

Breakage of Cellular Tissue by Pulsed Electric Field: Extraction of Polyphenols from Fresh Tea Leaves

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Pulsed electric field (PEF) is commonly understood as a fast, non-thermal, and highly effective method for extraction of intracellular compounds. This treatment involves discharge of high voltage electric pulses of a few microseconds into the food product which is placed or passed between two electrodes (Angersbach et al., 2000).

Results of experimental investigation of the electric breakage of a cellular material in pulsed electric fields and the influence of electric fields on polyphenols (PPs) extraction from fresh tea leaves are presented. Thin slices of the tissue are subjected to PEF treatment at different intensities and time. The disintegration of the cellular membrane is detected indirectly by a total PPs content measurement. When the cellular membrane is permeabilized, the overall amount of extracted PPs increases. Electric field strength is an important parameter that controls the efficiency of electroporation of the cellular tissue. Moreover, electroporation of the cellular tissue also depends on other parameters such as pulse duration or pulse width (PD), number of pulses (N) and pause between pulses (PBP). Dependencies of the electric field strength (E), the total treatment time (which considers the number of pulses (N) and the pulse width (PD) applied in the system) and the extraction yield of PPs at various modes of PEF treatment are studied.

Protocol I (N=30, PD=0.05 s and PBP (EXA)=0.5 s) results in a maximum value for the extraction yield of 27% when the electric field strength is 0.9 kV/cm. However, when the interval between pulses is equal to 3 s an electric field of 1.1 kV/cm is needed to obtain the same extraction yield (protocol II: N=30, PD=0.05 s and PBP (EXB)=3 s). The extraction yield (EY) values of the samples subjected to both pulse protocols increase with increasing electric field strength.

The effect of the total treatment time on the extraction yield is studied for two different electric field strengths, i.e. E=0.4 kV/cm and E=0.9 kV/cm. Experimental results show that longer pulses are more effective and their effect is particularly pronounced at a moderate electric field, E=0.4 kV/cm. When the total treatment time is 5 s (for both electric field strengths), experiment could not be performed due to problems with the PEF unit itself (low conductivity was detected in the PEF chamber). The PEF treatment accelerates kinetics of the extraction of polyphenolic compounds from fresh tea leaves.

1. Introduction

During the last decade, commercial interest in extraction of intracellular compounds and liquids from cellular plant tissue using various solid-liquid extraction methods has been growing. One of the factors influencing the extraction process is the degree of cell membrane disintegration. Different physical, chemical or biological treatments cause breakage of the cellular membrane (Bazhal, et al., 2003). Therefore, among different non-thermal processing methods used in the food industry, pulsed electric field (PEF) treatment was found to be a promising one and minimally invasive for breakage of cellular tissue (Huang et al., 2012).

The application of electric fields for a short duration of a few to several hundred microseconds is capable of inducing cell membrane permeabilization through a phenomenon called "electroporation" (Asavasanti et al., 2011). Applying an external electric field to the cells has shown to result in pore formation on the membrane. Pore formation is a dynamic process and can be reversible or irreversible depending on the intensity of the PEF treatment. When induced pores are small in comparison to the membrane area and if

they are generated with PEF treatment of low intensity the electric breakdown is reversible (Angersbach et al., 2000; Soliva-Fortuny et al., 2009). Increasing the intensity of the treatment by increasing electric field strength (E) and/or treatment time (t) (which considers the number of pulses and the pulse width applied in the system) will result in the formation of large pores and reversible permeabilization will turn into irreversible disruption of the cell membrane. The irreversible permeabilization of the cell membrane in the plant tissue provides a wide range of process applications where disruption of the cell membrane is required, including expression, extraction or diffusion of cell material (Knorr et al., 1994; Knorr and Angersbach, 1998).

Electric field strength is an important parameter that controls the efficiency of electroporation of the cellular tissue. Bazhal et al. (2003) presented classification of the PEF modes as low ($E \leq 100\text{-}200$ V/cm), moderate ($E = 300\text{-}1500$ V/cm) and high ($E > 1500$ V/cm). With a low electric field strength, the treatment time should be longer for electroporation of the cellular membranes. It has been found experimentally that time needed for electroporation of cellular membrane of the different biological tissues is inversely proportional to the electric field strength by factor dependency (Bouzzara and Vorobiev, 2003). In addition, Oliveira et al. (2010) investigated the effect of electric potential on production of lipase from yeast cells. In the literature, information about the most suitable range of electric field strength (E) is limited and data are mostly based on studies of secondary post-electric field effects, such as the inactivation rate of microorganisms, stress reaction on the biological tissue and diffusion of the material from the cells. These secondary effects also depend on other process parameters including pulse duration (PD), number of pulses (N) and pause between pulses (PBP).

It is noted that electric field induces a permeability variation of the initially electro-neutral cell membranes due to the pore formation. Based on this fact, the main hypothesis of the present work is that pulsed electric field applied on fresh tea leaves causes a secondary post treatment effect – extraction of polyphenols. Experimental results for the pulsed electric field treatment of fresh tea leaves are analysed and presented. By measuring the total polyphenols (PPs) content, which is a secondary post-electric field effect it was possible to monitor the process of pulse-induced membrane permeabilization. The effects of electric field strength and total treatment time during the PEF treatment on the extraction yield of PPs are studied and discussed in the Section 3.1. and 3.2.

2. Experimental section

2.1 Experimental method

Fresh tea leaves from Kenya (*Camellia Sinensis* variety) were used for experiments and stored at $+5$ °C until required. Before treatment, leaves were allowed to reach ambient temperature of approximately 20 °C. To determine the moisture content, fresh leaves were freeze dried and then subjected to a hot-air oven at 105 °C. Moisture content was within 75-80 %. Samples that were used for the moisture content were separated from the samples used for PEF treatment. For all experiments, thin slices of fresh tea leaves were used (approximately 1 cm width).

2.2 Pulsed electric field treatments

The discussed PEF treatment was performed using a Nutri-Pulse NP110-60 System (IXL Netherlands B.V.) which consists of PEF treatment chamber and generator of high voltage. Samples were placed in the treatment chamber between two stainless steel electrodes filled with an aqueous medium (Figure 1a). The aqueous medium was prepared with the same conductivity as the sample ($\sigma = 3.5$ mS/cm) using NaCl as a salt. The distance between electrodes was 4 cm. After cutting, leaves were subjected to different PEF treatments.

All experiments were carried out using an electric field strength E ranging from 0.1 to 1.1 kV/cm, a pulse duration PD from 0.0001 to 0.1 s, number of pulses N from 10 to 50 and pause between pulses from 0.5 to 5 s. The total treatment time was defined as the product of the number of pulses and the pulse width applied in the system (Figure 1b). Apart from the treatment time pause between pulses (PBP) is the interval between two pulses and represents the relaxation time. Based on the duration of a pause between pulses of 0.5 s, 3 s and 5 s experiments were divided into three groups EXA, EXB and EXC, respectively. The group of experiments where the PBP is 5 s (EXC) were not performed due to problems with the PEF unit (low conductivity was detected in the PEF chamber). This was unexpected, as Lebovka et al. (2001) showed that treatment with $\text{PBP} = 60$ s displayed accelerated kinetics of disintegration efficiency of the apple tissue in comparison with treatment with a PBP of 10^{-2} s for the fixed total treatment time. The

influence of the interval between pulses was reported on inactivation of *E. Coli* (Evrendilek and Zhang, 2005), the results were explained by accounting for the moisture transport processes inside the cell structure. However, the impact of the pause between pulses on PEF-induced effects is still doubtful. Further experiments are required to clarify the effect of PBP on the disintegration of cell membranes.

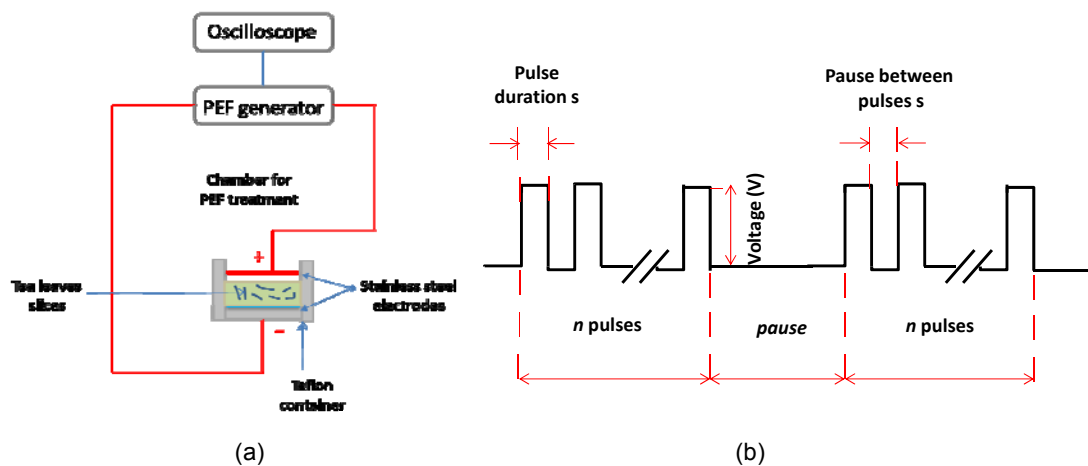


Figure 1: Experimental set up. Scheme of PEF treatment chamber (a) and PEF pulsing protocol (b).

The temperature was measured both at the inlet and outlet of the treatment chamber. In all experiments, the increment of the temperature due to the treatment never exceeded 5 °C. Experiments were duplicated.

2.3 Determination of extraction yield - measurement of total polyphenols content

The disintegration of cellular membrane was detected indirectly by a total PPs content measurement. Based on the fact that the cellular membrane is ruptured, diffusion of the cell materials together with polyphenols occurred and the extraction yield is defined as amount of extracted PPs. Total phenols were determined by direct reading of the absorbance at 725nm (SpectraMax 190 Absorbance Microplate Reader, USA) of diluted samples 1/10 (v/v). The total amount of PPs was expressed as gallic acid equivalents (GAE) by means of a corresponding calibration curve with standard gallic acid.

3. Results and discussion

3.1 Effect of electric field strength

Sale and Hamilton (1967) identified electric field strength E and the total treatment time (which considers the number of pulses and pulse width applied in the system) as the main variables determining the efficiency of the PEF damage of the plant tissue. Higher electric field strengths lead to a better damage efficiency (Canatella et al., 2001; Toepfl et al., 2007), but it was noticed that an optimal value of the electric field strength for many vegetables and fruit tissue is within 300 to 500 V/cm.

Figure 2 presents experimental results for the extraction yield of PPs from fresh tea leaves vs. electric field strengths for different pulse protocols: $N=30$, $PD=0.05$ s and $PBP(EXA)=0.5$ s (protocol I); $N=30$, $PD=0.05$ s and $PBP(EXB)=3$ s (protocol II). The only difference between protocol I and II is interval time between pulses.

The extraction yield (EY) values of the samples subjected to both protocols increase with increasing electric field strength. The PEF treatment accelerates kinetics of the extraction from fresh tea leaves. This is in agreement with the behaviour observed for different fruit and vegetable tissues (Vorobiev and Lebovka, 2008). Protocol I resulted in a maximum value for the extraction yield of 27% when the electric field strength is 0.9 kV/cm. However, when the interval between pulses is equal to 3 s an electric field of 1.1 kV/cm is needed to obtain the same extraction yield (protocol II).

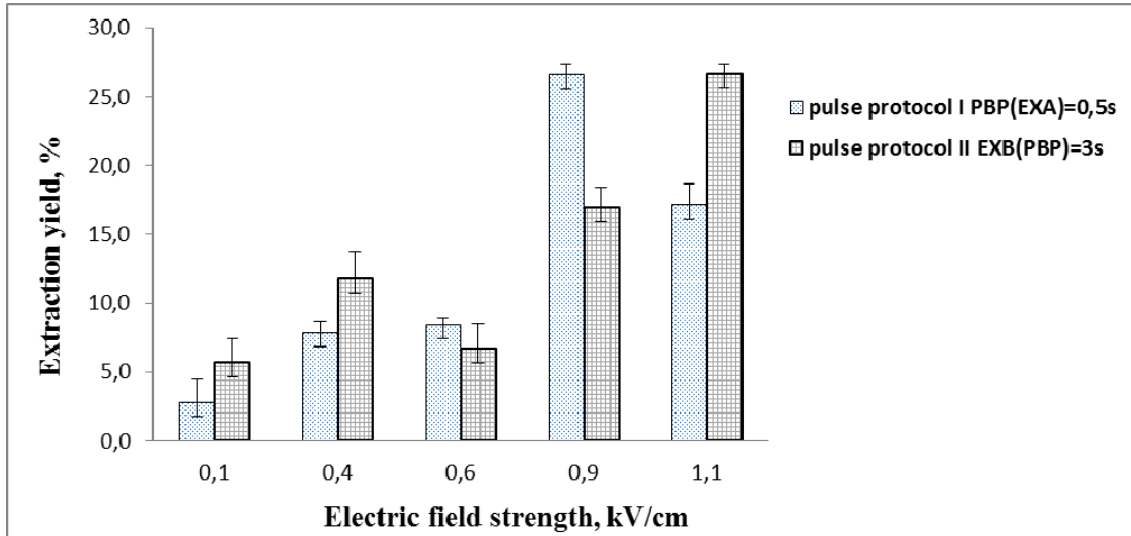


Figure 2. Experimental results for extraction yield of PPs from fresh tea leaves vs electric field strength at two pulse protocols: $N=30$, $PD=0.05$ s and $PBP=0.5$ s (protocol I) and $N=30$, $PD=0.05$ s and $PBP=3$ s (protocol II). Displayed error bars represent extraction yield values \pm standard experimental error. In all cases, a sample was taken 20 min after the end of the PEF treatment and stored at -30 °C until the extracted amount of total PPs was analysed. All experiments were duplicated.

For both protocols the same extraction yield was obtained, but at different electric field strengths. When the interval between pulses (relaxation time) is short (protocol I), a moderate electric field is enough to cause cell membrane rupture. However, protocol II resulted in a longer relaxation time and a higher electric field. Thus, it can be concluded that extraction kinetics strongly depends on the interval between pulses, which means that a longer interval between pulses requires a stronger electric field.

The variation in the electric field strength to obtain the same extraction yield between protocol I and II can also be related to the electrolysis between electrodes and tissue surface. Depending on the details of contact (geometry and size of the samples, orientation of the leaf slices, etc.) and composition of the samples (bud leaf, 1st, 2nd and 3rd open leaf), electrolysis may give rise to different amounts of stable ionic compounds which would result in an increase of conductivity and tissue disintegration.

3.2 Effect of total treatment time

The disintegration degree strongly depends on the treatment time and electric field strength (Vorobiev and Lebovka, 2008); at long times of PEF treatment a smaller electric field is required. Figure 3 presents experimental results for extraction yield at different treatment times for two electric field strengths 0.4 and 0.9 kV/cm, respectively.

Vorobiev and Lebovka (2008) studied the effect of pulse duration on the efficiency of PEF-treatment on sugar beet. Experiments showed that longer pulses were more effective and their effect was particularly pronounced at moderate electric field ($E=0.3$ kV/cm). This is partially in agreement with the observation because the highest extraction yield of 27 % is obtained for moderate electric field strength ($E=0.4$ kV/cm) and treatment time of 2.5 s (Figure 3). However, almost the same extraction yield ($EY=26.6$ %) is obtained for higher electric field ($E=0.9$ kV/cm) and shorter treatment time of 1.5 s. When the total treatment time was 5 s (for both electric field strengths) the experiment could not be performed due to problems with PEF unit itself (low conductivity was detected in the PEF chamber). Possible explanation lies in Ohm's law. Electrical resistance is the ratio of voltage over current by Ohm's law and conductivity is inversely proportional to resistance. Therefore, the resistance of the treatment chamber is an important parameter since the maximum allowed pulse current by the power switch is 600 A. This means that at 4kV the minimum resistance is 6 Ohm. If the resistance is lower than this the maximum pulse current of 600 A will be exceeded and if this situation continues for more than 5 pulses the system will automatically shut down to avoid damage to the high voltage switch. In this particular case, treatment time was 5 s $N=50$ pulses and $PD=0.1$ s. The electric field strength of 0.4 and 0.9 kV/cm, respectively, which exceed the critical situation mentioned above.

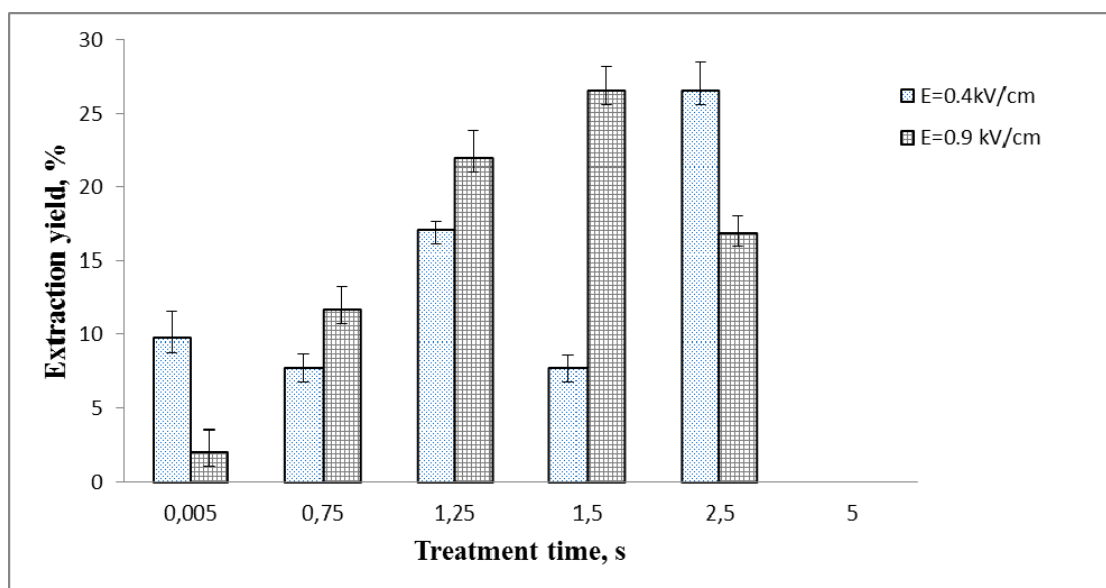


Figure 3. Experimental results for extraction yield of PPs from fresh tea leaves vs different treatment times at different values of electric field strength 0.4 and 0.9 kV/cm, respectively for the fixed value of PBP=0.5 s. Displayed error bars represent extraction yield values +/- standard experimental error. In all cases, sample was taken 20 min after the end of PEF treatment and stored at -30 °C until extracted amount of total PPs was analysed. All experiments were duplicated.

Existing works discuss mainly the effects of pulse duration in the PEF inactivation experiments with different microorganisms and some authors have demonstrated that inactivation was more efficient at higher pulse width (Belloso et al. 1997; Abram et al., 2003), but others observed little effect of the pulse width on inactivation (Raso et al. 2000; Manas et al. 2000; Sampedro et al. 2007). The effect of pulse width seems to vary depending on the electric field strength and a general relationship between PEF-treatment protocols, type and quality of soft tissue, contact parameters (geometry and size of the samples and orientation of the leaf slices), and the resulting degree of material disintegration requires more thorough study.

4. Conclusions

This work demonstrated the application of PEF that can exert several effects on the cell membrane. Efficiency of the PEF treatment was indirectly connected to secondary post electric field effect – extraction yield of polyphenols from fresh tea leaves. Different modes of PEF treatment (electric field strengths and total treatment times) were applied to investigate their effect on this complex phenomenon which comprises different levels of membranes, cells, tissue structure, and plant variety. Obtained results of extraction yield of polyphenols (Figures 2 and 3) could be explained by two key processes: resealing of cells; and diffusion of the cell material. The amount of extracted polyphenols from the leaves into the aqueous media strongly depends on the setting of PEF treatment.

Experimental results showed that extraction yield increases with increasing electric field strengths (two protocols (PBP (EXA)=0.5 s and PBP(EXB)=3 s) were studied). Protocol I (EXA) resulted in a maximum extraction yield of 27 % when the electric field strength is 0.9 kV/cm. However, when the interval between pulses is longer and equal to 3 s an electric field of 1.1 kV/cm is needed to obtain the same extraction yield (protocol II-EXB).

The total treatment time was presented as a product of the number of pulses and pulse width (duration). The effect of the total treatment time on the extraction yield was studied applying two different electric field strengths E=0.4 kV/cm and E=0.9 kV/cm. Experimental results showed that longer pulses which means longer treatment time=2.5 s were more effective for moderate electric field (E=0.4 kV/cm). Moreover, to achieve the same extraction yield of 27 % but with shorter total treatment time=1.5 s higher electric field (E=0.9 kV/cm) is required. When total treatment time was 5 s (for both electric field strengths) the experiment was not performed due to problems with PEF unit itself (low conductivity was detected in the PEF chamber).

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