

Disintegration Efficiency of Pulsed Electric Field Induced Effects on Onion (*Allium cepa* L.) Tissues as a Function of Pulse Protocol and Determination of Cell Integrity by $^1\text{H-NMR}$ Relaxometry

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Abstract: The influence of electrical pulse protocol parameters on cell rupture of onion tissues was investigated in order to improve fundamental understanding and to enhance the processing of plant tissues with pulsed electric fields (PEFs). The impact of PEF parameters on cell integrity of 20 mm dia, 4-mm thick disks of Don Victor onions (*Allium cepa* L.) was determined by ion leakage measurements. Electric field strength, pulse width, total pulse duration, and frequency effects were determined in relation to their effects on cell damage as a function of pulse protocol. Electric field strengths up to 500 V/cm increased the damage efficiency but there was no significant difference in efficiency beyond this field strength. Larger pulse widths increased the degree of tissue disintegration at a constant pulse number. Higher PEF efficiency was achieved with shorter pulse widths and a larger number of pulses at a constant total treatment time. Lower frequencies caused a greater degree of disintegration at constant number of pulses. $^1\text{H-NMR}$ experiments were performed to determine the proton relaxation components of the PEF-treated onion samples and to obtain cell damage information nondestructively. Paramagnetic ion uptake by the onion sample was used to identify different proton relaxation components. Five different proton relaxation components were observed and changes in the 2 components representing different proton environments showed high correlations with ion leakage results ($R^2 = 0.99$), indicating that T_2 distributions can be used to obtain information about cell membrane integrity in PEF-treated samples. $^1\text{H-NMR}$ proved to be an effective method for nondestructive quantification of cell membrane rupture in onions.

Keywords: cell disintegration, frequency, membrane integrity, nuclear magnetic resonance (NMR), onion tissue, pulsed electric fields, pulse width, T_2 distributions

Introduction

The irreversible permeabilization of cell membranes in plant tissues offers a wide range of process applications where cell membrane disruption is required, including drying, extraction or diffusion of plant metabolites, and permeabilization will also affect the mass and heat transfer of food products (Knorr and Angersbach 1998; Estiaghi and Knorr 1999; Bazhal and Vorobiev 2000; Bouzzara and Vorobiev 2000; Bazhal and others 2001; Eshtiaghi and Knorr 2002; Vorobiev and Lebovka 2006; Lebovka and others 2007). Electrical treatment of food materials, which may be referred to as electroporation (McLellan and others 1991) or electropermeabilization (Angersbach and others 2000), is an effective method of rupturing cellular membranes to facilitate subsequent extraction. The permeabilization of the plant cell membrane can be reversible (cell membrane reseals) or irreversible (cell ruptures) as a function of the electrical protocols used (Zimmermann and others 1974). The most important electric pulse

parameters are amplitude, duration, number, and repetition or frequency. To date, the basic mechanism for electroporation has been studied primarily at the single cell level, in cell suspensions, and most studies have been carried out with mammalian cells. These studies have focused on applications for electrochemotherapy and gene electrotransfer (Rols and Tessie 1990; Macek-Laber and Miklavcic 2001), and while other authors have studied pulsed electric field (PEF) effects on liquid foods (Zhang and others 1995; Barbosa-Cánovas and others 1998), there is limited information on electrical field applications to intact plant tissues in literature.

According to the aim of the application, the main challenge to utilization of electric field processing is the choice of an optimal PEF treatment mode, that is, electric field intensity (E), pulse shape, number of pulses (n), pulse duration (ti) and pulse repetition time (Δt). The goal of the process optimization procedure is in obtaining quantifiable correlations between the processing protocol and the resulting plasmolysis or degree of damage (Lebovka and others 2002). The secondary effects of electrical processing can also depend on other process parameters, such as pulse duration, energy input and the number of pulses utilized. Very little information is available regarding membrane permeabilization kinetics, or on the reversible-irreversible structural changes cells in real food systems during and after the application of high intensity electric field pulse. A fundamental understanding of these phenomena is essential for optimal process design and for the characterization

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of the critical process parameters of PEFs in the food industry (Angersbach and others 2000).

Estimation of the degree of damage can be defined as the ratio of the ruptured cells to the total number of cells. A conventional method of damage degree estimation is based on electrical conductivity measurements (Lebovka and others 2000; Angersbach and others 2002; Voroboev and Lebovka 2006). Another reasonable method for determination of the cell disintegration index of plant tissues, electrolyte leakage (ion leakage), has long been used as a measurement of the intactness and permeability of cell membranes (Salveit 1989; Vasquez-Tello and others 1990; Gonzalez 2009; Milczarek and others 2009; Ersus and Barrett 2010). Since the leakage of electrolytes is from high concentration inside the cell to a low concentration outside it, the efflux may be considered to be passive diffusion, but the influx must be due to active transport. Increased injury, as indicated by the net leakage, may result from either an increased efflux due to damage to the permeability of the plasmalemma, which surrounds the entire cell, or a decreased influx due to damage to the active transport system (Palta and others 1977). When the tonoplast membrane that surrounds the plant cell vacuole is ruptured, the ions located in the vacuole diffuse through the extracellular liquid and by measuring the change in conductivity it is possible to estimate the degree of cell rupture. Cell rupture can also be estimated by using nuclear magnetic resonance (NMR) relaxometry. ^1H NMR relaxation time measurements are being used extensively to investigate the water compartmentalization in plant tissues. Each compartment can be characterized by an intrinsic relaxation rate as long as there is no exchange of magnetization between the compartments (that is, immiscible phases such as water and oil) or the diffusive exchange between the compartments is extremely slow on the observational time scale of NMR experiments (Hills 1998). Within plant cells, several proton compartments have been identified previously by researchers (Hills and Duce 1990; Hills and others 1991; Snaar and Van As 1992; Raffo and others 2005). Between these compartments, water molecules or protons are in exchange, resulting in averaging of the intrinsic relaxation times, preventing the assignment of relaxation times to a particular compartment. In order to make the assignment of compartments, the change in relaxation times in different proton compartments after drying and freezing were investigated (Hills and Remigereau 1997). The effect of high pressure applications on strawberries (Marigheto and others 2004), starch and potato (Hills and others 2005), and onion (Gonzalez 2009) were studied by NMR relaxometry to investigate the intactness of cells. NMR relaxometry was also used to track cell permeability changes in plants during osmotic stress (Van Der Weerd and others 2001). Paramagnetic ions that enhance the relaxation rate were used for the assignments of proton compartments to cellular organelles based on the diffusion rate of the ion. (Snaar and Van As 1992; Marigheto and others 2004).

The objective of this work is to investigate the effect of PEF parameters, for example, external electric field strength, pulse width-pulse duration, and frequency, on the degree of cell rupture in intact onion tissues and to monitor the changes in membrane integrity of PEF-treated onion tissues using nondestructive NMR relaxation measurements.

Materials and Methods

Raw material

Spanish yellow onions cv. Don Victor (*Allium cepa* L.) were provided by Gills Onions (Oxnard, Calif., U.S.A.) and used for

PEF treatments. Onions were shipped to the UC Davis Food Science and Technology Dept. and stored at 4 °C until processed. Large yellow Spanish bulb cultivars that mature in late May were used. The bulb shape was globe to deep grano with the excellent firmness.

Sample preparation

The outer papery scales and the 1st and 2nd fleshy scale (layer) of the onions were removed. Starting from the 3rd scale, which is undamaged by the mechanical harvesting and postharvest conditions, samples were prepared by cutting tissue with a cork borer into disks that were 20 mm in dia and 4-mm thick. The lower epidermis of the onion tissues was separated manually. Each single disk was processed in the PEF system and was considered as one replicate sample. Eight replicate disks were processed for all experiments. Samples were frozen (−18 °C) and thawed twice, resulting in 100% ruptured cells for NMR and total conductivity measurements. Control samples were prepared by placing disks into the PEF sample chamber for the same time as those processed, but not applying the electric field treatment.

PEF treatment

PEF treatments were carried out using a system developed at the Univ. of California, Davis (Asavasanti and others 2010). The plexiglass cylindrical sample holder consists of a top and bottom chamber, and the bottom chamber has a well (gap) of a specific depth. The top chamber is assembled with a 2 cm dia flat stainless steel electrode. The well of the bottom chamber, used in these studies is 0.3 cm deep and 2 cm in dia and has a flat stainless steel electrode fixed inside the bottom. An onion sample of the same thickness is placed between the 2 electrodes of the sample chambers with the convex plane facing down. To ensure an airtight condition, o-ring gaskets are present between the top and the bottom electrode assemblies and a constant clamping force is applied to the sample holder using an Arbor press with a fixed deadweight. The PEF system consisted of a high voltage power supply (PowerPAC HV, Bio-Rad, Hercules, Calif., U.S.A.), a function generator (model 33220A, Agilent, Santa Clara, Calif., U.S.A.), a PEF generator, sample holder, and an oscilloscope (model TDS1012B, Tektronix, Beaverton, Oreg., U.S.A.) for signal monitoring. Experiments were carried out with monopolar positive pulses (for example, the current from one of the electrodes toward the grounding electrode) of rectangular shape.

Experimental design of PEF applications

The experimental design parameters are summarized in Table 1.

Influence of electric field strength, E (V/cm). To investigate the effect of electric field strength (E) on the cell disintegration efficiency, experiments were carried out using an E up to 1500 V/cm (125, 250, 500, 750, 1000, 1250, and 1500 V/cm) at one constant frequency (1 Hz), pulse number (10) and pulse width (100 μs).

Influence of pulse width (ti) alone and in combination with different pulse numbers (n) to result in constant pulse duration time. Experiments were performed using 5 different pulse widths (20, 40, 60, 80, and 100 μs) applied at constant electric field strength of 500 V/cm and frequency of 1 Hz for 10 pulses. In these trials, total pulse duration (pulse width \times 10 pulses) varied from 200, 400, 600, and 800 μs to 1000 μs for each trial, respectively.

In order to better understand the pulse width effect, another experiment was conducted. To separate the pulse width effect from the effect of the total pulse duration, the total pulse duration was

kept constant at 1000 μs by changing the pulse numbers for each pulse width. Protocols were designed such that the pulse width (μs) to pulse number combinations were 20/50, 50/20, 100/10, 500/2, and 1000/1.

Influence of frequency. To determine the frequency effect on disintegration of cells, the experiments were conducted in such a way that pulse width of 50 μs and 100 μs were chosen. Frequency trials were carried out at 0.5, 1, 2, 4, and 8 Hz and constant electric field strength of 500 V/cm. The pulse number was kept constant at 8 pulses.

Ion leakage (%) determination

One of each control and PEF-treated onion disks were placed directly into a 50 mL centrifuge tube containing 10 mL of an isotonic solution (0.2 M mannitol) following treatment. Ion leakage was measured as electrical conductivity (σ , mS/cm) changes over a 4 h time period at 25 °C (Salveit 1989) in a 0.2 M mannitol solution, which was found to be isotonic for onion tissues, using a conductivity meter (Accument portable AP65 Fisher Scientific). For all treatments (data not shown), the change in percent conductivity reaches a plateau after 240 min of ion leakage measurements. Ion leakage was calculated as a percentage of electrical conductivity of the sample. Total conductivity was measured from samples that were frozen (−18 °C) and thawed twice, resulting in 100% ruptured cells. Four replicates were used for the ion leakage analyses.

$$\text{Ion leakage}(\%) = \frac{\text{Conductivity}(t_i)}{\text{Total Conductivity}} \times 100 \quad (1)$$

NMR measurements

Relaxation measurements. The NMR relaxation measurements were carried out by using a 1.03T (43.8 MHz) NMR spectrometer (Aspect-AI, Netanya, Israel). Spin-spin relaxation times (T_2) were measured using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (Echo time: 0.5 ms; number of acquisition points: 16; number of echoes: 16383; number of averages: 16). Two onion disks, with one on top of the other, were utilized in order to increase the signal to noise ratio. The disks were placed in the same orientation for each measurement and experiments were repeated 4 times. The samples were located in the center of the coil. Multiexponential decay analysis of the CPMG curves was done by using the Nonnegative-Least-Square (NNLS) macro of the software PROSPA (Magritek, New Zealand). The macro is based on Lawson and Hanson algorithm that uses a regularization function that seeks to find a smooth spectrum of exponentials that satisfies the data in a chi-squared sense. It minimizes the function,

$$\min \left\{ \chi^2 = \sum_{j=1}^n \left(S_j - \sum_{i=1}^m A(T_{2i}) \exp(-t_j/T_{2i}) \right)^2 + C \sum_{i=1}^m (2A_i - A_{i-1} + A_{i+1})^2 \right\}$$

where S is the measured data, T_{2i} is the possible relaxation times, and A is the relaxation time distribution or the spectrum. The 2nd term in the expression corresponds to the regularization function and is an expression of the sharpness of the spectral features. The parameter C can be used to control the amount of smoothing applied to the spectrum. C is expressed in terms of $1/(\alpha)$, which is referred as the smoothing parameter. In order to prevent spurious peaks, α should be chosen such that the chi-squared value (χ^2) is approximately equal to the sum of the variances in the original dataset. In this study, another macro available in the software PROSPA was used to determine the value of α . More information about the macro can be found in PROSPA user manual (Magritek, New Zealand).

Paramagnetic ion study. In order to identify the proton relaxation components, the onion disks were soaked in 50 mM MnCl_2 to 200 mM mannitol solution. Thirty onion disks were placed into a constantly stirred mannitol solution of 750 mL. At each time, 2 onion disks were removed, blotted with tissue paper, placed into the magnet, and the relaxation times were measured. The experiment continued for 7 h.

Statistical analysis

Data were analyzed by using SPSS 11.5 package program (SPSS Inc., Chicago, Ill., U.S.A.). One-way ANOVA and Duncan's multiple range tests were used for determination of the differences between the PEF conditions ($P < 0.05$).

Results and Discussion

Effect of electric field strength on onion cell rupture

Electrical field strength is an important parameter that may be utilized to control the efficiency of plant and animal cell rupture. The influence of electric field strength, E (V/cm) on percent ion leakage is shown in Figure 1. Ion leakage of untreated (control) samples in an isotonic solution of 0.2 M mannitol is $7.8 \pm 1.7\%$. It is thought that the increase in electrical conductivity of control samples was due to leakage of electrolytes from cells cut during sampling and/or from the apoplastic region between the cells of the tissue. At a constant number of pulses (10) and frequency (1 Hz), application of electric field strengths of 250 and 500 V/cm both resulted in significant ($P < 0.05$) increases in ion leakage as compared to the control. Additional increases in field strength above 500 V/cm did not result in any increase in ion leakage, indicating that cells were ruptured already at 500 V/cm. Approximately 12% and 30% cell rupture were obtained with 125 and 250 V/cm PEF applications. Figure 1 indicates that PEF treatments between 500 and 1500 V/cm showed no significant difference in their effect on ion leakage ($P < 0.05$). In a separate report (Ersus and Barrett 2010), we evaluated the effect of PEF treatments with different electric field strengths and pulse numbers of various cell type that were distributed in a heterogeneous manner within plant tissues.

For different plant tissues, the critical level of electric field strength required for cell rupture is reported to be in the range of 200 to 500 V/cm, but this depends on the type of tissue (Bazhal

Table 1—Process conditions studied for PEF treatment of onion tissues.

PEF parameter studied	Units	Experimental variables	Constant parameters		
Electric field strength	E (V/cm)	125, 250, 500, 750, 1000, 1250, 1500	Pulse number = 10	$f = 1$ Hz	Pulse width = 100 μs
Pulse width	t_i (μs)	20, 40, 60, 80, 100	Pulse number = 10	$f = 1$ Hz	$E = 500$ V/cm
Pulse width/pulse number	t_i (μs): n	20/50, 50/20, 100/10, 500/2, 1000/1	Total pulse duration = 1000 μs	$f = 1$ Hz	$E = 500$ V/cm
Frequency	f (Hz)	0.5, 1.2, 4.8	Pulse number = 8	$f = 1$ Hz	$E = 500$ V/cm
	t_i (μs)	50 or 100			

and others 2003). It was also reported previously by these authors that the additional increase of E beyond that required for plant cell rupture results in a progressive increase in energy consumption but gives no additional increase in the disintegration index. However, in our experiments because all of the cells are already ruptured at 500 V/cm, it was impossible to achieve higher conductivity rates at higher electric field strengths.

It is known that when using moderate PEF treatments (for example, 0.5 to 2 kV/cm, $t_i = 10^{-4}$ to 10^{-5} s), the integrity of cells rapidly decreases; but due to a resealing or recovering process, some of the cell membranes lose their permeability. This resealing phenomenon has been reported to take place anywhere from 1 s to 10^2 sat 25 °C (Chang and Reese 1990). Resealing time for vegetable cells has been reported as 1 s (Knorr and others 2000). Living plant cells have a natural potential difference of approximately -150 mV (Coster 1965). Rupture occurs when the transmembrane potential of plant cell membranes reaches between 0.7 and 2.2 V under various field strength intensities (Angersbach and others 2000). Application of field strengths higher than the critical electric field strength results in irreversible rupture to vary-

ing degrees, according to magnitude of E and the pulse duration that is directly related to the energy density (Q) given to the sample. With low levels of E, a relatively long total electric current application time is required for electroporation of cellular tissue (Bazhal and others 2003). Ranchero and Sabroso onions treated at 333 V/cm showed total cell rupture at 333 V/cm with a 100 pulse application (Asavasanti and others 2010). When the electric field strength was increased up to 500 V/cm, however, the number of pulses required to achieve membrane permeabilization decreased 10 times, for example, to 10 pulses.

Previous studies have also found that plant tissue disruption under PEF treatments can be achieved within 10^{-4} to 10^{-2} s at moderate electric field strengths between 0.5 and 5.0 kV/cm (Dunn 2001; Lebovka and others 2001, 2002). Other authors have reported slight membrane breakdown of potato, apple, and fish tissues occurred at 15 to 200 V/cm, whereas significant membrane breakdown was observed when the field strength was increased to 400 to 800 V/cm (Angersbach and others 2000). Bazhal and others (2003) stated that the optimal value for the field strength E_{opt} corresponds to the minimum amount of total energy

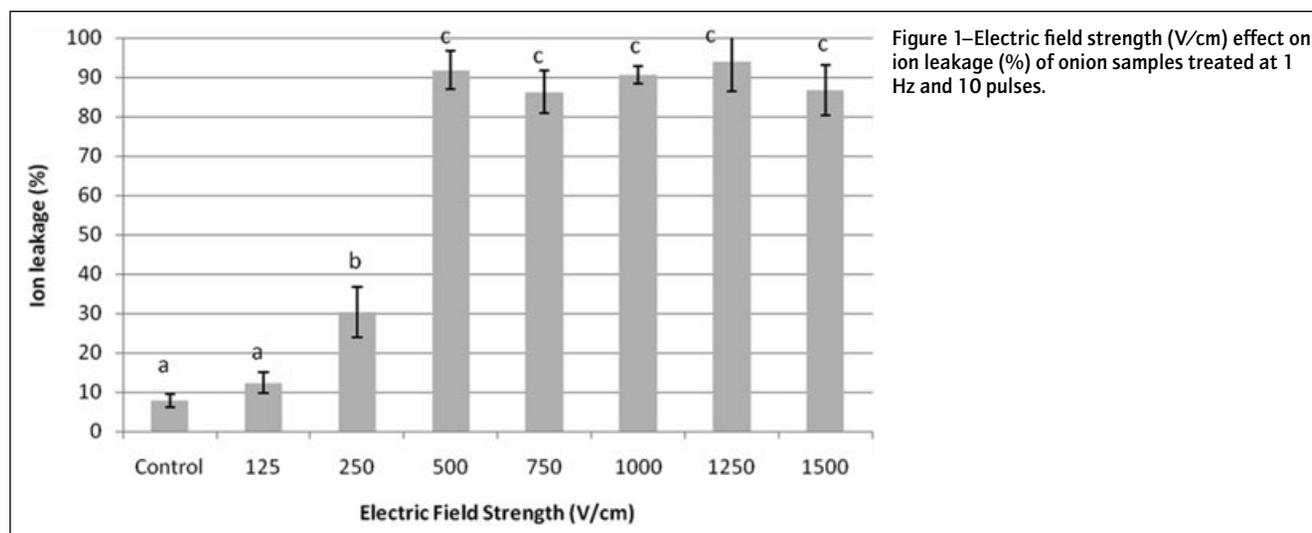


Figure 1—Electric field strength (V/cm) effect on ion leakage (%) of onion samples treated at 1 Hz and 10 pulses.

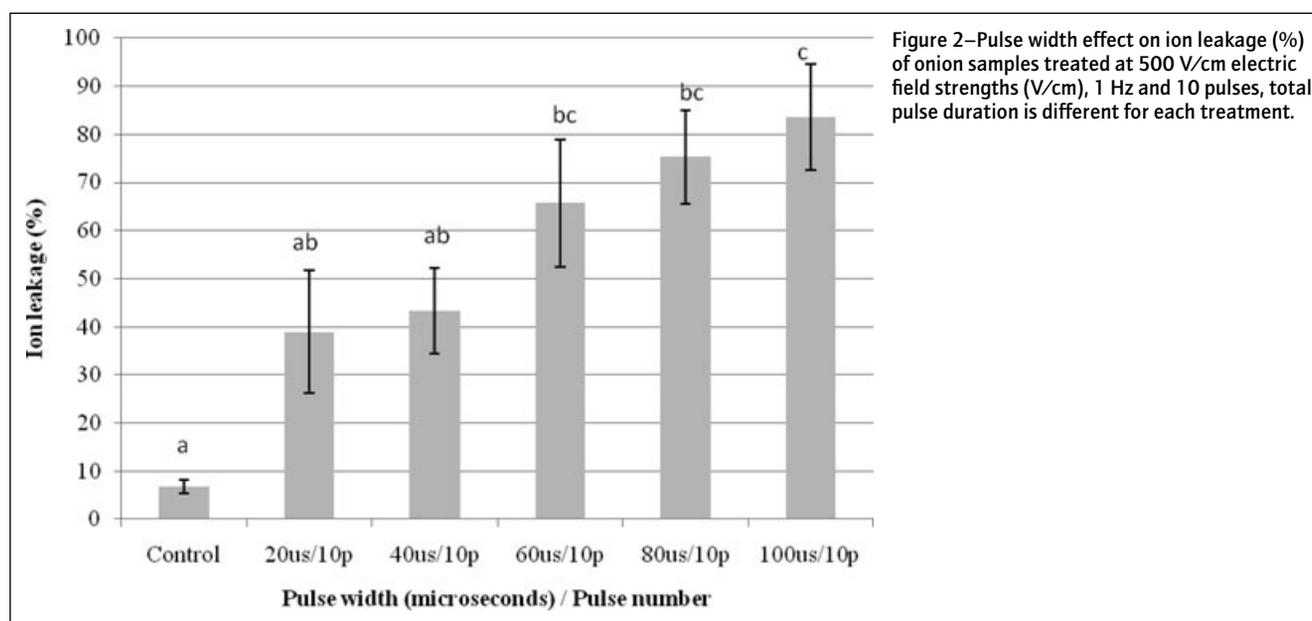


Figure 2—Pulse width effect on ion leakage (%) of onion samples treated at 500 V/cm electric field strengths (V/cm), 1 Hz and 10 pulses, total pulse duration is different for each treatment.

consumption. Our results showed that the optimal value of applied PEF strength for rupture of onion tissue is treatment at 500 V/cm for 10 pulses, with 100 μ s pulse width monopolar rectangular pulses applied at 1 Hz.

Effect of pulse width (ti) and pulse number (n) applied at a constant pulse duration time on onion cell rupture

The pulse width effect on cell rupture of onion tissue was investigated and results were shown in Figure 2. The pulse width was increased from 20 to 100 μ s, while pulse number (10), E (500 V/cm), and frequency (1 Hz) were kept constant. The total pulse durations for each treatment were different as 200, 400, 600, 800, and 1000 μ s, which indicates that the total electrical energy applied to the system increased for each increasing pulse width experiment. In this study, at a constant pulse number with varying pulse duration and increasing pulse width, the degree of cell rupture as measured by ion leakage, increased significantly with pulse width. Percent ion leakage was 39.0 ± 12.7 , 65.7 ± 13.2 , and $83.6 \pm 10.9\%$ for the 20, 60, and 100 μ s pulse width treatments, respectively. Ion leakage for the control was found to be $6.8 \pm 1.5\%$, which is likely due to electrolytes leaking from

cell surfaces cut during sampling. Increases in pulse width from 20 to 100 μ s and resultant increases in total pulse duration caused a significant ($P < 0.05$) approximately 114% increase in cell rupture of onion tissue.

De Vito and others (2008) showed the effect of different pulse durations (10, 100, and 1000 μ s), with interpulse durations Δt of 100 μ s and different numbers of pulses applied on the efficiency of the PEF treatment of sugar beet and apple tissues at electric field strengths ranging from 100 to 400 V/cm. Both the degree of and the time evolution for tissue damage were quantified by the electrical conductivity disintegration index (Z) and the characteristic damage time (τ), respectively. Samples exposed to the same PEF treatment time ($n \times t_i$) showed noticeably higher disintegration efficiency for the greater pulse duration. These authors also reported that the general relationships between the PEF treatment protocols, type and quality of soft plant tissues, process parameters (temperature, geometry, and size of samples, and so on) and the resulting degree of material disintegration are not completely clear and require more thorough study in the future (De Vito and others 2008). It has been reported that the pulse amplitude results in a larger area of membrane electroporation with a smaller extent

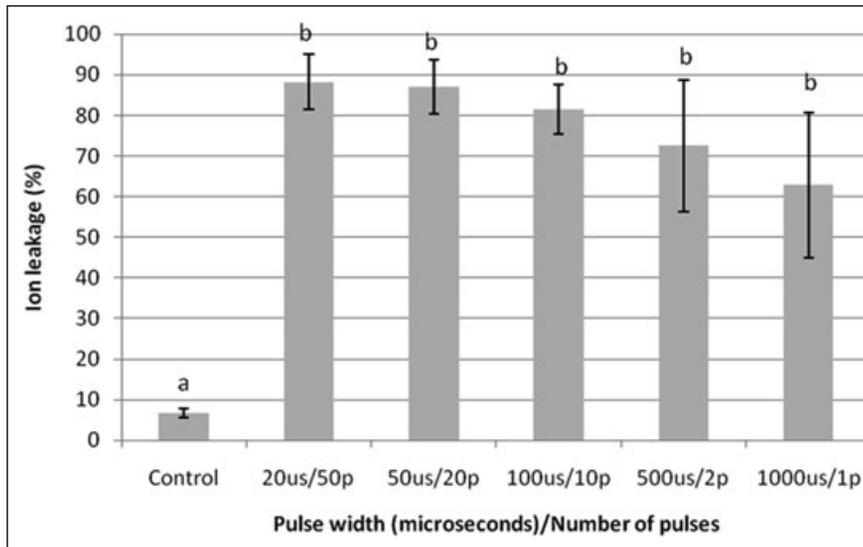


Figure 3—Pulse width and pulse number effect on ion leakage (%) of onion samples treated at 500 V/cm electric field strengths (V/cm), 1 Hz and 10 pulses for 1000 μ s total pulse duration.

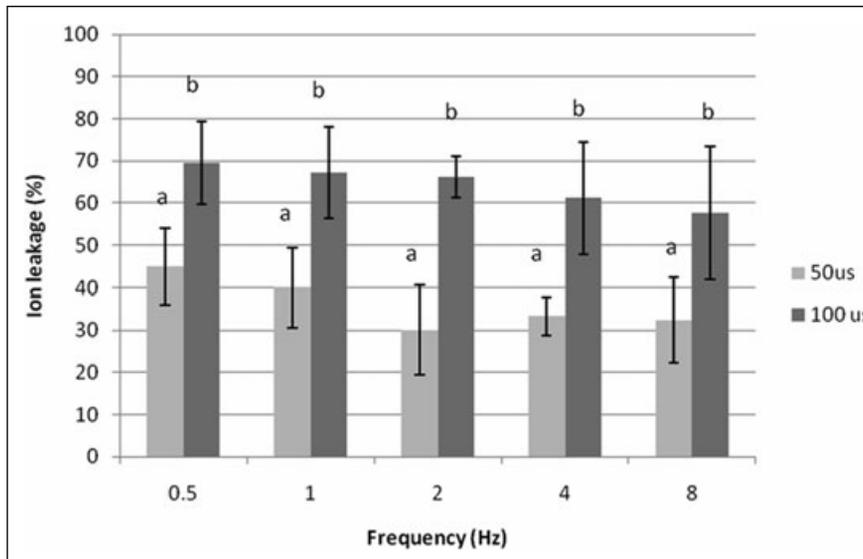


Figure 4—Hertz effect on ion leakage (%) of onion samples treated at 500 V/cm electric field strengths (V/cm) at a constant pulse number ($n = 8$) for 50 and 100 μ s pulse widths.

of electroporation, while increases in pulse number and duration do not affect the electroporated membrane area but increased the extent of electroporation (Rols 2006).

There is insufficient literature available regarding the effect of pulse duration on PEF-induced disintegration of plant tissues at a fixed total treatment time (Vorobiev and Lebovka 2008). Some authors have demonstrated that microbial inactivation was more efficient at higher pulse width and a constant quantity of applied energy (Martin-Belloso and others 1997; Abram and others 2003) but others observed little effect of pulse width (Raso and others 2000; Sampedro and others 2007; Bazhal and others 2003). In or-

der to better understand the pulse width effect and separate it from total pulse duration, a different experiment was conducted where total pulse duration was kept constant at 1000 μ s by changing the number of pulses for each pulse width experiment (Figure 3). Protocols were designed such that pulse width (μ s)/pulse number were 20/50, 50/20, 100/10, 500/2, and 1000/1. With the lowest pulse width (20 μ s) and highest pulse number of pulse (50) combination, ion leakage was found to be $88.2 \pm 6.9\%$. When the pulse width increased to 100 μ s and 10 pulses were applied to the tissue, ion leakage was $81.5 \pm 6.0\%$. Application of pulse width of 500 μ s for only 2 pulses caused $72.5 \pm 16.2\%$ ion leakage, whereas

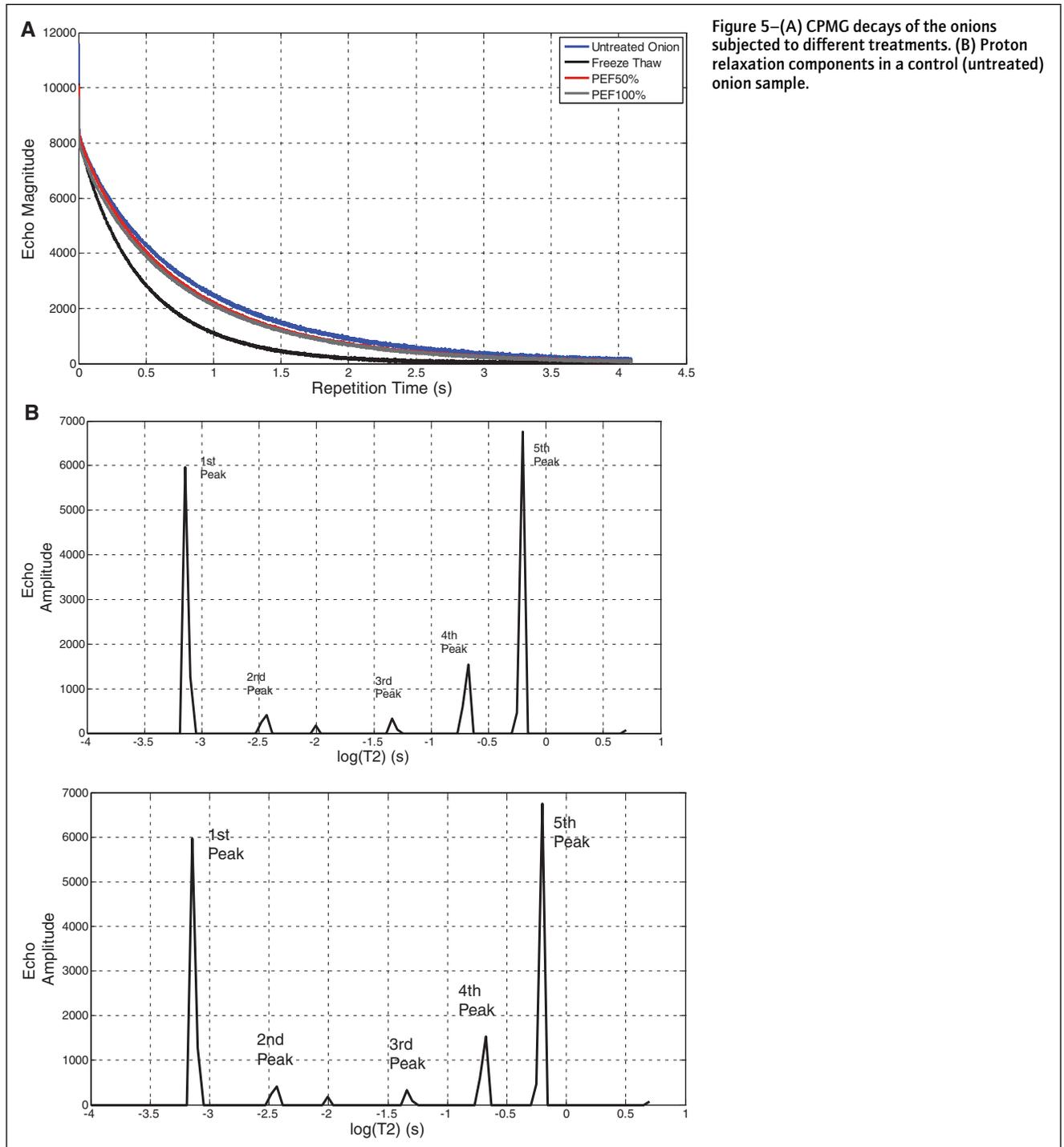


Figure 5–(A) CPMG decays of the onions subjected to different treatments. (B) Proton relaxation components in a control (untreated) onion sample.

the 1000 μs treatment for 1 pulse resulted in a lower ion leakage of $62.9 \pm 17.9\%$. Even though the ion leakage values decreased with decreasing pulse numbers and increasing pulse widths, no significant change was observed between each treatment ($P < 0.05$). This may be due to the relatively large standard deviations observed, particularly when few pulses were applied. The standard deviation of single-pulse treatment was also found to be the largest. Biological variability of the raw material may explain these large standard deviations, even though 4 replicate disks were treated for each process combination.

It is known that the insulating properties of the cell membrane can be completely recovered within several seconds after the termination of an electrical pulse (Angersbach and others 2000). The membrane charging time may be rather large, for example, 10^{-5} to 10^{-4} s, for cellular tissues with large cells. An efficient cell rupture requires pulses of a longer duration, as compared to the membrane charging time, in order to reach the maximum transmembrane voltage (Bazhal and others 2003). Also, large cells and an extracellular medium with a relatively low electrical conductivity results in deceleration of the membrane charging processes (Kotnik and others 1998). Hence, with the highest pulse width application for a single-pulse treatment, the lowest level of cell rupture in onion tissues was obtained, while the highest cell rupture was accomplished using the smallest pulse width for the greatest number of pulses. In onion tissues, the cell types and distribution can also have an effect on the response of different cells to rupture (Ersus and Barrett 2010).

Effect of frequency on cell rupture

The effect of frequency on the percentage of onion cells ruptured (Figure 4) was determined at 500 V/cm with an 8 pulse application and 2 different pulse widths (50 to 100 μs). Although not statistically significant, decreases in ion leakage were observed with increasing frequency (0.5 to 8 Hz) for both pulse widths applied at constant field strength and pulse number. The effect of frequency using 50 μs pulse width treatments appeared to be more effective than the 100 μs pulse width treatment. The effect of pulse width (50 to 100 μs) on ion leakage values was significant ($P < 0.05$). Ion leakage results following 100 μs treatments for 8 pulses at 500 V/cm were found to be 69.5 ± 9.8 and $57.6 \pm 15.7\%$ for frequencies of 0.5 and 8 Hz, respectively. Following the 50 μs treatment, however, under similar conditions the ion leakage results were 45.1 ± 9.1 and $32.3 \pm 10\%$. The decrease in ion leakage ratio was determined as 17.1% for 100 μs and

28.4% for 50 μs treatments, which is calculated from 0.5 to 8 Hz applications.

The main difference between the 0.5 to 8 Hz applications, was that when the same amount of pulses were given to the system, different treatment times result, that is 16, 8, 4, 2, and 1 s for 0.5, 1, 2, 4, and 8 Hz, respectively. It is expected that sufficient efficiency will be accomplished by the PEF protocol for long pulse duration (t_i) long as compared to the membrane charging time (De Vito and others 2008). For shorter treatment times, the membrane charging may not be completed. When pulses are applied with high repetition frequency, the pause between 2 consecutive pulses is too short for membrane charging and therefore this prevents cell membrane rupture. Similar findings were reported for shorter duration ($t_i = 10 \mu\text{s}$) PEF applications, where the membrane charging may be unfinished and PEF damage effects can be suppressed (De Vito and others 2008). The effect of different frequency applications, between 0.5 and 8 Hz, on ion leakage rates following 50 or 100 μs pulse width treatments was not significantly significant ($P < 0.05$).

Relaxation time measurements

Paramagnetic ion study. CPMG decay plots for the control (untreated) and treated samples of onion tissue are shown in Figure 5A along with the components calculated from a multiexponential decay analysis for the untreated tissue in Figure 5B, the relaxation

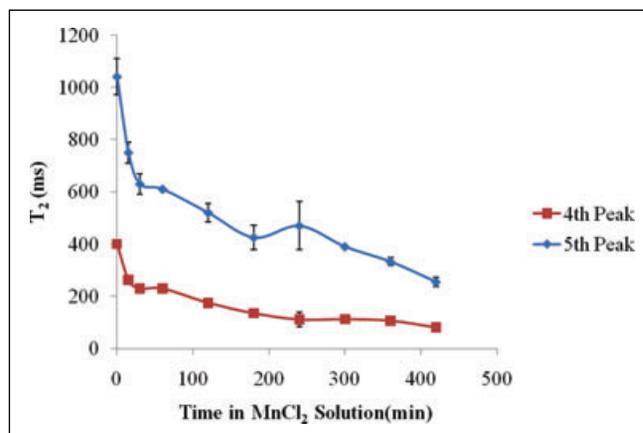


Figure 7—Effect of the paramagnetic ion on the 4th and 5th proton relaxation components.

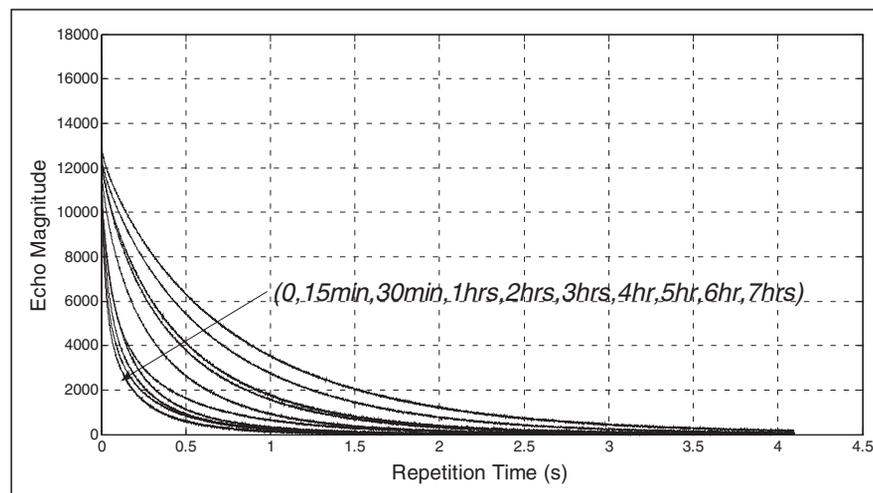


Figure 6—CPMG curves obtained at different times of paramagnetic ion soaking.

spectrum. The CPMG curves obtained during paramagnetic ion uptake are also shown in Figure 6. The decay of the signal is very rapid initially and then slows. The complete decay is well described by 5 components in Figure 5B (the small peak in the middle was thought to be spurious since the contribution was so small and not present in all experiments).

The paramagnetic ions were used to decrease proton relaxation times enabling peaks in the relaxation spectrum to be assigned to specific cell compartments (Snaar and Van As 1992). Shown in Figure 7 are the changes observed in the relaxation spectra for peaks 4 and 5 (Figure 5B) as a function of soaking time in MnCl₂-mannitol solution. The percentage change of peaks 4 and 5 are shown in Figure 8. It is found that the change in the peaks are significantly different from each other and with respect to soaking times ($P < 0.05$).

The largest compartments in an onion tissue are the cytoplasm and the vacuole. The cytoplasm compartment should be impacted first by paramagnetic ion diffusion and the vacuole second.

The vacuole will contain the largest number of protons and will have greater amplitude in the relaxation spectrum. Peak 4 is assigned to cytoplasm since the changes in T_2 values are faster and the amplitude lower than peak 5, which is assigned to vacuole. However, it is important to note that a change in the relaxation time of vacuole was observed at the end of 15 min in contrast to findings of Snaar and Van As (1992). They found out that in apple parenchyma tissue it took almost 16 h to see the change in the relaxation time of the vacuole in the presence of paramagnetic ions. On the other hand, Hills and Duce (1990) found out that in an onion tissue, in contrast to an apple tissue, relaxation is dominated by the various combinations of fast proton exchange between water and biopolymers. These findings are consistent with the behavior observed for peak 4 and peak 5.

Peaks 1, 2, and 3 are virtually unchanged by paramagnetic ion diffusion and hence associated with the protons in solid-like compartment in the cell (for example, macromolecules and membranes).

Relaxation time measurements on PEF-treated and freeze-thawed samples. The T_2 values of the 4th and 5th peaks for different processing conditions together with the ion leakage

results are shown in Figure 9A and B. As can be seen from the figure, the ion leakage results correlate well ($R^2 > 0.85$) with the relaxation time results. The T_2 values for the freeze-thawed onions are approximately 180 ms and approximately 600 ms for the 4th and 5th peaks, respectively. The relaxation times decreased 44% and 38% for the 4th and 5th peaks, respectively. It is known that (Salveit 1989) in freeze-thawed onions, cells are completely ruptured and the ion leakage for a freeze-thawed onion sample is considered to be 100%. So as long as the moisture loss in freeze-thawed sample is insignificant, in terms of relaxation times, the T_2 values of the freeze-thawed samples can be considered as the

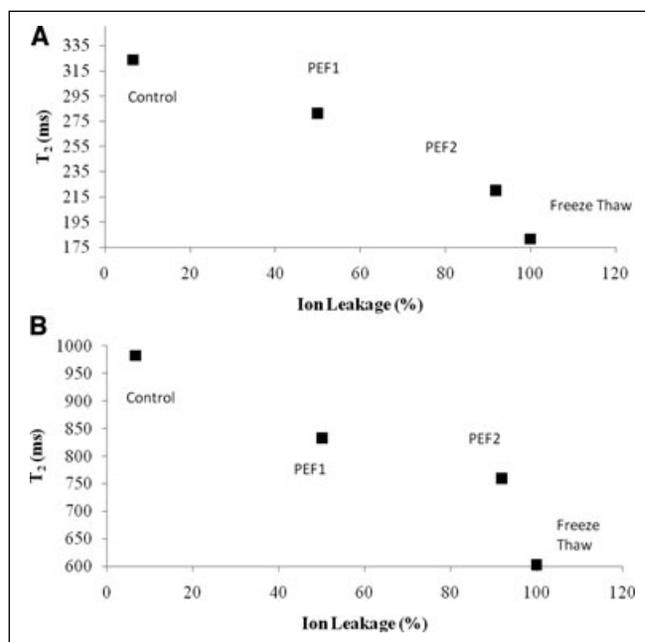


Figure 9—(A) Correlation between T_2 of 4th peak and ion leakage. (PEF1: 500 V/cm, 50 μ s pulse width, 1 Hz, 10 pulse, PEF2: 500 V/cm, 100 μ s pulse width, 1 Hz, 10 pulse). (B) Correlation between T_2 of 5th peak and ion leakage (PEF1: 500 V/cm, 50 μ s pulse width, 1 Hz, 10 pulse, PEF2: 500 V/cm, 100 μ s pulse width, 1 Hz, 10 pulse).

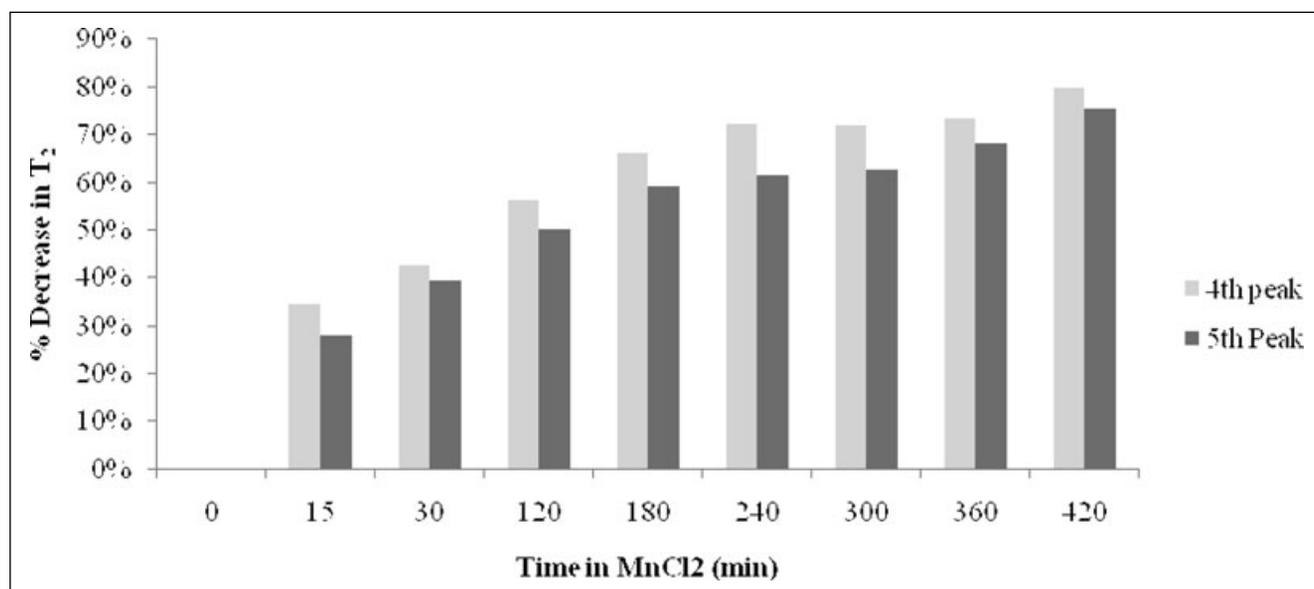


Figure 8—Percentage change in T_2 of the 4th and 5th peaks in MnCl₂-mannitol solutions.

limit for a ruptured cell and can be used as an intactness measure for the cells. On the other hand, it is interesting to note that the relaxation time measurements for the freeze-thawed samples give still the same number of peaks as the untreated onions but with shorter relaxation times. The presence of the same peaks shows that the exchange of water by molecular diffusion between the different relaxation components is slow on the NMR relaxation time scale and implies that there are still significant permeability barriers to water exchange (Hills and Remigereau 1997) although the cell is significantly damaged.

Relaxation time measurements were done for 2 different PEF conditions (PEF1: 500 V/cm, 50 μ s pulse width, 1 Hz, 10 pulse, PEF2: 500 V/cm, 100 μ s pulse width, 1 Hz, 10 pulse). According to ion leakage results, PEF2 conditions resulted in $91.83 \pm 4.86\%$ rupture that is consistent with higher T_2 values compared to freeze-thawed samples. PEF1 treatment that resulted in 50% rupture had higher T_2 values for both peaks compared to PEF1 and freeze-thawed samples. The high correlation between ion leakage and the T_2 values indicates that relaxation time measurements can be used for characterizing cell intactness.

Conclusion

PEF processing can be optimized to achieve varying levels of disruption to cellular tissues. PEF systems designed to achieve high levels of cellular damage should employ high voltage applications for short times with high repetition rates or frequencies. Validation of PEF efficiency can be achieved using either ion leakage measurements or nondestructive NMR relaxation spectrum analysis. In-line control of PEF processing may be achieved by employing NMR relaxation measurements to optimize PEF processing parameters.

Nomenclature

E	electric field strength (V/cm)
n	number of pulse
f	frequency (Hz)
t_i	pulse width (μ s)
n_{ti}	total pulse duration (μ s)

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