 402-405.

Guinebretiere, M.H., Nguyen-The, C., Morrsion, N., Reich, M. & Nicot, P. (2000). Isolation and characterization of antagonists for the biocontrol of the postharvest wound pathogen *Botrytis cinerea* on strawberry fruits. *Journal of Food Protection, 63,* 386-394.

Gurtler, J.B., Rivera, R.B., Zhang, H.Q. & Geveke, D.J. (2010). Selection of surrogate bacteria in place of *E. coli* O157:H7 and *Salmonella* Typhimurium for pulsed electric field treatment of orange juice. *International Journal of Food Microbiology, 139,* 1-8.

Gurtler, J.B., Bailey, R.B., Geveke, D.J. & Zhang, H.Q. (2011). Pulsed electric field inactivation of *E. coli* O157:H7 and non-pathogenic surrogate *E. coli* in strawberry juice as influence by sodium benzoate, potassium sorbate, and citric acid. *Food Control, 22,* 1689-1694.

Hamilton, A. (2009). Squeezed: what you don't know about the orange juice. Yale University Press.

Hamilton, W.A. & Sale, J.H. (1967). Effects of high electric fields on microorganisms. II. Mechanism of action of the lethal effect. *Biochimica et Biophysica Acta, 148,* 789-800.

Harding, P.L., Winston, J.R., & Fisher, D.F. (1940). Seasonal changes in Florida oranges. In: US Department of Agriculture, *Technical Bulletin, 753,* 1-97.

Hartyáni, P., Dalmadi, I., Cserhalmi, Z., Kántor, D-B., Tóth-Markus, M. & Sass-Kiss, A. (2011). Physical–chemical and sensory properties of pulsed electric field and high hydrostatic pressure treated citrus juices. *Innovative Food Science and Emerging Technologies, 12,* 255-260.

Hauben, K.J.A., Bartlett, D.H., Soontjens, C.C.F., Cornelis, K., Wuytack, E.Y. & Michiels, C.W. (1997). *Escherichia coli* mutants resistant to inactivation by High Hydrostatic Pressure. *Applied and Environmental Microbiology, 63,* 945-950.

Heinz, V., Phillips, S.T., Zenker, M. & Knorr, D. (1999). Inactivation of *bacillus subtilis* by high intensity pulsed electric fields under close to isothermal conditions. *Food Biotechnology, 13,* 155-168.

Heinz, V., Alvarez, I., Angersbach, A. & Knorr, D. (2002). Preservation of liquid foods by high intensity pulsed electric fields — basic concepts for process design. *Trend in Food Science and Technology, 12,* 103-111.

Heinz, V., Toepfl, S. & Knorr, D. (2003). Impact of temperature on lethality and energy efficiency of apple juice pasteurization by pulsed electric fields treatment. *Innovative Food Science and Emerging Technologies, 4,* 167-175.

Hendrickx, M., Ludikhuyze, L., Van den Broeck, I. & Weemaes, C. (1998). Effects of High Pressure on enzymes related to food quality. *Trends in Food Science & Technology, 9,* 197-203.

Hirsch, A.R., Försch, K., Neidhart, S., Wolf, G. & Carle, R. (2008). Effects of thermal treatments and storage on pectin methylesterase and peroxidase activity in freshly squeezed orange juice. *Journal of Agricultural and Food Chemistry, 56,* 5691-5699.

Ho, S.Y., Mittal, G.S. & Cross, J.D. (1997). Effect of high field electric pulses on the activity of selected enzymes. *Journal of Food Engineering, 31,* 69-84.

Holland, R.R., Reeder, S.K. & Pritchett, D.E. (1976). Cloud stability test for pasteurised citrus juices. *Journal of Food Science, 41,* 812-815.

Huang, K., Yu, L., Wang, W., Gai, L. & Wang, J. (2014). Comparing the pulsed electric field resistance of the microorganisms in grape juice: application of the Weibull model. *Food Control, 35,* 241-251.

Hülsheger, H. & Niemann, E.-G. (1980). Lethal effect of High-voltage pulses on *E. coli* k12. *Radiation and Environmental Biophysics, 18,* 281-288.

Hülsheger, H., Potel, J. & Niemann, E-G (1981). Killing of bacteria with electric pulses of High Field Strength. *Radiation and Environmental Biophysics, 20,* 53-65.

Hülsheger, H., Potel, J., & Niemann, E-G (1983). Electric field effects on bacteria and yeast cells. *Radiation and Environmental Biophysics, 22,* 149-162.

ICMSF (1998). Soft drinks, fruit juices, concentrates and food preserves. In: ICMSF (Eds.). Micro-organisms in Foods. Springer, Boston.

Irving, D. (2012). We zijn nu al aan het opschalen. *VMT Voedingsmiddelenindustrie, 16/17*, 11-13.

ISO 21527-1 (2008). Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of yeasts and moulds. Part 1: colony count technique in products with water activity greater than 0.95.

Jacob, H.E., Förster, W. & Berg, H. (1981). Microbiological implications of electric field effects. II. Inactivation of yeast cells and repair of their envelope. *Zeitschrift für Allgemeine Mikrobiologie, 21,* 225-233.

Jaeger, H., Meneses, N. & Knorr, D. (2009). Impact of PEF treatment inhomogeneity such as electric field distribution, flow characteristics and temperature effects on the inactivation of *E. coli* and milk alkaline phosphatase. *Innovative Food Science and Emerging Technologies, 10,* 470-480.

Jaeger, H., Schulz, A., Karapetkov, N. & Knorr. D. (2009). Protective effect of milk constituents and sublethal injuries limiting process effectiveness during PEF inactivation of *Lb. rhamnosus. International Journal of Food Microbiology, 134,* 154-161.

Jaeger, H., Meneses, N., Moritz, J. & Knorr, D. (2010). Model for the differentiation of temperature and electric field effects during thermal assisted PEF processing. *Journal of Food Engineering, 100,* 109-118.

Jaeger, H., Meneses, N. & Knorr, D. (2014). Food technologies: Pulsed Electric Field Technologie. In: Motarjemi, Y., Moy, G.G. & Todd, E.C. (Eds.). *Encyclopaedia of Food Safety,* 239-244. Academic Press.

Jaeger, H. Roth, A., Toepfl, S., Holzhauser, S., Engel, K-H., Knorr, D., Vogel, R., Bandick, N., Kulling, S., Heinz, V. & Steinberg, P. (2016). Opinion on the use of ohmic heating for the treatment of foods. *Trends in Food Science and Technology, 55,* 84-97.

Jarvis, B., Seiler, D.A.L., Ould, A.J.L. & Williams, A.P. (1983). Observations on the enumeration of moulds in food and feedingstuffs. *Journal of Applied Bacteriology, 55,* 325-336.

Jay, S. & Anderson, J. (2001). Fruit juice and related products. In: Moir, C.J., Andrew-Kabilafkas, C., Arnold, G., Cox, A.D., Hocking, A.D., Jenson, I. (Eds). *Spoilage of processed foods: causes and diagnosis.* Southwood Press, Sydney.

Jayaram, S., Castle, G. S. P. & Margaritis, A. (1992). Kinetics of sterilization of *Lactobacillus brevis* cells by the application of high voltage pulses. *Biotechnology and Bioengineering*, *40*, 1412–1420.

Jayaram, S., Castle, G.S.P. & Margaritis, A. (1993). The effects of high field DC pulse and liquid medium conductivity on survivability of *Lactobacillus brevis. Applied and Environmental Biotechnology, 40,* 117-122.

Jeantet, R., Baron, F., Nau, F., Roignant, M. & Brulé, G. (1999). High intensity pulsed electric fields applied to egg white: effects on *Salmonella* Enteritidis inactivation and protein denaturation. *Journal of Food Protection, 62,* 1381-1386.

Jia, M., Zhang, Q.H. & Min, D.B. (1999). Pulsed electric field processing effects on flavour compounds and microorganisms of orange juice. *Food Chemistry, 65,* 445-451.

Jordán, M.J., Goodner, K.L. & Laencina, J. (2003). Deaeration and pasteurization effects on the orange juice aromatic fraction. *LWT Food Science and Technology, 36,* 391-396.

Joslyn, M.A. & Pilnik, W. (1961). Enzymes and enzyme activity. In: Sinclair, W.B. (Ed.) *The orange, its biochemistry and physiology.* University of California Press, Berkeley, California.

Kabak, B. (2009). The fate of mycotoxins during thermal food processing. *Journal of the Science of Food and Agriculture, 89,* 549-554.

Kemeny, G. & Rosenberg, B. (1973). Compensation law in thermodynamics and thermal death. *Nature, 243*, 400.

Kempkes, M.A. (2010). PEF systems for commercial food and juice processing. In: Doona, C.J., Kustin, K. & Feeherry, F.E. (Eds.). *Case studies in novel food processing technologies. Innovations in processing, packaging and predictive modelling.* Woodhead Publishing.

Kew, T.J., Veldhuis, M.K., Bissett, O.W., & Patrick, R. (1957). The effect of time and temperature of pasteurisation on the quality of canned citrus juices. *USDA Agricultural* Research Service, ARS-72-6, Winter Haven.

Klimczak, I., Malecka, M., Szlachta, M., & Gliszczynska-Swiglo, A. (2007). Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. *Journal of Food Composition and Analysis, 20*, 313–322.

Kováts, E. (1958). Gas-chromatographische Charakterisierung organischer Verbindungen. Teil 1: Retentionsindices aliphatischer Halogenide, Alkohole, Aldehyde und Ketone. *Helvetica Chimica Acta, 41, 1915-1932.*

Kroll, S. & Klostermeyer, H. (1984). Proteolytic activities of *Ps. fluorescens* in milk: Determination with azocasein in comparison HPA. *Zeitschrift fúr Lebensmittel-Untersuchung und Forschung, 178,* 179-186.

Krop, J.J.P. (1974). The mechanism of cloud loss phenomena in orange juice. PhD thesis. Agricultural University Wageningen, The Netherlands.

Lado, B.H. & Yousef, A.E. (2003). Selection and identification of a *Listeria monocytogenes* target strain for pulsed electric field process optimisation. *Applied and Environmental Microbiology, 69,* 2223-2229.

Lebovka, N.I., Praporscic, I., Ghnimi, S. & Vorobiev, E. (2005). Does electroporation occur during the ohmic heating of food? *Food Engineering and Physical Properties, 70,* 308-311.

Lee, H.S. & Coates, G.A. (2003). Effect of thermal pasteurization on Valencia orange juice colour and pigments. *LWT Food Science and Technology, 36,* 153-156.

Leizerson, S. & Shimoni, E. (2005a). Effect of ultrahigh-temperature continuous ohmic heating treatment on fresh orange juice. *Journal of Agricultural and Food Chemistry, 53,* 3519-3524.

Leizerson, S. & Shimoni, E. (2005b). Stability and sensory shelf life of orange juice pasteurized by continuous ohmic heating. *Journal of Agricultural and Food Chemistry, 53,* 4012-4018.

Liu, J., Sui, Y., Wisniewski, M., Droby, S. & Liu, Y. (2013). Review: Utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. *International Journal of Food Microbiology, 167,* 153-160.

Loghavi, L. & Sastry, S.K. (2009). Effect of moderate electric field frequency and growth stage on the cell membrane permeability of *Lactobacillus acidophilus. Biotechnology Progress, 25,* 85-94.

Lumry, R. & Eyring, H. (1954). Conformation changes of proteins. *Journal of Physical Chemistry, 58,* 111-120.

Mackey, B.M., & Derrick, C.M. (1986). Elevation of the heat resistance of *Salmonella typhimurium* by sublethal heat shock. *Journal of Applied Bacteriology, 61,* 389-393.

Mañas, P., Barsotti, L. & Cheftel, J.C. (2002). Microbial inactivation by pulsed electric fields in a batch treatment chamber: effects of some electrical parameters and food constituents. *Innovative Food Science & Emerging Technologies, 2,* 239-249.

Manso, T. & Nunes, C. (2011). *Metschnikowia andauensis* as a new biocontrol agent of fruit postharvest diseases. *Postharvest Biology and Technology, 61,* 64-71.

Marsellés-Fontanet, Á.R. & Martín-Belloso, O. (2007). Optimization and validation of PEF processing conditions to inactivate oxidative enzymes of grape juice. *Journal of Food Engineering, 83,* 452-462.

Marsellés-Fontanet, A.R., Puig, A., Olmos, P., Mínguez-Sanz, S. & Martín-Belloso, O. (2009). Optimising the inactivation of grape juice spoilage organisms by pulse electric fields. *International Journal of Food Microbiology, 130,* 159-165.

Martín, O., Qin, B-L., Chang, F.J., Barbosa-Cánovas, G.V. & Swanson, B.G. (1997). Inactivation of *Escherichia coli* in skim milk by high intensity pulsed electric fields. *Journal of Food Process Engineering, 20,* 317-336.

Martín-Belloso, O. & Elez-Martínez, P. (2005). Enzymatic inactivation by pulsed electric fields. In: Sun, D.W. (Ed.). *Emerging technologies for food processing,* Elsevier, London.

Mastello, R.B., Capobiango, M., Chin, S-T, Monteiro, M., & Marriott, P.J. (2015). Identification of odour-active compounds of pasteurized orange juice using multidimensional gas chromatography techniques. *Food Research International, 75,* 281-288.

Mastwijk, H. (2006). Pulsed power systems for application of pulsed electric fields in the food industry. In: Raso, J. & Heinz, V. (Eds.). *Pulsed Electric Fields Technology for the Food Industry. Fundamentals and application.* Springer, New York.

Mastwijk, H.C., Gulfo-van Beusekom, K., Pol-Hofstad, I.E., Schuten, H., Boonman, M. & Bartels, P.V. (2007). Definitions and guidelines for reporting on pulsed electric field experiments. In: Lelieveld, H.L.M., Notermans, S. & de Haan, S.W.H. (Eds.). *Food preservation by pulsed electric fields: from research to application.* Woodhead Publishing, Cambridge UK.

Mastwijk, H.C., Timmermans, R.A.H. & Van Boekel, M.A.J.S. (2017). The Gauss-Eyring model: a new thermodynamic model for biochemical and microbial inactivation kinetics. *Food Chemistry, 237,* 331-341.

Mathys, A., Toepfl, S., Siemer, C., Favre, L., Benyacoub, J. & Hansen, C.E. (2011). Pulsed electric field treatment process and dairy product comprising bioactive molecules obtainable by the process. *European Patent Application,* no. EP 2543254A1.

Maxwell, J.C. (1904). A treatise on Electricity and Magnetism. Third edition, Oxford University Press, London, 435-441.

Mazzotta, A.S. (2001). Thermal inactivation of stationary-phase and acid-adapted *Escherichia coli* O157:H7, *Salmonella,* and *Listeria monocytogenes* in fruit juices. *Journal of Food Protection, 64*, 315-320.

McDonald, L.C., Hackney, R.C. & Ray, B. (1983). Enhanced recovery of injured *Escherichia coli* by compounds that degrade hydrogen peroxide or block its formation. *Applied and Environmental Microbiology, 45,* 360-365.

McDonald, K. & Sun, D-W. (1999). Predictive food microbiology for the meat industry: a review. *International Journal of Food Microbiology, 52,* 1-27.

McDonald, C.J., Lloyd, S.W., Vitale, M.A., Petersson, K. & Innings, F. (2000). Effects of pulsed electric fields on microorganisms in orange juice using electric field strengths of 30 and 50 kV/cm. *Food Engineering and Physical Properties, 65,* 984-989.

Meléndez-Martínez, A. J., Vicario, I. M., & Heredia, F. J. (2007a). Provitamin A carotenoids and ascorbic acid contents of the different types of orange juices marketed in Spain. *Food Chemistry, 101*, 177–184.

Meléndez-Martínez, A.J., Vicario, I.M. & Heredia, F.J. (2007b). Rapid assessment of vitamin A activity through objective colour measurements for the quality control of orange juices with diverse carotenoid profiles. *Journal of Agriculture and Food Chemistry, 55,* 2808-2815.

Meneses, N., Jaeger, H., Moritz, J. & Knorr, D. (2011). Impact of insulator shape, flow rate and electrical parameters on inactivation of *E. coli* using a continuous co-linear PEF system. *Innovative Food Science and Emerging Technologies, 12,* 6-12.

Meneses, N., Jaeger, H. & Knorr, D. (2011). pH-changes during pulsed electric field treatments – numerical simulation an in situ impact on polyphenoloxidase inactivation. *Innovative Food Science and Emerging Technologies, 12,* 499-504.

Metselaar, K.I., den Besten, H.M.W., Abee, T., Moezelaar, R. & Zwietering, M.H. (2013). Isolation and quantification of highly resistant variants of *Listeria monocytogenes. International Journal of Food Microbiology, 166,* 508-514.

Metselaar, K.I. (2016). Quantitative and ecological aspects of *Listeria monocytogenes* population heterogeinity. PhD thesis, Wageningen University, Wageningen, NL.

Metselaar, K.I., Abee, T., Zwietering, M.H. & Den Besten, H.M.W. (2016). Modeling and validation of the ecological behaviour of wild-type *Listeria monocytogenes* and stress-resistant variants. *Applied and Environmental Microbiology, 82,* 5389-5401.

Michalski, C.B., Brackett, R.E., Hung, Y.C. & Ezeike, O.I. (1999). Use of capillary tubes and plate heat exchanger to validate U.S. Department of Agriculture Pasteurization protocols for elimination of *Salmonella* Enteritidis from liquid egg products. *Journal of Food Protection, 62,* 112-117.

Min, S., Min, S.K., & Zhang, Q.H. (2003). Inactivation kinetics of tomato juice lipoxygenase by pulsed electric fields. *Food Engineering and Physical Properties, 68,* 1995-2001.

Min, S., Jin, Z.T., Min, S.K., Yeom, H. & Zhang, Q.H. (2003). Commercial-scale pulsed electric field processing of orange juice. *Food Chemistry and Toxicology, 4,* 1265-1271.

Morren, J., Roodenburg, B. & De Haan, S.W.H. (2003). Electrochemical reaction and electrode corrosion in pulsed electric field (PEF) treatment chambers. *Innovative Food Science and Emerging Technologies, 4,* 285-295.

Mosqueda-Melgar, J. Raybaudi-Massilia, R.M. & Martín-Belloso, O. (2007). Influence of treatment time and pulse frequency on *Salmonella* Enteritidis, *Escherichia coli* and *Listeria monocytogenes* populations inoculated in melon and watermelon juices treated by pulsed electric fields. *International Journal of Food Microbiology, 117,* 192-200.

Moss, M.O. (2008). Fungi, quality and safety issues in fresh fruits and vegetables. *Journal of Applied Microbiology, 104,* 1239-1243.

Motulsky, H.J. & Christopoulos, A. (2003). Fitting models to biological data using linear and nonlinear regression. A practical guide to curve fitting. Oxford University Press, New York.

Nagy, S. & Smoot, J.M. (1977). Temperature and storage effect on percent retention and percent U.S. recommended dietary allowance of vitamin C in canned single-strength orange juice. *Journal of Agricultural and Food Chemistry, 25,* 135-138.

Nagy, S., Rouseff, R.L. & Lee, H.S. (1989). Thermally degraded flavours in citrus juice products. In: Parliment, T.H., McGorrin, R.J., & Ho, C-T (Eds.). *Thermal degradation of aromas.* American Chemical Society, Washington DC.

Nahku, R., Peebo, K., Valgepea, K., Barrick, J.E., Adamberg, K. & Vilu, R. (2011). Stock culture heterogeneity rather than new mutational variation complicates short-term cell physiology studies of *Escherichia coli* K-12 MG1655 in continuous culture. *Microbiology, 157*, 2604-2610.

Nath, N. & Ranganna, S. (1977). Time/temperature relationship for thermal inactivation of pectinesterase in mandarin orange (*Citrus reticulata*) juice. *Journal of Food Technology, 12,* 411-419.

Neidhardt, F.C., Ingram, J.L. & Schaechter, M. (1990). The effects of temperature, pressure and pH. In: Neidhardt, F.C., Ingram, J.L. & Schaechter, M. (Eds). *Physiology of the bacterial cell. A molecular Approach.* Sinauer Associates, Sunderland.

Neidhardt, F.C. & Umbarger, H.E. (1996). Chemical composition of *Escherichia coli.* In: F.C. Neidhardt (Ed.). *Escherichia coli and Salmonella: Cellular and Molecular Biology.* Second edition. American Society of Microbiology Press.

Neumann, E. & Rosenheck, K. (1972). Permeability changes induced by electric impulses in vesicular membranes. *Journal of Membrane Biology, 10,* 279-290.

Neumann, E., Sprafke, A., Boldt, E., & Wolf, H. (1992). Biophysical considerations of membrane electroporation. In: Chang, D.C., Saunders, J.A., Chassy, B.M. & Sowers, A.E. (Eds). Guide to Electroporation and Electrofusion. Academic Press, Inc. San Diego.

Neumann, E. (1989). The relaxation hysteresis of membrane electroporation. In: Neumann, E., Sowers, A.E. & Jordan, C.A. (Eds.). *Electroporation and Electrofusion in Cell Biology. Plenum Press*, New York.

Ng, H., Bayne, H.G., Garibaldi, J.A. (1969). Heat resistance of *Salmonella*: the uniqueness of *Salmonella* Senftenberg 775. *Applied Microbiology*, *17*, 78-82.

Noël, H., Hofhuis, A., De Jonge, R., Heuvelink, A.E., De Jong, A., Heck, M.E.O.C., De Jager, C. & Van Pelt, W. (2010). Consumption of fresh fruit juice: how a healthy food practice caused a national outbreak of *Salmonella* Panama Gastroenteritis. *Foodborne pathogens and disease, 7,* 375-381.

Ohshima, T., Okuyama, K. & Sato, M. (2002). Effect of culture temperature on high-voltage pulse sterilization of *Escherichia coli. Journal of Electrostatics, 55,* 227-235.

O'Neil, C.E. & Nicklas, T.A. (2008). A review of the relationship between 100% fruit juice consumption and weight in children and adolescents. *American Journal of Lifestyle Medicine, 2,* 315-354.

Pagán, R. & Mañas, P. (2006). Fundamental aspects of microbial membrane electroporation. In: Raso, J. & Heinz, V. (Eds.). *Pulsed Electric Fields Technology for the Food Industry. Fundamentals and application.* Springer.

Palaniappan, S., Sastry, S.K. & Richter. E.R. (1990). Effects of electricity on microorganisms: a review. *Journal of Food Processing and Preservation, 14,* 393-414.

Paredes-Sabja, D., Setlow, P. & Sarker, M.R. (2011). Germination of spores *Bacillales* and *Clostridiales* species: mechanisms and proteins involved. *Trends in Microbiology, 19,* 85-94.

Peleg, M. (1995). A model of microbial Survival after exposure to pulsed electric fields. *Journal of Science of Food and Agriculture, 67,* 93-99.

Peleg, M. & Cole, M.B. (1998). Reinterpretation of Microbial Survival Curves. *Critical Reviews in Food Science and Nutrition, 38,* 353-380.

Peleg, M. & Normand, M. (2004). Calculating Microbial Survival Parameters and Predicting Survival Curves from Non-Isothermal Inactivation Data. *Critical Reviews in Food Science and Nutrition, 44,* 409-418.

Peleg, M., Normand, M.D. & Corradini, M.G. (2012). The Arrhenius equation revisited. *Critical Reviews in Food Science and Nutrition*, 52, 830-851.

Pérez, O.E. & Pilosof, A.M.R. (2004). Pulsed electric fields effects on the molecular structure and gelation of β-lactoglobulin concentrate and egg white. *Food Research International, 37,* 102-110.

Pérez, M.C.P., Aliaga, D.R., Bernat, C.F., Enguidanos, M.R. & López, A.M. (2007). Inactivation of *Enterobacter sakazakii* by pulsed electric field in buffered peptone water and infant food formula. *International Dairy Journal, 17,* 1441-1449.

Perez-Cacho, P.R. & Rouseff, R. (2008a). Fresh squeezed orange juice odor: a review. *Critical Reviews in Food Science and Nutrition, 48,* 681-695.

Perez-Cacho, P.R. & Rouseff, R. (2008b). Processing and storage effects on orange juice aroma: a review. *Journal of Agricultural and Food Chemistry, 56,* 9785-9796.

Picart, L., Dumay, E. & Cheftel, J.C. (2002). Inactivation of *Listeria innocua* in dairy fluids by pulsed electric fields: influence of electric parameters and food composition. *Innovative Food Science & Emerging Technologies, 3,* 357-369.

Pillet, F., Formosa-Dagui, C., Baaziz, H., Dague, E. & Rols, M.P. (2016). Cell wall as a target for bacteria inactivation by pulsed electric fields. *Nature Scientific Reports, 6,* 19778.

Pitt, J.I. & Hocking, A.D. (2009). Fresh and Perishable Foods. In: Pitt, J.I., Hocking, A.D. (Eds). *Fungi and Food Spoilage,* third edition. Springer, New York.

Popham, D.L. (2002). Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. *Cellular and Molecular Life Science, 59,* 426-433.

Poschet, F., Geeraerd, A.H., Scheerlink, N., Nicolai, B.M. & Van Impe, J.F. (2003). Monte Carlo analysis as a tool to incorporate variation on experimental data in predictive microbiology. *Food Microbiology, 20,* 285-295.

Pothakamury, U.R., Vega, H., Zhang, Q., Barbosa-Canovas, G.V. & Swanson, B.G. (1996). Effect of growth stage and processing temperature on the inactivation of *E. coli* by pulsed electric fields. *Journal of Food Protection, 59,* 1167-1171.

Puértolas, E., López, N., Condón, S., Raso, R. & Álvarez, I. (2009). Pulsed electric fields inactivation of wine spoilage yeast and bacteria. *International Journal of Food Microbiology, 130,* 49-55.

Put, H.M.C, De Jong, J., Sand, F.E.M.J. & Van Grinsven, A.M. (1976). Heat resistance studies on yeast spp. causing spoilage in soft drinks. *Journal of Applied Bacteriology, 40,* 135–152.

Qin, B.L. Zhang, Q., Barbosa-Cánovas, G.V., Swanson, B.G. & Pedrow, P.D. (1994). Inactivation of microorganisms by pulsed electric fields of different voltage waveforms. *IEEE Transactions on Dielectrics and Electrical Insulation, 1,* 1047-1057.

Qin, B-L., Zhang, Q., Barbosa-Cánovas, G.V. Swanson, B.G. & Pedrow, P.D. (1995a). Pulsed electric field treatment chamber design for liquid food pasteurization using a finite element method. *Transactions of the ASAE, 38*, 557-565.

Qin, B-L., Chang, F-J., Barbosa-Cánovas, G.V. & Swanson, B.G. (1995b). Nonthermal inactivation of *Saccharomyces cerevisiae* in apple juice using pulsed electric fields. *LWT- Food Science and Technology, 28,* 564-568.

Qin, B.L., Barbosa-Cánovas, G.V., Swanson, B.G., Pedrow, P.D. & Olsen, R.G. (1998). Inactivating microorganisms using pulsed electric field continuous treatment system. *IEEE Transactions on industry applications, 34,* 43-50.

Qin, Z., Balasubramanian, S.K., Pearce, J.A. & Bischof, J.C. (2014). Correlated parameter fit of Arrhenius model for thermal denaturation of proteins and cells. *Annals of Biomedical Engineering, 42*, 2392-2404.

Qiu, X., Sharma, L., Tuhela, L., Jia, M. & Zhang, Q.H. (1998). An integrated PEF pilot plant for continuous nonthermal pasteurization of fresh orange juice. *Transactions of the ASAE, 41,* 1069-1074.

Rampersaud, G.C. (2015). 100% Fruit juice: perspectives amid the sugar debate. *Public Health Nutrition, 19,* 906-913.

Rampersaud, G.C. & Valim, M.F. (2017). 100% citrus juice: Nutritional contribution, dietary benefits, and association with anthropometric measures. *Critical Reviews in Food Science and Nutrition, 57,* 129-140.

Raso, J., Calderón, M.L., Góngora, M., Barbosa-Cánovas, G.V. & Swanson, B.G. (1998). Inactivation of mold ascospores and conidiospores suspended in fruit juices by pulsed electric fields. *Lebensmittel-Wissenschaft und Technologie, 31,* 668-672.

Raso, J., Álvarez, I., Condón, S. & Sala, F.J. (2000). Predicting inactivation of *Salmonella senftenberg* by pulsed electric fields. *Innovative Food Science & Emerging Technologies, 1,* 21-29.

Raso, J. & Barbosa-Cánovas, G.V. (2003). Nonthermal preservation of foods using combined processing techniques. C*ritical Reviews in Food Science and Nutrition, 43,* 265-285.

Raso, J., Condón, S. & Álvarez, I. (2014). Pulsed electric field. In: Batt, C.A. (Ed.). *Encyclopedia of Food Microbiology*, second ed. Elsevier ltd.

Raso, J. (2016). Fundamental and applied aspects of pulsed electric fields for microbial inactivation. In: Jarm, T. & Kramar, P. (Eds.). *1st World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine and Food & Environmental Technologies.* Springer Science and Business Media.

Raso, J., Frey, W., Ferrari, G., Pataro, G., Knorr, D., Teissié, J. & Miklavcic, D. (2016). Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes. *Innovative Food Science and Emerging Technologies, 37,* 312-32.

R Development Core Team. (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Redd, B.J., Hendrix, C.M., & Hendrix, D.L. (1986). Pectinesterase enzyme activity. In: Redd, B.J., Hendrix, C.M. & Hendrix, D.L. (Eds.). *Quality Control Manual for Citrus Processing Plants. Book 1: regulations, citrus methodology, microbiology, conversion charts, tables, other*. Intercit.

Reiff, F. (1965). Fundamentals of Statistical and Thermal Physics, McGraw-Hill.

Reina, L.D., Jin, Z.T., Zhang, H. & Youseff, A.E. (1998). Inactivation of *Listeria monocytogenes* in milk by pulsed electric field. *Journal of Food Protection, 61,* 1203-1206.

Reineke, K., Schottroff, F., Menesis, N. & Knorr, D. (2015). Sterilization of liquid foods by pulsed electric fields – an innovative ultra-high temperature process. *Frontiers in Microbiology, 6,* 400.

Roberts, T.A., Cordier, J-L., Gram, L., Tompkin, R.B., Pitt, J.I., Gorris, G.M. & Swanson, K.M.J. (2005). Soft drinks, fruit juices, concentrates, and fruit preserves. In: Roberts, T.A., Cordier, J-L., Gram, L., Tompkin, R.B., Pitt, J.I., Gorris, G.M. & Swanson, K.M.J. (Eds.). *Micro-organisms in Foods 6. Microbial Ecology of Food Commodities*. Springer-Verlag, US.

Rodrigo, D., Martínez, A., Harte, F., Barbosa-Cánovas, G.V. & Rodrigo, M. (2001). Study of inactivation of *Lactobacillus plantarum* in orange-carrot juice by means of pulsed electric fields: comparison inactivation kinetics models. *Journal of Food Protection, 64,* 259-263.

Rodrigo, D., Barbosa-Cánovas, G.V., Martínez, A. & Rodrigo, M. (2003a). Pectin methyl esterase and natural microflora of fresh mixed orange and carrot juice treated with pulsed electric fields. *Journal of Food Protection, 66,* 2336-2342.

Rodrigo, D., Ruíz, P., Barboas-Cánovas, G.V., Martínez, A. & Rodrigo, M. (2003b). Kinetic model for the inactivation of *Lactobacillus plantarum* by pulsed electric fields. *International Journal of Food Microbiology, 81,* 223-229.

Rodrigo, D., Barbosa-Cánovas, G.V., Martínez., A. & Rodrigo, M. (2003c). Weibull distribution function based on an empirical mathematical model for inactivation of *Escherichia coli* by pulsed electric fields. *Journal of Food Protection, 66*, 1007-1012.

Roodenburg, B. (2007). Electrochemistry in pulsed electric field treatment chambers. In: Lelieveld, H.L.M., Notermans, S. & de Haan, S.W.H. (Eds.). *Food preservation by pulsed electric fields. From research to application.* Woodhead publishing, CRC Press, Boca Raton.

Rosenberg, B., Kemeny, G., Switzer, R.C. & Hamilton, Th.C. (1971). Quantitative evidence for protein denaturation in the case of thermal death. *Nature*, 232**,** 471-473.

Rotschild, G., Van Vliet, C. & Karsenty, A. (1975). Pasteurization conditions for juices and comminuted products of Israeli citrus fruits. *Journal of Food Technology, 10,* 29-38.

Rouse, A.H. & Atkins, C.D. (1953). Further results from a study on heat inactivation of pectinesterase in citrus juices. *Food Technology,* 7, 221-223.

Russell, N.J. (2002). Bacterial membranes: the effects of chill storage and food processing. An overview. *International Journal of Food Microbiology, 79,* 27-34.

Ryall, B., Eydallin G. & Ferenci T. (2012). Culture history and population heterogeneity as determinants of bacterial adaptation: the adaptomics of a single environmental transition. *Microbiology and Molecular Biology Reviews, 76*, 597-625.

Saldaña, G., Puértolas, E., Álvarez, I., Meneses, N., Knorr, D. & Raso, J. (2010a). Evaluation of static treatment chamber to investigate kinetics of microbial inactivation by pulsed electric fields at different temperatures at quasi-isothermal conditions. *Journal of Food Engineering, 100,* 349-356.

Saldaña, G., Puértolas, E., Condón, S., Álvarez, I. & Raso, J. (2010b). Inactivation kinetics of pulsed electric field-resistant strains of *Listeria monocytogenes* and *Staphylococcus aureus* in media of different pH. *Food Microbiology, 27,* 550-558.

Saldaña, G., Puértolas, E., Condón, S., Álvarez, I. & Raso, J. (2010c). Modeling inactivation kinetics and occurence of sublethal injury of a pulsed electric field-resistant strain of *Escherichia coli* and *Salmonella* Typhimurium in media of different pH. *Innovative Food Science and Emerging Technologies, 11,* 290-298.

Saldaña, G., Puértolas, E., Monfort., S., Raso, J. & Álvarez, I. (2011). Defining treatment conditions for pulsed electric field pasteurization of apple juice. *International Journal of Food Microbiology, 151*, 29-35.

Saldaña, G., Álvarez, I., Condón, S. & Raso, J. (2013). Microbial aspects related to the feasibility of PEF technology for food pasteurization. *Critical Reviews in Food Science and Nutrition, 54,* 1415-1426.

Sale, A.J.H. & Hamilton, W.A. (1967). Effects of high electric fields on micro-organisms. I. Killing of bacteria and yeast. *Biochimica et Biophysica Acta, 148,* 781-788.

Sale, A.J.H. & Hamilton, W.A. (1968). Effects of high electric fields on micro-organisms. III. Lysis of erythrocytes and protoplasts. *Biochimica et Biophysica Acta, 163,* 37-43.

Samaranayake, C.P., Sastry, S.K. & Zhang, H. (2005). Pulsed Ohmic heating – A Novel Technique for minimization of electro-chemical reactions during processing. *Food Engineering and Physical Properties, 70,* 460-465.

Sánchez-Moreno, C., Plaza, L., Elez-Martínez, P., De Ancos, B., Martín-Belloso, O. & Cano, M.P. (2005). Impact of high pressure and pulsed electric fields on bioactive compounds and antioxidant activity of orange juice in comparison with traditional thermal processing. *Journal of Agricultural and Food Chemistry, 53,* 4403-4409.

Sastry, S.K. (2016). Towards a philosophy and theory of volumentric Nonthermal processing. *Journal of Food Science, 81,* E1431-E1446.

Saulis, G. (2010). Electroporation of Cell Membranes: The Fundamental Effects of Pulsed Electric Fields in Food Processing. *Food Engineering Reviews, 2,* 52-73.

Schilling, S., Schmid, S., Jaeger, H., Ludwig, M. Dietrich, H., Toepfl, S., Knorr, D., Neidhart, S., Schieber, A. & Carle, R. (2008). Comparative study of pulsed electric field and thermal processing of apple juice with particular consideration of juice quality and enzyme deactivation. *Journal of Agricultural and Food Chemistry, 56,* 4545-4554.

Schoenbach, K.H., Peterkin, F.E., Alden, R.W. & Beebe, S.J. (1997). The effect of pulsed electric fields on biological cells: experiments and applications. *IEEE transactions on plasma science, 25,* 284-292.

Schokker, E.P. (1997). Kinetic of heat inactivation of the extracellular proteinase from Pseudomonas fluorescens 22F at 35-70°C. In: Kinetic Modelling of Enzyme Inactivation. Kinetics of heat inactivation of the extracellular proteinase from Pseudomonas fluorescens 22F. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.

Schokker, E.P. & Van Boekel, M.A.J.S. (1997). Kinetic modeling of enzyme inactivation: kinetics of heat inactivation at 90-110°C of extracellular proteinase from *Pseudomonas fluorescens* 22F. *Journal of Agricultural and Food Chemistry, 45,* 4740-4747.

Schroeder, S., Buckow, R. & Knoerzer, K. (2009). Numerical simulation of pulsed electric fields (PEF) processing for chamber design and optimisation. *Seventh International Conference on CDF in the minerals and process industries,* CSIRO, Melbourne, Australia. 9-11 December 2009.

Sensoy, I., Zhang, H., & Sastry, S.K. (1997). Inactivation kinetics of *Salmonella Dublin* by pulsed electric fields. *Journal of Food Process Engineering, 20,* 367-381.

Sharma, M., Adler, B.B., Harrison, M.D. & Beuchat, L.R. (2005). Thermal tolerance of acid-adapted and unadapted *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* in cantaloupe juice and watermelon juice. *Letters in Applied Microbiology, 41,* 448-453.

Sharma, P. Oey, I. & Everett, D.W. (2014). Effect of pulsed electric field processing on the functional properties of bovine milk. *Trends in Food Science & Technology, 35,* 87-101.

Sharma, P., Bremer, P., Oey, I. & Everett, D.W. (2014). Bacterial inactivation in whole milk using pulsed electric field processing. *International Dairy Journal, 35,* 49-56.

Shaw, P.E. & Wilson, C.W. (1980). Importance of selected volatile components to natural orange, grapefruit, tangerine, and mandarin flavors. In: Nagy, S. & Attaway, J.A. (Eds). *Citrus Nutrition and Quality*, American Chemical Society, Washington D.C.

Shaw, P.E. (1991). Fruits ii. In: Maarse, H. (Ed.). *Volatile compounds in foods and beverages.* Marcel Dekker, Inc. New York.

Sieiro, C., García-Franga, B., López-Seijas, J., da Silva, A.F. & Villa, T.G. (2012). Microbial pectic enzymes in the food and wine industry. In: Valdez (Ed.). *Food Industrial Processes – Methods and Equipment.* InTech, Croatia.

Siemer, C., Toepfl, S. & Heinz, V. (2014). Inactivation of *Bacillus subtilis* spores by pulsed electric fields (PEF) in combination with thermal energy – I. Influence of process- and product parameters. *Food Control, 39,* 163-171.

Smelt, J.P.P.M., Hellemons, J.C., Wouters, P.C. & Van Gerwen, S.J.C. (2002). Physiological and mathematical aspects in setting criteria for decontamination of foods by physical means. *International Journal of Food Microbiology, 78,* 57-77.

Smelt, J.P.P.M. & Brul, S. (2014). Thermal Inactivation of Microorganisms. *Critical Reviews in Food Science and Nutrition, 54,* 1371-1385.

Snir, R., Koehler, P.E., Sims, K.A. & Wicker, L. (1996). Total and thermostable pectinesterases in citrus juices. *Journal of Food Science, 61*, 379-382.

Somolinos, M., García, D., Mañas, P., Condón, S. & Pagán, R. (2008). Effect of environmental factors and cell physiological state on Pulsed Electric Fields resistance and repair capacity of various strains of *Escherichia coli. International Journal of Food Microbiology, 124,* 260-267.

Somolinos, M., Espina, L., Pagán, R. & Garcia, D. (2010). *sigB* absence decreased *Listeria monocytogenes* EGD-e heat resistance but not its Pulsed Electric Fields resistance. *International Journal of Food Microbiology, 141,* 32-38.

Stanley, D.W. & Parkin, K.L. (1991). Biological membrane deterioration and associated quality losses in food tissues. *Critical Reviews in Food Science and Nutrition, 30*, 487–553.

Stoeckel, M., Lidolt, M., Stressler, T., Fischer, L., Wenning, M. & Hinrichs, J. (2016). Heat stability of indigenous milk plasmin and proteases from *Pseudomonas:* a challenge in the production of ultra-high temperature milk products. *International Dairy Journal, 61,* 250-261.

Tatum, J.H., Nagy, S. & Berry, R.E. (1975). Degradation products formed in canned single-strength orange juice during storage. *Journal of Food Science, 40*, 707-709.

Teissié, J., Golzio, M. & Rols, M.P. (2005). Mechanisms of cell membrane electropermeabilization: a minireview of our present (lack of ?) knowledge. *Biochimica et Biophysica Acta – General Subjects, 1724*, 270–280.

Terefe, N.S., Buckow, R. & Versteeg, C. (2013). Quality related enzymes in plant based products: effects of novel food processing technologies. Part 2: pulsed electric field processing. *Critical Reviews in Food Science and Nutrition, 55,* 1-15.

Tetra Pak (2004). The orange book. Tetra Pak Processing Systems AB.

Timmermans, R.A.H., Mastwijk, H.C., Knol, J.J., Quataert, M.C.J., Vervoort, L., Van der Plancken, I., Hendrickx, M.E. & Matser, A.M. (2011). Comparing equivalent thermal, high pressure and pulsed electric field processes for mild pasteurization of orange juice. Part I: Impact on overall quality attributes. *Innovative Food Science and Emerging Technologies, 12,* 235-243.

Timmermans, R.A.H., Nierop Groot, M.N., Nederhoff, A.L., Van Boekel, M.A.J.S., Matser, A.M. & Mastwijk, H.C. (2014). Pulsed electric field processing of different fruit juices: Impact of pH and temperature on inactivation of spoilage and pathogenic micro-organisms. *International Journal of Food Microbiology, 173,* 105-111.

Timmermans, R.A.H., Nederhoff, A.L., Nierop Groot, M.N., Van Boekel, M.A.J.S. & Mastwijk, H.C. (2016). Effect of electrical field strength applied by PEF processing and storage temperature on the outgrowth of yeasts and moulds naturally present in a fresh fruit smoothie. *International Journal of Food Microbiology, 230,* 21-30.

Timmermans, R.A.H., Mastwijk, H.C., Nierop Groot, M.N. & Van Boekel, M.A.J.S. (2017). Evaluation of the Gauss-Eyring model to predict thermal inactivation of micro-organisms at short holding times. *International Journal of Food Microbiology, 263,* 47-60.

Ting, S.V. (1980). Nutrients and Nutrition of Citrus Fruits. In: Nagy, S. & Attaway, J.A. (Eds). *Citrus Nutrition and Quality*, American Chemical Society, Washington D.C.

Toepfl, S., Mathys, A., Heinz, V. & Knorr, D. (2006). Review: Potential of High Hydrostatic Pressure and Pulsed Electric Fields for Energy Efficient and Environmentally Friendly Food Processing. *Food Reviews International, 22,* 405-423.

Toepfl, S., Heinz, V. & Knorr, D. (2007a). History of pulsed electric field treatment. In: Lelieveld, H.L.M., Notermans, S. & de Haan, S.W.H. (Eds.). *Food preservation by pulsed electric fields. From research to application.* Woodhead publishing.

Toepfl, S., Heinz, V. & Knorr, D. (2007b). High intensity pulsed electric fields applied for food preservation. *Chemical Engineering and Processing, 46,* 537-546.

Toepfl, S. (2011). Pulsed Electric Field food treatment – scale up from lab to industrial scale. *Procedia Food Science, 1,* 776-779.

Toepfl, S., Siemer, C., Saldaña-Navarro, G. & Heinz, V. (2014). Overview of Pulsed Electric Fields Processing for Food. In: Sun, D-W. (Ed.) *Emerging Technologies for Food Processing,* second edition. Academic Press, 93-114.

Torregrosa, F., Esteve, M.J., Frígola, A. & Cortés, C. (2006). Ascorbic acid stability during refrigerated storage of orange-carrot juice treated by high pulsed electric field and comparison with pasteurized juice. *Journal of Food Engineering, 73,* 339-345.

Tournas, V.H. (1994). Heat-resistant fungi of importance to the food and beverage industry. *Critical Reviews in Microbiology, 20,* 243-263.

Tournas, V.H. & Katsoudas, E. (2005). Mould and yeast flora in fresh berries, grapes and citrus fruits. *International Journal of Food Microbiology, 105,* 11-17.

Trammel, D.J., Dalsis, D.E. & Malone, C.T. (1986). Effect of oxygen on taste, ascorbic acid loss and browning for HTST-pasteurized, single-strength orange juice. *Journal of Food Science, 51,* 1021-1023.

Tribst, A.A.L., Sant' Ana, A.S. & Massaguer, P.R. (2009). Review: Microbiological quality and safety of fruit juices – past, present and future perspectives. *Critical Reviews in Microbiology, 35*, 310-339.

Tsong, T.Y. (1990). On electroporation of cell membranes and some related phenomena. *Bioelectrochemistry and Bioenergetics, 24,* 271-295.

Tsong, T.Y. (1991). Electroporation of cell membranes. *Biophysical society, 60,* 297-306.

Ulmschneider, M.B., Bagnéris, C., McCusker, E.C., DeCaen, P.G., Delling, M., Clapham, D.E., Ulmschneider, J.P. & Wallace (2013). Molecular dynamics of ion transport through the open conformation of a bacterial voltage-gated sodium channel. *Proceedings of the National Academy of Sciences, 110,* 6364-6369.

USFDA, United States Food and Drug Administration (2001). Hazard Analysis and Critical Control Point (HACCP): procedures for safe and sanitary processing and importing of fruit juice; Final Rule (21 CFR 120). Federal Register, *66,* 6137-6202.

USFDA, United States Food and Drug Administration (2004). Guidance for Industry Juice HACCP Hazards and Controls Guidance. First edition; final guidance. *US Department of Health and Human Services Food and Drug Administration. Center for Food Safety and Applied Nutrition.* https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Juice/ucm072557.htm

Valero, M., Micol, V., Saura, D., Muñoz, N., Lorente, J. & Marti, N. (2010). Comparison of antimicrobial treatments for citrus juices. In: Hemming, D. (Ed.). *Plant Science Reviews 2010,* S R Nova Pvt Ltd, Bangalore, India. pp. 63-74.

Van Asselt, E.D. & Zwietering, M.H. (2006). A systematic approach to determine global thermal inactivation parameters for various food pathogens. *International Journal of Food Microbiology, 107,* 73-82.

Van Boekel, M.A.J.S. (2002). On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *International Journal of Food Microbiology, 74,* 139-159.

Van Boekel, M.A.J.S. & Zwietering, M.H. (2007). Experimental design, data processing and model fitting in predictive microbiology. In: Brul, S., Van Gerwen, S. & Zwietering M (Eds.). *Modelling microorganisms in food.* CRC Press, Woodhead Publishing Limited, Boca Raton.

Van Boekel, M.A.J.S. (2008). Kinetic Modeling of Food Quality: a Critical Review. *Comprehensive reviews in Food Science and Food Safety, 7,* 144-158.

Van Boekel, M.A.J.S. (2009a). Kinetics and Statistics. In: Van Boekel, M.A.J.S. (Ed.). *Kinetic Modeling of Reactions in Foods.* CRC Press, Taylor & Francis Group, Boca Raton.

Van Boekel, M.A.J.S. (2009b). Chemical Thermodynamics in a nutshell. In: Van Boekel, M.A.J.S. (Ed.). *Kinetic Modeling of Reactions in Foods.* CRC Press, Taylor & Francis Group, Boca Raton.

Van Boekel, M.A.J.S. (2009c). Enzyme kinetics. In: Van Boekel, M.A.J.S. (Ed.). *Kinetic Modeling of Reactions in Foods.* CRC Press, Taylor & Francis Group, Boca Raton.

Van den Bosch, H.F.M. (1997). Chamber design and process conditions for pulsed electric field treatment of food. In: Lelieveld, H.L.M., Notermans, S. & de Haan, S.W.H. (Eds.). *Food preservation by pulsed electric fields. From research to application.* Woodhead publishing.

Van den Broeck, I., Ludikhuyze, L.R., Weemaes, C.A., Van Loey, A.M. & Hendrickx, M.E. (1999). Thermal inactivation kinetics of pectinesterase extracted from oranges. *Journal of Food Processing Preservation, 23,* 391-406.

Van der Veen, S., Wagendorp, A., Abee, T. & Wells-Bennik, M.H. (2009). Diversity assessment of heat resistance of *Listeria monocytogenes* strains in a continuous-flow heating system. *Journal of Food Protection, 5*, 999-1004.

Van Heesch, E.J.M., Pemen, A.J.M., Huijbrechts, P.A.H.J., Van der Laan, P.C.T., Ptasinski, K.J., Zanstra, G.J. & De Jong, P. (2000). A fast pulsed power source applied to treatment of conducting liquids and air. *IEEE Transitions on Plasma Science, 28,* 137-143.

Van Loey, A., Verachtert, B. & Hendrickx, M. (2002). Effect of high electric field pulses on enzymes. *Trends in Food Science & Technology, 12,* 94-102.

Vega-Mercado, H., Pothakamury, U.R., Chang, F-J, Barbosa-Cánovas, G.V. & Swanson, B.G. (1996). Inactivation of *Escherichia coli* by combining pH, ionic strength and pulsed electric fields hurdles. *Food Research International, 29,* 117-121.

Vega-Mercado, H., Powers, J.R., Martín-Belloso, O., Barbosa-Cánovas, G.V. & Swanson, B.G. (2001). Change in susceptibility of proteins to proteolysis and the inactivation of an extracellular protease from *Pseudomonas fluorescens* M3/6 when exposed to pulsed electric fields. In: Barbosa-Cánovas, G.V. & Zhang, Q.H. (Eds.). *Pulsed Electric Fields in Food Processing. Fundamental Aspects and Applications.* Lancaster, USA, Technomic Publishing Company, Inc.

Vega-Mercado, H., Gongora-Nieto, M.M., Barbosa-Canovas, G.V. & Swanson, B.G. (2007). Pulsed Electric Fields in Food Preservation. In: Shafiur, R.M. (Ed.). *Handbook of food preservation.* 2nd ed. CRC Press, Boca Raton.

Versteeg, C. (1979). Pectinesterase from the orange fruit – their purification, general characteristics and juice cloud destabilizing properties. PhD thesis. Agricultural University Wageningen, The Netherlands.

Vervoort, L., Van der Plancken, I., Grauwet, T., Timmermans, R.A.H., Mastwijk, H.C., Matser, A.M., Hendrickx, M.E. & Van Loey, A. (2011). Comparing equivalent thermal, high pressure and pulsed electric field processes for mild pasteurization of orange juice. Part II: Impact on specific chemical and biochemical quality parameters. *Innovative Food Science and Emerging Technologies, 12,* 466-477.

Vervoort, L., Grauwet, T., Kebede, B.T., Van der Plancken, I., Timmermans, R., Hendrickx, M. & Van Loey, A. (2012). Headspace fingerprinting as an untargeted approach to compare novel and traditional processing technologies: a case-study on orange juice pasteurisation. *Food Chemistry*, 134, 2303-2312.

Vorobiev, E. & Lebovka, N. (2008). Pulsed-Electric-Fields-Induced Effects in Plant Tissues: Fundamental Aspects and Perspectives of Applications. In: Vorobiev, E. & Lebovka, N. (Eds.). *Electrotechnologies for Extraction from Food plants and Biomaterials.* Springer Science.

Vorobiev, E. & Lebovka, N. (2010). Enhanced extraction from solid foods and biosuspensions by Pulsed Electrical Energy. *Food Engineering Reviews, 2,* 95-108.

Walker, M. & Phillips, C.A. (2008). *Alicyclobacillus acidoterrestris*: an increasing threat to the fruit juice industry? *Internation Journal of Food Science and Technology, 43,* 250-260.

Walkling-Ribeiro, M., Noci, F., Cronin, D.A., Riener, J., Lyng, J.G. & Morgan, D.J. (2008a). Reduction of *Staphylococcus aureus* and quality changes in apple juice processed by ultraviolet irradiation, pre-heating and pulsed electric fields. *Journal of Food Engineering, 89,* 267-273.

Walkling-Ribeiro, M., Noci, F., Cronin, D.A., Lyng, J.G. & Morgan, D.J. (2008b). Inactivation of *Escherichia coli* in a tropical fruit smoothie by a combination of heat and pulsed electric fields. *Food Microbiology and Safety, 73,* M395-M399.

Walkling-Ribeiro, M., Noci, F., Cronin, D.A., Lyng, J., & Morgan, D.J. (2010). Shelf life and sensory attributes of a fruit smoothie-type beverage processed with moderate heat and pulsed electric fields. *LWT – Food Science and Technology, 43,* 1067-1073.

Walsh, M., Rouseff, R. & Naim, M. (1997). Determination of furaneol and *p*-vinylguaiacol in orange juice employing differential UV wavelight and fluorescence detection with a unified solid phase extraction. *Journal of Agricultural and Food Chemistry, 45,* 1320-1324.

Weaver, J.C. & Chizmadzhev, Y.A. (1996). Theory of electroporation: a review. *Bioelectrochemistry and Bioenergetics, 41*, 135–160.

Weisstein, E.W. (2016a). Log Normal Distribution. *MathWorld, A Wolfram Web Resource.* http://mathworld.wolfram.com/LogNormalDistribution.html

Weisstein, E.W. (2016b). Erfc. *MathWorld, A Wolfram Web Resource*. http://mathworld.wolfram.com/Erfc.html

Whitaker, J.R. (1996). Enzymes. In: Fennema, O.R. (Ed.). *Food Chemistry,* third edition. CRC Taylor & Francis Group.

Whiting, R.C. (1993). Modeling bacterial survival in unfavourable environments. *Journal of Industrial Microbiology, 12,* 240-246.

WHO, World Health Organization (2015). Healthy diet. Factsheet no. 394. May 2015.

Wibowo, S., Grauwet, T., Santiago, J.S., Tomic, J., Vervoort, L., Hendrickx, M. & Van Loey, A. (2015). Quality changes of pasteurised orange juice during storage: a kinetic study on specific parameters and their relation to colour instability. *Food Chemistry,* 187, 140-151

Wisnieuwski, M., Biles, C., Droby, S., McLaughlin, R., Wilson, C. & Chalutz, E. (1991). Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii.* I. Characterization of attachment to *Botrytis cinerea. Physiological and Molecular Plant Pathology, 39,* 245-258.

Wolfram (2016). https://www.wolframalpha.com/input/?i=solve+(a*T*exp(-((b%2FT-c)))t-1%3D0+for+T. accessed March 14, 2016.

Wouters, P.C., Dutreux, N., Smelt, J.P.P.M. & Lelieveld, H.L.M. (1999). Effects of Pulsed Electric Fields on Inactivation Kinetics of *Listeria innocua. Applied and Environmental Microbiology, 65,* 5364-5371.

Wouters, P.C., Alvarez, I. & Raso, J. (2001). Critical factors determining inactivation kinetics by pulsed electric field food processing. *Trends in Food Science & Emerging Technologies, 12,* 112-121.

Xi, B., Li, S., Liu, Z., Tian, H., Yin, X., Huai, P., Tang, W, Zhou, D. & Steffen, L.M. (2014). Intake of fruit juice and incidence of type 2 diabetes: a systematic review and meta-analysis. *PLOS One, 9,* e93471.

Xiang, Y., Gubian, S., Suomela, B. & Hoeng, J. (2013). Generalized Simulated Annealing for Efficient Global Optimization: the GenSA Package for R. The R Journal, 5, URL http://journal.r-project.org/.

Yang, R.J., Li, S.Q. & Zhang, Q.H. (2004). Effects of pulsed electric fields on the activity of enzymes in aqueous solution. *Journal of Food Science, 69,* 241-248.

Yeom, H.W., Zhang, Q.H. & Dunne, C.P. (1999). Inactivation of papain by pulsed electric fields in a continuous system. *Food Chemistry, 67,* 53-59.

Yeom, H.W., Streaker, C.B., Zhang, Q.H. & Min, D.B. (2000). Effects of pulsed electric fields on the quality of orange juice and comparison with heat pasteurization. *Journal of Agricultural and Food Chemistry, 48,* 4597-4605.

Yeom, H.W., Zhang, Q.H. & Chism, G.W. (2002). Inactivation of pectin methyl esterase in orange juice by pulsed electric fields. *Food Engineering and Physical Properties, 67,* 2154-2159.

Yoshioka, S., Aso, Y., Izutsu, K. & Koijma, S. (1994). Is stability prediction possible for protein drugs? Denaturation kinetics of beta-Galactosidase in solution, *Pharmaceutical Research*, *11,* 1721-1725.

Zhang, Q., Monsalve-González, A., Qin, B-L, Barbosa-Cánovas, G.V. & Swanson, B.G. (1994). Inactivation of *Saccharomyces cerevisiae* in apple juice by square-wave and exponential decay pulsed electric fields. *Journal of Food Process Engineering, 17,* 469-478.

Zhang, Q, Barbosa-Cánovas, G.V. & Swanson, B.G. (1995). Engineering aspects of pulsed electric field pasteurization. *Journal of Food Engineering, 25,* 261-281.

Zhong, K., Chen, F., Wu, J., Wang, Z., Liao, X., Hu, X. & Zhang, Z. (2005). Kinetics of inactivation of *Escherichia coli* in carrot juice by pulsed electric field. *Journal of Food Process Engineering, 28,* 595-609.

Zhong, K., Wu, J., Wang, Z., Chen, F., Liao, X., Hu, X. & Zhang, Z. (2007). Inactivation kinetics and secondary structural change of PEF-treated POD and PPO. *Food Chemistry, 100,* 115-123.

Zimmerman, U., Pilwat, G. & Riemann, F. (1974). Dielectric breakdown of cell membranes. *Biophysical Journal, 14,* 881-899.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M. & Van 't Riet, K. (1990). Modeling of the bacterial growth curve. *Applied and Environmental Microbiology, 56*, 1875-1881.

Summary

Pulsed Electric Field (PEF) processing has gained a lot of interest the last decades as mild processing technology as alternative to thermal pasteurisation, and is suitable for preservation of liquid food products such as fruit juices. PEF conditions typically applied at industrial scale for pasteurisation are high intensity pulsed electric fields aiming for minimal heat load, with an electric field strength (E) in the range of 15 – 20 kV/cm and pulse width (τ) between 2 – 20 µs. Alternatively, moderate intensity pulsed electric fields with an electric field strength in the order of E = 0.5 – 5.0 kV/cm are used as a pre-step to disintegrate plant cells or electroporate micro-organisms for mass transfer in food and biotechnological, but not as an alternative preservation technology. The work described in this thesis investigated how moderate and high intensity PEF processing conditions, product matrix and characteristics of target species affect microbial inactivation, shelf life, enzyme activity and quality of fruit juices. This was performed by a systematic evaluation of the impact of most important process parameters in PEF processing, including electric field strength, pulse width and temperature, in a continuous-flow configuration. Microbial inactivation after PEF treatment was compared to that of an equivalent thermal process to distinguish the electroporation and thermal effects responsible for inactivation.

In Chapter 1, an overview of the current status in the scientific field on fruit juice processing, pulsed electric field processing and kinetic modelling is provided, knowledge gaps were identified and the aim and research questions following from that are described.

In Chapter 2, the impact of a high intensity PEF process (E = 20 kV/cm and τ = 2 µs) was studied on the inactivation of *Escherichia coli, Salmonella* Panama, *Listeria monocytogenes* and *Saccharomyces cerevisiae* in apple, orange and watermelon juice. Kinetic data showed that for identical process and matrix conditions, the yeast *S. cerevisiae* was the most sensitive micro-organism, followed by *S.* Panama and *E. coli,* which displayed comparable inactivation kinetics. *L. monocytogenes* was most resistant towards the treatment conditions tested. A synergistic effect between temperature and electric pulses was observed at inlet temperatures above 35 °C, hence less electrical energy for inactivation was required at higher temperatures. The different juice matrices resulted in a different degree of inactivation, predominantly determined by pH, where more acidic conditions led to more inactivation.

In Chapter 3, the effects of high intensity PEF processing conditions (E = 13.5 – 24.0 kV/cm) and storage temperature on the outgrowth of surviving yeast and mould populations naturally present in a fresh fruit smoothie were assessed over time. Results showed that untreated smoothie was predominantly spoiled by the outgrowth of yeasts, typically after 8 days (stored at 4 or 7 °C), while initial number of moulds present in the smoothie declined during storage. PEF inactivated most yeasts present in the smoothie, thereby providing outgrowth opportunities for the moulds, which were visually observed after 14 days (stored at 7°C) or 18 days (stored at 4°C). The intensity of the electric field strength both affected the $\log_{10}$ reduction of yeasts and

the lag-time, the period the cell required to grow out. A similar effect of electric field strength on the degree of inactivation has been observed for moulds, although electric field strength did not influence the period to visual mould growth.

Chapter 4 and 5 focussed on the development of a model to be used to fit and predict non-linear log-time inactivation of a thermal process at a holding time comparable to a PEF process. In Chapter 4, a rational thermodynamic model based on Gaussian distribution and Eyrings rate constant has been developed that can accurately model non-linear log-time inactivation kinetics of enzymes and micro-organisms exposed to a thermal and/or chemical (acid) treatment. This so-called Gauss-Eyring model is a bivariate log-normal distribution with temperature and time as independent variables. Model parameters standard activation enthalpy and entropy are directly related to reference temperature *Tr* and *Z*-value, commonly used in kinetic analysis in food microbiology. An essential feature of the kinetic model is that its parameters are treated as stochastic variables, owing to the underlying physics, based on the Lumry-Eyring model for unfolding of proteins using transition state theory. The performance of the model was evaluated using published data on enzyme inactivation and microbial inactivation including a wide range of temperatures and pH.

In Chapter 5, this Gauss-Eyring model was used to fit inactivation data of *E. coli, L. monocytogenes, Lactobacillus plantarum, Salmonella* Senftenberg and *S. cerevisiae* in orange juice. Thermal inactivation data was collected by exposing capillary tubes with target organisms to different temperature time combinations using a water bath to obtain inactivation kinetics, either via isothermal (a fixed temperature with varying holding time) or isotime (a fixed holding time with varying temperature) series. The model fitted well to the inactivation data of individual cultures. Variability between the different cultures of the five tested micro-organisms was observed. Therefore, an average value of the parameters of the individual cultures was used to predict inactivation as a function of temperature for a chosen (short) holding time.

In Chapter 6, a systematic evaluation of the individual effects of electric field strength and pulse width in combination with heat on the inactivation of *E. coli, L. monocytogenes*, *S.* Senftenberg, *L. plantarum* and *S. cerevisiae* in orange juice was carried out. A wide range of conditions has been tested, including both moderate intensity as well as high intensity PEF. Both electric field strength and pulse width were shown to be important for microbial inactivation. Inactivation kinetics of the tested conditions were compared to an equivalent thermal reference process, based on parameter estimates obtained in Chapter 5. A non-thermal pulse effect was observed for three specific sets of conditions in addition to the thermal effect responsible for inactivation. A non-thermal pulse effect was found for high intensity PEF treatment at E = 15 or 20 kV/cm and τ = 2 µs, but also at moderate intensity PEF condition of E = 2.7 kV/cm and τ = 1000 µs. The effectivity of this moderate intensity PEF condition was evaluated for *E. coli* and *L. monocytogenes* in watermelon juice and coconut water, varying in pH and conductivity. Interestingly, this moderate intensity PEF condition showed the same effectivity for all matrices in the pH range of 3.8 to 6.0, while high intensity PEF conditions at E = 20 kV/cm did show a strong dependence on product pH for microbial inactivation (Chapter 2). This suggests that a different mechanism is responsible for inactivation at moderate intensity conditions

compared to high intensity conditions. Speculations on the mechanism responsible for inactivation are made in Chapter 8.

In Chapter 7, the impact of moderate intensity PEF (E = 0.9 – 2.7 kV/cm) and long pulse width (τ = 1000 µs) at variable maximum temperatures was evaluated on quality attributes of freshly squeezed orange juice, and compared to the impact of two thermal processes using either mild or severe pasteurisation conditions. No differences for pH and soluble solids were found after application of any treatment, and only small differences were observed for colour and vitamin C content after PEF and thermal treatment, mainly for the conditions applied at higher temperature. A large processing effect was measured in the enzyme activity of pectin methylesterase (PME), responsible for undesired cloud instability. Reduction of the remaining enzyme activity depended on the maximum applied temperature, and levels below the critical value to obtain shelf stable juices were found, showing that it is possible to select moderate intensity PEF conditions for adequate pasteurisation of fruit juices, both with respect to micro-organisms and enzymes. The impact of processing on volatile flavour compounds was moderate when compared to untreated, although some deviations between moderate intensity PEF treated and thermally processed orange juice were found for individual compounds, with a better retention of the flavour compounds after application of moderate intensity PEF.

In Chapter 8, the main results of this thesis were discussed and concluding remarks and recommendations were presented. In conclusion, this thesis provided novel insight in the use of pulsed electric field processing as a mild pasteurisation process with improved quality as alternative to thermal pasteurisation of fruit juice. The work described in this thesis resulted in more insight in the individual effects of electric field strength and pulse width, and presented an effective combination of long pulse width and moderate intensity electric field strength as alternative PEF condition to the currently used high intensity electric field strength conditions for preservation. In addition, these moderate intensity/long pulse duration conditions are promising for industrial application, as they showed to be less sensitive for differences in the characteristics of the micro-organisms than high intensity PEF conditions and they are effective in both high-acid as well as low-acid products.

Samenvatting

Het gebruik van pulserende elektrische velden, oftewel Pulsed Electric Fields (PEF) heeft de afgelopen decennia veel belangstelling gekregen als milde conserveringstechniek met als doel om voor microbiologische stabiliteit te zorgen, waarbij tegelijkertijd de karakteristieken van verse producten behouden blijven. PEF kan gebruikt worden als alternatief voor thermische pasteurisatie en is geschikt voor vloeibare levensmiddelen waaronder vruchtensappen. PEF procescondities die typisch toegepast worden voor pasteurisatie op industriële schaal, zijn pulserende elektrische velden met een hoge intensiteit en korte duur gericht op een minimale warmtebelasting, met een elektrische veldsterkte (E) in het bereik van 15 – 20 kV/cm en puls duur (τ) tussen 2 – 20 µs. PEF procescondities met een matige intensiteit in de orde van E = 0.5 – 5.0 kV/cm worden gebruikt als een processtap om plantencellen te desintegreren om massatransport in voedsel- en biotechnologie te verbeteren of voor toepassingen op micro-organismen anders dan milde conservering. Het doel van het onderzoek beschreven in dit proefschrift is om te evalueren hoe matige en hoge intensiteit PEF procescondities, producteigenschappen en eigenschappen van het micro-organisme van invloed zijn op de microbiële inactivatie, houdbaarheid, enzymactiviteit en kwaliteit van vruchtensappen. Om dit doel te bereiken is een systematische evaluatie van de belangrijkste procesparameters, inclusief elektrische veldsterkte, puls duur en temperatuur, in een continue debietconfiguratie uitgevoerd. De microbiële inactivatie na PEF behandeling is vergeleken met die van een gelijkwaardig thermisch proces om een onderscheid te maken tussen de elektroporatie en thermische effecten in een PEF-proces.

In hoofdstuk 1 van dit proefschrift is een overzicht gegeven van de huidige stand van zaken op het gebied van vruchtensapverwerking, het PEF-proces en kinetisch modelleren. Kennis hiaten zijn geïdentificeerd en de daaruit voortvloeiende onderzoeksvragen zijn beschreven.

In hoofdstuk 2 is de invloed van een PEF-proces met hoge intensiteit (E = 20 kV/cm en τ = 2 µs) op de inactivatie van *Escherichia coli*, *Salmonella* Panama, *Listeria monocytogenes* en *Saccharomyces cerevisiae* in appel-, sinaasappel- en watermeloensap onderzocht. Met kinetische gegevens is aangetoond dat voor identieke procescondities en producteigenschappen de gist *S. cerevisiae* het meest gevoelige was voor PEF, gevolgd door *S.* Panama en *E. coli*, die vergelijkbare inactivatie kinetiek vertoonde. *L. monocytogenes* was het meest resistent onder de geteste behandelingsomstandigheden. Een synergistisch effect tussen temperatuur en elektrische pulsen werd waargenomen bij voorverwarmtemperaturen boven 35 °C, dit betekent dat minder elektrische energie nodig was voor inactivatie bij hogere voorverwarmtemperaturen. De verschillende sap matrices resulteerden in een andere mate van inactivatie, voornamelijk bepaald door de pH, waarbij meer zure omstandigheden tot meer inactivatie leidden.

In hoofdstuk 3 zijn de effecten van PEF procescondities met hoge intensiteit (E = 13.5 – 24.0 kV/cm) op de uitgroei van overlevende gist- en schimmelpopulaties, die van nature aanwezig zijn in een verse fruitsmoothie, beoordeeld gedurende een bewaarperiode. Resultaten toonden aan dat onbehandelde smoothie voornamelijk bedorven werd door de uitgroei van gisten, over het algemeen na 8 dagen (opgeslagen bij 4 of 7 °C), terwijl het aantal schimmels in de smoothie tijdens de opslag daalde. PEF behandeling

inactiveerde de meeste gisten van nature aanwezig in de smoothie, waardoor er uitgroeimogelijkheden voor de schimmels ontstonden, die visueel werden waargenomen na 14 dagen (opslagtemperatuur van 7 °C) of 18 dagen (opslagtemperatuur van 4 °C). De intensiteit van de elektrische veldsterkte beïnvloedde zowel de $\log_{10}$ reductie van het aantal aanwezige gisten als de duur van de lag-fase, de periode die een cel nodig heeft voordat de celdeling begint. Ook voor schimmels was de elektrische veldsterkte van invloed op de mate van inactivatie, echter werd de periode tot visuele schimmelgroei niet beïnvloedt door de elektrische veldsterkte.

Hoofdstuk 4 en 5 richten zich op de ontwikkeling van een model te gebruiken om niet-lineaire log-tijd inactivatie van een thermisch proces met een houdtijd vergelijkbaar aan die van een PEF-proces te beschrijven en te voorspellen. In hoofdstuk 4 is een rationeel thermodynamisch model op basis van Gauss-verdeling en Eyrings reactiesnelheidsconstante ontwikkeld dat nauwkeurig de niet-lineaire log-tijd-inactivatiekinetiek van enzymen en micro-organismen die blootgesteld worden aan thermische en/of chemische (zure) behandeling kan beschrijven. Dit zogenaamde Gauss-Eyring-model is een stochastisch model op basis van de lognormale verdeling met temperatuur en tijd als onafhankelijke variabelen. Modelparameters standaard activerings-enthalpie en -entropie zijn direct gerelateerd aan referentietemperatuur *Tr* en *Z*-waarde. Het concept van een referentietemperatuur en *Z*-waarde wordt ook toegepast bij kinetische analyse in voedselmicrobiologie. Een essentieel kenmerk van dit kinetische model is dat zijn parameters worden behandeld als stochastische variabelen. De onderliggende fysica is gebaseerd op het Lumry-Eyring-model, een kwantum mechanisch model voor het ontvouwen van eiwitten op basis van Eyrings *transition-state* theorie. De werking van het Gauss-Eyring model werd geëvalueerd met behulp van gepubliceerde data van zowel enzym inactivatie als microbiële inactivatie, over een breed temperatuur, tijd en pH bereik. In hoofdstuk 5 is dit Gauss-Eyring model verder gebruikt om inactivatie van *E. coli*, *L. monocytogenes*, *Lactobacillus plantarum*, *Salmonella* Senftenberg en *S. cerevisiae* in sinaasappelsap te beschrijven. Thermische inactivatiedata zijn verzameld door het blootstellen van capillaire buizen met micro-organismen aan verschillende temperatuur-tijdcombinaties in een waterbad om inactivatiedata te verkrijgen voor kinetische modellering, hetzij via een isothermische (vaste temperatuur met verschillende houdtijd) of een isotijd (een vaste houdtijd met variabele temperatuur) serie. Het Gauss-Eyring model beschreef de inactivatie van individuele culturen goed. Variabiliteit tussen de verschillende kweek-culturen van de vijf geteste micro-organismen werd waargenomen. Om toch tot één functie voor inactivatie bij een vast gekozen (korte) houdtijd te komen, is de gemiddelde waarde van de parameters van de afzonderlijke, individuele kweken genomen.

In Hoofdstuk 6 is een systematische evaluatie uitgevoerd van de individuele effecten van elektrische veldsterkte en puls duur in combinatie met temperatuur op de inactivatie van *E. coli*, *L. monocytogenes*, *S.* Senftenberg, *L. plantarum* en *S. cerevisiae* in sinaasappelsap. Een groot bereik aan procescondities is getest, waaronder zowel matige intensiteit als hoge intensiteit PEF procescondities. Zowel de elektrische veldsterkte als de puls duur bleken belangrijk te zijn voor microbiële inactivatie. Inactivatiekinetiek van de geteste condities werd vergeleken met een equivalent thermisch proces, gebaseerd op parameterschattingen verkregen in hoofdstuk 5. Naast het thermische effect dat verantwoordelijk is voor inactivatie werd een additioneel, niet-

thermisch puls effect waargenomen voor drie specifieke sets van condities. Een niet-thermisch puls effect werd gevonden voor PEF behandelingen met hoge intensiteit bij E = 15 of 20 kV/cm en τ = 2 µs, maar ook bij een matige PEF intensiteit van E = 2.7 kV/cm en τ = 1000 µs. De effectiviteit van deze matige intensiteit PEF procesconditie werd ook geëvalueerd voor *E. coli* en *L. monocytogenes* in watermeloensap en kokosnootwater, variërend in pH en geleidbaarheid. Opvallend is dat deze matige intensiteit PEF procesconditie dezelfde effectiviteit voor alle matrices in het pH-bereik van 3.8 tot 6.0 vertoonde, terwijl hoge intensiteit PEF procescondities bij E = 20 kV/cm een sterke afhankelijkheid van product-pH voor microbiële inactivatie tonen (hoofdstuk 2). Dit suggereert dat een ander mechanisme verantwoordelijk is voor inactivatie bij matige intensiteit condities in vergelijking met hoge intensiteit condities. In hoofdstuk 8 worden een aantal hypotheses benoemd ten aanzien van het mechanisme dat verantwoordelijk is voor inactivatie.

In hoofdstuk 7 is de invloed van matige intensiteit PEF procescondities (E = 0.9 – 2.7 kV / cm) en lange puls duur (τ = 1000 µs) bij variabele maximum temperaturen geëvalueerd op kwaliteitsattributen van versgeperst sinaasappelsap. De effecten op specifieke kwaliteitsattributen zijn vergeleken met de effecten na een milde of een intense thermische pasteurisatie. Er zijn geen verschillen waargenomen voor pH en oplosbare vaste stoffen, en er werden slechts kleine verschillen waargenomen voor kleur en vitamine C-gehalte tussen PEF en thermische behandeling, voornamelijk bij de omstandigheden waarbij een hogere temperatuur werd gebruikt. Een groot behandelingseffect werd wel gemeten in de enzymactiviteit van pectine methylesterase (PME), dat verantwoordelijk is voor de troebelheidstabiliteit van vruchtensap. De resterende enzymactiviteit hing af van de maximaal toegepaste temperatuur, waarbij er PEF condities zijn gevonden met een enzymactiviteit beneden de kritische waarde om sappen te krijgen die stabiel zijn bij kamertemperatuur. Op basis van deze resultaten en de resultaten die gepresenteerd zijn in hoofdstuk 6, blijkt dat matige intensiteit PEF procescondities geschikt zijn om vruchtensappen te pasteuriseren, zowel met betrekking tot micro-organismen als enzymen. De invloed van de PEF behandeling op vluchtige aromacomponenten was gering in vergelijking met onbehandeld sap, terwijl er verschillen tussen matige intensiteit PEF behandeld en thermisch behandeld sinaasappelsap werden gevonden voor individuele stoffen, waarbij een beter behoud van de aromacomponenten na toepassing van matige intensiteit PEF werd gevonden.

In Hoofdstuk 8 zijn de belangrijkste resultaten van dit proefschrift besproken en zijn concluderende opmerkingen en aanbevelingen gepresenteerd. Concluderend levert dit proefschrift nieuwe inzichten op over het gebruik van het PEF-proces als conserveringstechniek als alternatief voor thermische pasteurisatie van vruchtensap, waarbij de verse karakteristieken beter behouden blijven. Het werk dat beschreven staat in dit proefschrift resulteerde in meer inzicht in de individuele effecten van elektrische veldsterkte en puls duur, en presenteert een effectieve combinatie van lange puls duur en matige intensiteit elektrische veldsterkte als alternatieve PEF procesconditie voor de momenteel gebruikte hoge intensiteit elektrische veldsterkte condities voor conservering. Daarnaast zijn deze matige intensiteit/ lange puls duur condities veelbelovend voor industriële toepassing, omdat ze minder gevoelig zijn voor verschillen in de eigenschappen van de micro-organismen dan hoge intensiteit PEF procescondities, en ze zijn effectief in de inactivatie van micro-organismen in producten met zowel een hoge als lage pH-graad.

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Rian

About the author

Rian Timmermans was born on 15 February, 1985 in Weert and grew up in Soerendonk, The Netherlands. In 2003, she graduated from secondary school at Philips van Horne S.G. in Weert. In the same year, she started her Bachelor Food Technology at Wageningen University. The thesis that was part of the BSc program was conducted at NIZO Food Research in 2006 with the topic 'identification of minor components separated by capillary zone electrophorese in raw milk', and Rian graduated in 2006. In the same year, she started her Master Food Technology at Wageningen University, with specialisation Product Functionality. Her thesis was conducted at the Laboratory of Food Chemistry and focussed on the influence of fibre addition on arabinoxylan-gluten protein interaction during dough making. The internship that was part of the MSc program, was conducted at Cargill Texturizing Solutions, Baupte, France. During this internship, she worked on the imitation of pizza cheese with reduced protein content. After graduation of the MSc program in 2008, she started as a researcher food technology at Agrotechnology & Food Innovations, currently known as Wageningen Food & Biobased Research. During this period, she worked on different mild preservation technologies in various (international) projects with universities and food industry. Rian initiated the PhD project in 2012, entitled, "*Moderate and High Intensity Pulsed Electric Fields* effect on microbial inactivation, shelf life and quality of fruit juices" at the chair group Food Quality and Design (Wageningen University), besides her job as a researcher. The results of this work performed at Wageningen Food & Biobased Research are described in this thesis. During her PhD, Rian received "PhD Student of the Year Award", 2nd place at European Federation of Food Science and Technology (EFFoST) conference in Uppsala, Sweden in 2014. Currently, Rian is continuing her job as a project leader and scientist mild preservation at Wageningen Food & Biobased Research.

List of publications

Peer-reviewed journals

Timmermans, R.A.H., Mastwijk, H.C., Knol, J.J., Quataert, M.C.J., Vervoort, L., Van der Plancken, I., Hendrickx, M.E. & Matser, A.M. (2011). Comparing equivalent thermal, high pressure and pulsed electric field processes for mild pasteurization of orange juice. Part I: Impact on overall quality attributes. *Innovative Food Science and Emerging Technologies, 12,* 235-243.

Vervoort, L., Van der Plancken, I., Grauwet, T., **Timmermans, R.A.H.**, Mastwijk, H.C., Matser, A.M., Hendrickx, M.E. & Van Loey, A. (2011). Comparing equivalent thermal, high pressure and pulsed electric field processes for mild pasteurization of orange juice. Part II: Impact on specific chemical and biochemical quality parameters. *Innovative Food Science and Emerging Technologies, 12,* 466-477.

Vervoort, L., Grauwet, T., Kebede, B.T., Van der Plancken, I., **Timmermans, R.**, Hendrickx, M. & Van Loey, A. (2012). Headspace fingerprinting as an untargeted approach to compare novel and traditional processing technologies: a case-study on orange juice pasteurisation. *Food Chemistry*, 134, 2303-2312.

Timmermans, R.A.H., Nierop Groot, M.N., Nederhoff, A.L., Van Boekel, M.A.J.S., Matser, A.M. & Mastwijk, H.C. (2014). Pulsed electric field processing of different fruit juices: Impact of pH and temperature on inactivation of spoilage and pathogenic micro-organisms. *International Journal of Food Microbiology, 173,* 105-111.

Timmermans, R.A.H., Nederhoff, A.L., Nierop Groot, M.N., Van Boekel, M.A.J.S. & Mastwijk, H.C. (2016). Effect of electrical field strength applied by PEF processing and storage temperature on the outgrowth of yeasts and moulds naturally present in a fresh fruit smoothie. *International Journal of Food Microbiology, 230,* 21-30.

't Lam, G.P., Postma, P.R., Fernandes, D.A., **Timmermans, R.A.H.**, Vermuë, M.H., Barbosa, M.J., Eppink, M.H.M., Wijffels, R.H. & Olivieri, G. (2017). Pulsed Electric Field for protein release of the microalgae *Chlorella vulgaris* and *Neochloris oleabundans. Algal Research, 24*, 181-187.

Mastwijk, H.C., **Timmermans, R.A.H.** & Van Boekel, M.A.J.S. (2017). The Gauss-Eyring model: a new thermodynamic model for biochemical and microbial inactivation kinetics. *Food Chemistry, 237,* 331-341.

Timmermans, R.A.H., Mastwijk, H.C., Nierop Groot, M.N. & Van Boekel, M.A.J.S. (2017). Evaluation of the Gauss-Eyring model to predict thermal inactivation of micro-organisms at short holding times. *International Journal of Food Microbiology, 263,* 47-60.

Timmermans, R.A.H., Mastwijk, H.C., Berendsen, L.B.J.M., Nederhoff, A.L., Matser, A.M., Van Boekel, M.A.J.S. & Nierop Groot, M.N. Effect of Moderate and High intensity Pulsed Electric Fields (PEF) on microbial inactivation in fruit juice. *Submitted for publication.*

Timmermans, R.A.H., Roland, W.S.U., Van Kekem, C., Matser, A.M. & Van Boekel, M.A.J.S. Effect of pasteurisation by Moderate Intensity Pulsed Electric Field treatment compared to heat treatment on quality attributes of fresh orange juice. *Submitted for publication.*

Peer reviewed book-chapter

Matser, A.M. & **Timmermans, R.A.H.** (2016). High-pressure effects on fruits and vegetables. In: Balasubramaniam, V.M., Barbosa-Cánovas, G.V. & Lelieveld, H.L.M. (Eds.) *High Pressure Processing of Food. Principles, Technology and Applications.* Springer, New York.

Patents

Timmermans, R.A.H., de Moraes, R.E., Mastwijk, H.C. & Matser, A.M. Process for fast and homogeneously heating a liquid product and apparatus for such process. WO 2013 141703. Applicant: Wageningen Food & Biobased Research.

Timmermans, R.A.H., de Moraes, R.E. & Mastwijk, H.C. Process for liquid food preservation using pulsed electric fields treatment. WO 2017086784. Applicant: Wageningen Food & Biobased Research.

Overview of completed training activities

Discipline specific activities

Courses

Reaction Kinetics, VLAG, Wageningen, 2012
Electroporation based Technologies and Treatment, COST, Ljubljana, Slovenia, 2012 (oral presentation)
PEF School for Food Processing, COST, Zaragoza, Spain, 2014 (oral presentation)

Conferences

iFood, Hannover, Germany, 2013 (poster presentation)
28th EFFoST International Conference, Uppsala, Sweden, 2014 (oral & poster presentation)
1st World Congress on Electroporation and Pulsed Electric Fields, Portorož, Slovenia, 2015
29th EFFoST International Conference, Athens, Greece, 2015 (oral presentation)
2015 International Nonthermal Processing Workshop, Athens, Greece, 2015
2nd World Congress on Electroporation and Pulsed Electric Fields, Norfolk, USA, 2017 (oral presentation)

General courses

Reviewing a scientific paper, WGC, Wageningen, 2013
Professioneel samenwerken en communiceren, ICM, Wageningen, 2013
Philosophy and Ethics of Food Science & Technology, VLAG, Wageningen, 2014
Techniques for Writing and Presenting a Scientific Paper, WGC, Wageningen, 2014
Multivariate analysis for food data/scientists, VLAG, Wageningen, 2014
Project & Time Management, WGC, Wageningen, 2015

Other activities

Preparation of PhD research proposal, 2012
Board Young AFSG, Young WUR, 2012-2013
PPS Mild Preservation consortium meetings, 2013-2016
Work discussion meetings and scientific meetings, Food Technology & Fresh Food & Chains, 2012-2017

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