Pulsed Electric Field Treatment of Plant Tissue

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Doctoral dissertation

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Front cover: Permeabilization of onion epidermal cells by pulsed electric field

Back cover: Diffusion of vacuolar content into cell wall following permeabilization

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To the memories of

Ömer Osmanlı *...for his honesty in life*
&
Adeviye *and* ***Fatma Fincan****...for their love and sacrifice*

Pulsed Electric Field Treatment of Plant Tissue

Fincan, M. 2003, Doctoral dissertation, Lund University

Abstract

Plant cells like other cells contain water and various cell components surrounded by membranes. Cell membranes constitute a barrier in removing the intracellular substances from the plant tissue. The transport of such substances out of the tissue is highly influenced by how intact the cell membrane is. Methods for disintegration of cell membranes should be effective, energy saving and minimize the product deterioration.

A pulsed electric field (PEF) is a newly emerged non-thermal food processing technology which has characteristic effects on the cell membrane. PEF processing involves subjecting food to short, repeated pulses of a high electric field between an anode and a cathode. The main result of this process is the disruption of the cell membrane leading to the formation of either temporary or permanent pores. The area of interest in the treatment of plant tissue with PEF is facilitation of certain processes such as extraction, drying and osmotic dehydration, non-thermally and economically. For PEF to be employed in commercial plant tissue processing, a better understanding of the changes brought about by PEF in the structure of plant tissue at the cellar level is required. After the formation of pores in the cell membranes, a number of changes are initiated inside the tissue. Most important of these is the loss of turgor, and subsequent diffusion of intracellular content out of the cell. This is reflected in the mechanical and electrical conductive properties of the tissue.

In the study undertaken here, a microscale method was developed making the study of PEF-induced permeabilization possible at cell level. We demonstrated that permeabilization of plant tissue starts to occur with a single pulse of 100 μs at 0.35 kV/cm field strength. The timescale for the diffusion of intracellular content into the extracellular region is in the range of 2 min. When changes in the mechanical properties of plant tissue (potato) exposed to a PEF, with or without osmotic treatment, were studied using stress relaxation, the short-term (time) modulus was found to be little affected. The residual elasticity was the parameter most affected even at lower levels of PEF treatment. Maximal PEF treatment had a similar effect to exposure to a 0.7 M hyperosmotic solution implying that the effect of PEF on relaxation behavior can be explained by loss of turgor. Neither hypoosmotic nor hyperosmotic pretreatment appeared to enhance the effect of PEF treatment. When the effect of a PEF on extraction of red pigment from red beetroot was investigated, it was found that PEF treatment level at 1 kV/cm field strength led to the extraction of about 90 % of the total red beetroot pigment following 1 h aqueous extraction. The energy consumption was 7 kJ/kg. The increase in tissue conductivity after PEF treatment correlated linearly with the extraction yield of up to an extraction level of 60-70 %. Diffusion of red pigment and ionic species in the extraction process was also studied in terms of a bimodal Fickian diffusion model. The process appeared to be governed by a slow- and a fast apparent diffusion coefficient which were independent of the treatment level. However, the fraction of the yield associated with the faster diffusion coefficient increased with treatment intensity, and at maximum PEF treatment and in frozen/thawed tissue it accounted fully for the transport. The faster apparent diffusion coefficient was 2-5 times lower than the corresponding diffusion coefficient in dilute solution.

Key words; Pulsed electric field, PEF, Cell permeabilization, electroporation, plant tissue, epidermis, potato, red beetroot, visualization, onion Neutral Red, generalized Maxwell model, bimodal Fickian diffusion model

Behandling av Växtvävnad med Pulserande Elektriska Fält

Fincan, M. 2003, Doktorandsavhandling, Lunds Universitet

Sammanfattning

Växtceller liksom andra celler innehåller vatten plus olika cellkomponenter, vilka är omslutna av ett membran. Cellmembranet utgör dock ett hinder för att utvinna substanser från växtvävnaden. Transporten av dessa substanser ut ur vävnaden påverkas mycket av hur intakt cellmembranet är. Metoder för att ta sönder cellmembran bör vara effektiva, energibesparande samt minimera produktförsämringen.

Elektriska pulser (PEF) är en nyligen introducerad icke-termisk livsmedelsprocess, som har en karakteristisk påverkan på cellmembranet. PEF-behandling innebär att livsmedlet utsätts för korta och upprepade pulser emellan en anod och en katod. Det huvudsakliga resultatet av denna process är en skada på cellmembranet, som leder till bildandet av antingen tillfälliga eller permanenta porer. Det intressanta med behandling av växtvävnad med elektriska pulser är att ingen värmebehandling behövs och på så sätt underlättas vissa processer såsom extraktion, torkning och osmotisk torkning.

För att använda pulser i en kommersiell processning av växtvävnad krävs en bättre förståelse för de förändringar i strukturen hos växtvävnad på cellnivå orsakat av dessa pulser. Efter att porer i cellmembranet bildats, sker ett antal förändringar inuti vävnaderna. Den viktigaste av dessa är förlust av turgor och påföljande spridning av intracellulärt innehåll ut ur cellen. Detta reflekteras i att de mekaniska och elektriska egenskaperna förändras i vävnaden.

I denna undersökning har utvecklats en metod som möjliggör studier av PEF-permeabilisering på cellnivå. Vi har demonstrerat att permeabilisering av växtvävnad börjar ske vid en enda puls av 100 μs vid 0.35 kV/cm fältstyrka. Tidsskalan för diffusion av intracellulärt innehåll ut i extracellulärt området är 2 min. Vid stress relaxationsstudier undersökte vi förändringar i de mekaniska egenskaperna hos växtvävnad, som var utsatta för pulser med eller utan osmotisk förbehandling. Vi kom fram till att den initiala styvheten påverkades lite medan kvarvarande elasticitet var den parameter som påverkades mest, även vid lägre nivå av pulsbehandling. Maximal pulsbehandling hade en likadan effekt som 0.7 M hyperosmotisk lösning, vilket innebär att inverkan av pulser på relaxationsbeteende kan förklaras som förlust av turgor. Varken hypoosmotisk eller hyperosmotisk förbehandling visade sig förbättra inverkan av pulsbehandling. När effekten av elektriska pulser på extraktion av färg från rödbetor undersöktes, visade det sig att en pulsbehandlingsnivå vid 1 kV/cm`s fältstyrka ledde till 90 % utbyte av ett rödbetsfärgämne vid efterföljande vattenextraktion under 1 timme. Energiförbrukningen var 7kJ/kg. En ökning av vävnadens konduktivitet efter pulsbehandling korrelerade linjärt med en extraktionsnivå upp till 60-70 %. Genom att använda bimodal Fick's diffusionsmodell undersökte vi också extraktionshastigheten av rödbetsfärgämnet och några salter. Processen visade sig var styrd av en långsam och en snabb diffusionskoefficient, vilka var oberoende av behandlingsnivån. Men extraktionsandelen som skedde med den högre hastigheten, ökade med behandlingsnivån. Den snabbare diffusionskoefficienten var 2-5 gånger lägre än den motsvarande diffusionskoefficienten i utspädd lösning.

Bitki dokusunun kesikli elektrik alan ile işlem edilmesi

Fincan, M. 2003, Doktora tezi, Lund University

Özet

Bitki hücrelerinde diğer hücreler gibi su ve muhtelif bir çok hücre bileşenleri zarlarla çevrelenmiş olarak bulunur. Hücre zarları hücre içi maddeleri ayrıştırma işlemlerinde engel teşkil ederler. Bu tür maddelerin doku dışına taşınımı hücre zarının ne derece sağlam olduğu ile oldukça alakalıdır. Hücre zarını parçalamak için kullanılan metodlar etkin, enerji sarfiyatı az ve aynı zamanda ürün bozulmasını en aza indirgeyecek şekilede olmalıdır.

Hücre zarı üzerine karakteristik etkisi olan *Kesikli Elektirik Alan* (PEF) uygulaması, ısısal olmayan, yeni bir gıda teknolojisidir. PEF uygulaması gıdanın anod ve katod arasında kısa, tekrarlı elektrik alanlarına maruz bırakılma işlemidir. Bu işlemin en önemli sonucu hücre zarında geçici veya kalıcı deliklerin oluşumu ile hücre zarının bozunumudur. Bu işlemin bitki bazlı gıdaya uygulama da esas ilgi kurutma, ekstraksiyon ve osmotik kurutma gibi bazı prosesleri ısısal olmayan bir şekilde, ekonomik olarak kolaylaştırmaktır.Bununla birlikte, PEF'in bitki dokusunu kulanan proseslerde kullanılabilmesi için, dokuda PEF uygulaması sonucu ortaya çıkan değişikliklerin hücre seviyesinde daha iyi anlaşılması gerekir. Hücre zarında deliklerin oluşumu ile, doku içerisinde bir çok değişiklikler meydana gelir. Bu değişikliklerin en önemlisi hücre iç basınçının kaybolması ve hücre içindeki maddelerin hücre dışına doğru difuzyonudur. Bu aynı zamanda dokunun mekanik ve elektrik iletkenlik özelliklerinede yansır.

Bu tezde yapılan çalışmada, PEF etkisiyle hücre zarının bozunumunu inceleyen mikro bir metod geliştirildi. Bu metod ile, bitki dokusunda bozunum başlangıçının 0.35 kV/cm lik elektrik alan kuvvetinin 100 μs lik tek bir vurgusu sonrası oluştuğu gösterildi. Hücre içi sıvısının hücre dışına difuzyon zamanı ise 2 dakika olarak saptandi. PEF, osmotik işlem ile veya osmotik işlemsiz, bitki dokusuna (Patates) uygulandığında, dokuda ortaya çıkan mekaniksel değişiklikler, ' *stress relaxation* ' deneyi ile çalışıldı. Bu çalışmada başlangıç sertlik parametresinin çok az etkilendiği fakat kalıcı sertlik parametresinin en düşük PEF uygulamalarında bile en fazla etkilendiği bulundu. Maksimum şiddette PEF uygulamasının, 0.7 M lik osmotik işlemi ile aynı etkiye sahip olması, PEF in hücre içi basınçının kaybolmasına yol açtığı şeklinde açıklanabilir. Osmotik işlem PEF ile beraber uygulandığında ise, düşük ve yüksek seviyedeki osmotik işlem PEF ile etkileşmedi. PEF in kırmızı pancardan renk maddesi ekstraksiyonu üzerine etkisi incelendiğinde, 1 kV/cm lik elektrik alan uygulamasının ekstraksiyon verimini 90 % artırdığı bulundu. Bunun enerjı maliyeti 7 kJ/kg'dir. PEF uygulaması sonucunda bitki dokusunda ortaya çıkan elektrik iletkenlik artışı, ekstraksiyon verimi ile 60-70 % seviyesine kadar doğrusal olarak arttığı ayrica belirlendi. Renk ve elektriksel maddelerin ekstraksiyon hızı, '*Bimodal Fick* ' difuzyon modeli ile çalışıldığında, ekstraksiyon hızının, biri hızlı diğeri yavaş fakat PEF'den etkilenmeyen difuzyon katsayıları ile ifade olunduğu görüldü. Bununla birlikte hızlı difuzyondaki ekstraksiyon fraksiyonu PEF uygulamasının şiddeti ile artış gösterdi. Hızlı difuzyon katsayıları, seyrek çözeltidekine nispeten 2-5 kat daha düşük idi.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. **In situ visualization of the effect of a pulsed electric field on plant tissue**
Mustafa Fincan and Petr Dejmek,
Journal of Food Engineering, vol. 55(3), 223-230 (2002)

II. **Effect of osmotic pretreatment and pulsed electric field on the viscoelastic properties of potato tissue.**
Mustafa Fincan and Petr Dejmek,
Journal of Food Engineering (in press) (2003)

III. **Pulsed electric field treatment for solid-liquid extraction of red beetroot pigment.**
Mustafa Fincan, Francesca De Vito and Petr Dejmek
Journal of Food Engineering (submitted)(2003)

IV. **Pulsed electric field treatment for solid-liquid extraction of red beetroot pigment: Mathematical modeling of mass transfer**
Yongyut Chalermchat, Mustafa Fincan and Petr Dejmek
(Manuscript)

Content

INTRODUCTION

During the processing of cellular plant food, one or more stages are often intended to modify the tissue structure so as to facilitate the transfer of the intracellular contents into an external medium. The processes employed for such a purpose should be economic and safe from health and product point of view.

The pulsed electric field (PEF) method is a non-thermal method of treatment with characteristic effects on the cell membrane. PEF processing of food containing cells involves the application of short, repeated pulses of a high electric field to material between an anode and a cathode. Main result of this is the disruption of the cell membrane leading to the formation of either temporary or permanent pores. The exact mechanism of this pore formation remains to be elucidated. Despite this, permeabilization of the membrane has been shown to be influenced mainly by the electrical conditions employed and the inherent characteristics of the material treated.

The formation of temporary pores in the cell membrane using PEF or " electroporation ", as it is also called, was initially used in biotechnology to transfer molecules into a cell, with the aim of obtaining new cell properties without causing significant disturbance to the functionality of the living cells. There have also been attempts in medicine to cure tumors via the transfer of drugs into the cells.

The impact of pore formation on food research has been considerable during the past decade. Interest has largely been on food processing, where the modification of the membrane can be significant or advantageous. PEF treatment in this area has been considered as a single processing alternative and in combination with other food processing methods. The area in which most effort has been devoted so far is whether or not PEF treatment can be a non-thermal processing alternative to the heat pasteurization/sterilization of liquid food. PEF treatment of liquid food was found to have a less detrimental effect on nutritional quality while inactivating microorganisms via irreversible permeabilization of the organisms`cell membrane. Another area in which PEF has awakened interest is in the treatment of plant material. Neither this area of research nor the effects of PEF on plant tissue have however

been explored extensively at a basic level. Particular interest originated from the claims that PEF in combination with other processes can facilitate the mass transport of intercellular compounds from plant tissue in processes such as the extraction of fruit juice and sugar, and in drying processes.

In order to gain evidence to this hypothesis, studies on PEF in food processing should be aimed at acquiring further knowledge on the PEF-induced changes at cell level. Plant material can be regarded as a heterogeneous, semi-solid material in which cells are arrayed in a given structural organization. Limited methods are available for studying the changes inside tissue during and after PEF treatment. In most studies reported in the literature, the measurement of the tissue conductivity during and after PEF treatment is a common method of expressing the effects on the tissue. PEF treatment mainly targets charged molecules/ions and the plant cell has a large vacuole rich in ionic content. The conductivity of plant tissue is thus a relevant measure of membrane permeabilization, although it cannot be used to express effects such as the change of ionic concentration across heterogeneous material.

The effect of PEF on cellular plant tissue is the topic of this thesis. We concentrated initially on the effect on a simple plant tissue. To do this, staining was used to visualize the different cell regions in order to follow the changes under a light microscope. Image analysis was employed to study the internal mass transport inside the tissue after permeabilization. Thereafter, studies on the effects on more complex tissue were undertaken. One particular aspect studied was how the mechanical properties of the tissue were affected by the release of intracellular liquid or after the loss of turgor following PEF treatment with and without osmotic treatment. Finally, the effect on extraction of intracellular compounds into an external liquid medium from PEF treated tissue was investigated, with regard to the degree of extraction that could be achieved with PEF alone. In connection with this study, the kinetics of diffusion were also studied via mathematical modeling.

BACKGROUND

Theory

Exposing a cell to an electric pulse of high intensity (1-20 kV/cm) and short duration (μs to ms) results in disruption of the cell membrane. The mechanism of disintegration in the membrane during and after PEF has not yet been fully elucidated. Many theoretical models have been suggested to explain the phenomena. Of the suggested models, the dielectric breakdown model proposed by Zimmerman (1986) has gained overwhelming acceptance.

The model considers the cell membrane to be a capacitor filled with a dielectric material of a low dielectric constant, and exposure to external electric field pulses initially induces polarization of the cell interior and exterior charges across the membrane, building up a potential difference across the membrane. Further increase in the membrane potential, in response to an increase in the external electric field yields a compression pressure on the membrane due to the attraction of surface charges of opposite signs and when a critical membrane potential of about 1 V is reached, significant perturbations in the membrane occur. (Figure.1) The theory assumes that, due to the perturbations at the critical membrane potential, the membrane looses its impermeability to ions due to the formation of transmembrane pores, which in turn leads to an immediate electrical discharge of the membrane and, in turn to decompression of the membrane. The membrane breakdown is thus thought to be as a consequence of an electrostatic compressing force balanced against the elastic counter force of the membrane. Pore formation is a dynamic process and can be reversible if the external electric field is removed when the transmembrane potential reaches the critical level of 1 V, and if the pores are small in relation to the total membrane surface. Exposing a cell to a continuous stream of external electric field pulses or imposing too high an electric field, results in larger and larger areas of the membrane becoming porous, and when the size and number of pores become large compared with the total membrane surface, reversible breakdown turns into irreversible breakdown, i.e. mechanical destruction of the cell (Zimmerman, 1986).

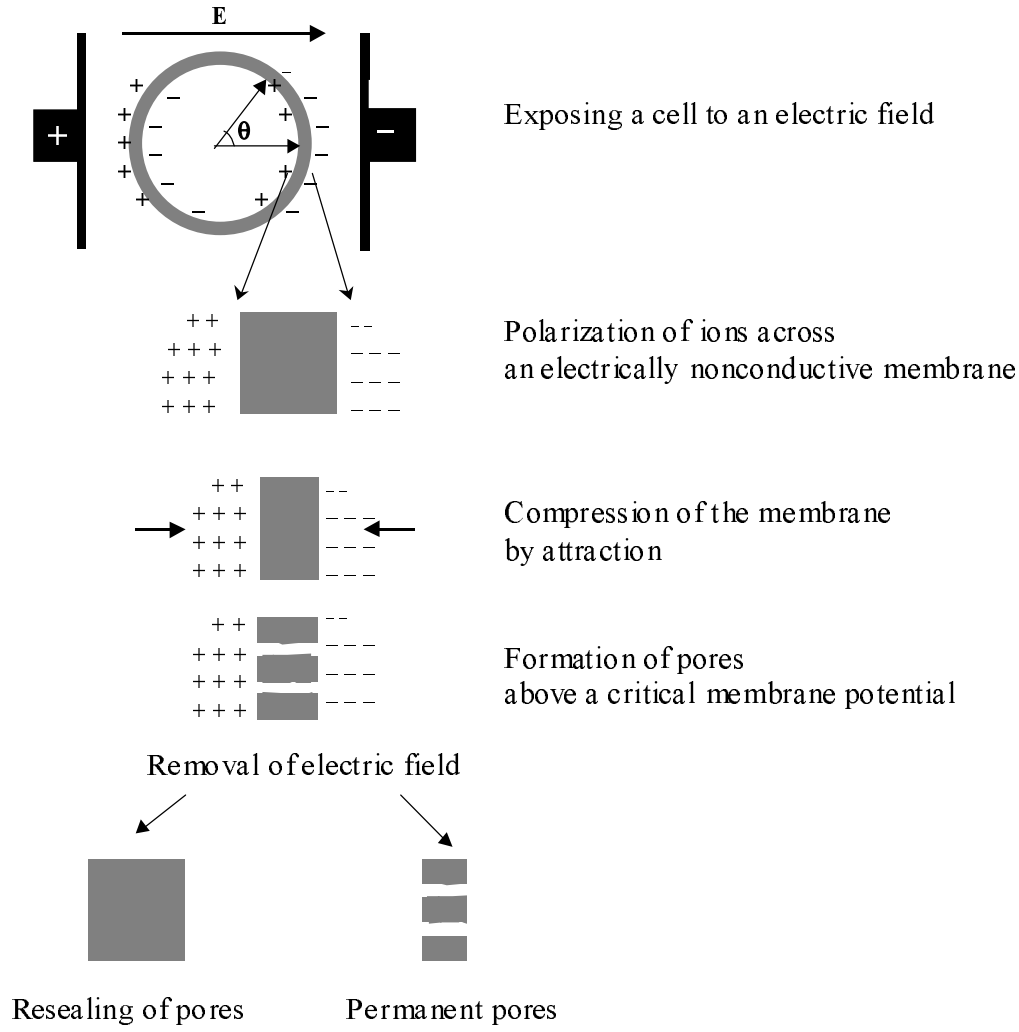

Figure 1. *Schematic description of dielectric breakdown of cell membrane.*

One difficulty in the study of mechanism of pore formation is that the process of permeabilization is extremely rapid. The pores formed in the membrane are not static structures, but dynamic, fluctuating in size and relaxing tending to reseal. Pore formation takes place within 10 ns at sites where the membrane potential reached 1 V (Dimitrov and Jain, 1984). Using rapid-freezing electron microscopy technique, Chang and Reese (1990) studied changes in membrane structure induced by radio-frequency (RF) electric field. In this study, significant structural changes in the membrane after the RF treatment appeared as deep, pore-like membrane openings in the external surface of the membrane. The sizes of the openings were not uniform at any particular time. They first appeared 20-40 nm in diameter, and at a later time expanded to 20-120 nm, becoming more abundant on the surface. The

study also reported that some of the openings disappeared in 10 s, due to the resealing process of the membrane. The resealing process is also found to be temperature dependent, which is slow at low temperate and faster at higher temperature. Zimmerman (1982) proposed that the restoration of the original membrane impermeability occurs within a few microseconds if the breakdown occurred in the lipid domains, and within seconds to 30 min (depending on temperature) if in the protein regions or at the lipid-protein junctions. Based on more recently available information from other researchers, the time scales of the events involved in the pore formation, are shown in Table 1 (Ho and Mittal, 1996).

Time elapsed (s)	Events
ns molecular	molecular collision within lipid membrane
0.1 to 1 μs	cell and cell membrane charging times
0.1 to 1 μs	membrane discharging times: reversible
1 to 10 μs	membrane discharging times: irreversible
0.1 ms to 2.8 h	membrane recovery: metastable pore

Table 1. *Time scales of the events involved in the pore formation (Ho and Mittal, 1996).*

Membrane polarization is due to accumulation of negative and positive charges within the cell at membrane areas closest to the cathode and anode, respectively. For that reason, the induced transmembrane potential is not evenly distributed over the cell surface, that is, the potential difference is the highest at the membrane sites closest to the electrodes and the lowest at the membrane sites most distanced to the electrodes.

Following Hamilton and Sale (1967) who first time showed that the breakdown of membranes occurs when transmembrane voltage reaches about 1 V, more studies concentrated on relation between transmembrane voltage and membrane permeabilization. Several equations have been established to relate the PEF-induced transmembrane potential to various properties of the membrane, external medium and electrical parameters. For a cell surrounded by a nonconducting membrane, transmembrane potential (φ) (Volt) is described as:

$$\varphi(E, M, t_a) = -kr_c Eg \cos\theta(M)(1-\exp(-\frac{t_a}{\alpha}) \quad \text{Eq. (1)}$$

where k is a dimensionless shape factor, r_c is the cell radius (μm), E is the electric field strength (V/μm), M is the point of interest on the membrane, g is the relative electric permeability of the membrane (dimensionless), θ is the angle between the direction of E and M, t_a is the time (μs) after the field was applied and α is the characteristic time (μs) constant/relaxation time of the membrane (Ho and Mittal, 1996). In addition to factors accounted for in the equation, the breakdown voltage is influenced by other conditions, particularly temperature. Zimmerman (1980) measured breakdown voltage in planar lipid bilayer and in *Valonia utricularis* and reported that the breakdown voltage of the membrane was 2 V at 4 ^{o}C, 1 V at 20 ^{o}C and 0.5 at 30-40 ^{o}C.

Apart from the compression model, there are further candidate models regarding the mechanism of molecular reorientation, which leads to pore formation, and the transport of ionic or aqueous solution through these pores. Tsong (1991) theorized that pore formation could occur both through protein channels and lipid domains. Since protein channels (0.05 V gating potential) have a smaller dielectric strength than lipid bilayer (0.15-0.50 V), it is reasonable to assume that many voltage- sensitive protein channels would be opened before the lipid bilayer. As a result, protein channels might be irreversibly denatured by Joule heating or electric modification of functional groups (Tsong 1991). Due to the impermeability restored within seconds to a couple of minutes when temperature of permeabilized membrane was increased, Zimmerman (1982) suggested that the breakdown occurs at protein sites, arguing that resealing time of lipid domain is less than μs. Another point of view was that the direct electric field effects on the membrane structure are of minor extent and that interfacial polarization leads to strong transmembrane fields which, in turn, induce major structural rearrangements (Ho and Mittal, 1996). It was also believed that the initial stage of the permeabilization is based on the occurrence of hydrophobic pores formed by the lateral thermal fluctuations of lipid molecules in a lipid bilayer and hydrophilic pores form later when the energy absorbed increased beyond a certain level. The consensus is, however, that hydrophilic pathways (pores) after PEF must eventually form, so that ions and water readily cross the membrane (Weaver, 1993).

Fundamentals of processing fluid food with PEF

In the late 1960s, Hamilton first showed that the killing of bacteria, protoplasts and yeast in suspension was a direct effect of the electric pulses and that the individual enzyme activities were not inhibited by the treatment. They attributed the cell death of vegetative bacterial cells to an irreversible loss of membrane function as a semipermeable barrier and demonstrated the membrane damage via the lysis of erythrocytes and protoplasts, and leakage of intracellular content. Following this, the possibility of employing PEF as a non-thermal food preservation or cold pasteurization/sterilization method in food processing has been an exciting research topic. To investigate better or optimize the permeabilization of membranes in food, various systems have also been developed for PEF treatment of food, mainly for fluid foods.

In relation to food, PEF treatment can be defined as a process in which high electric fields, from a pulse generator, are delivered in the form of short pulses (μs-ms) of direct current to the food material in the treatment chamber. In its simplest form, the generation of pulses involves slow charging of a capacitor in an electrical circuit and fast discharging of the electrical energy in the capacitor over the treatment chamber. This gives rise to an exponentially decaying pulse. A simplified PEF system for food processing is shown in Figure.2.

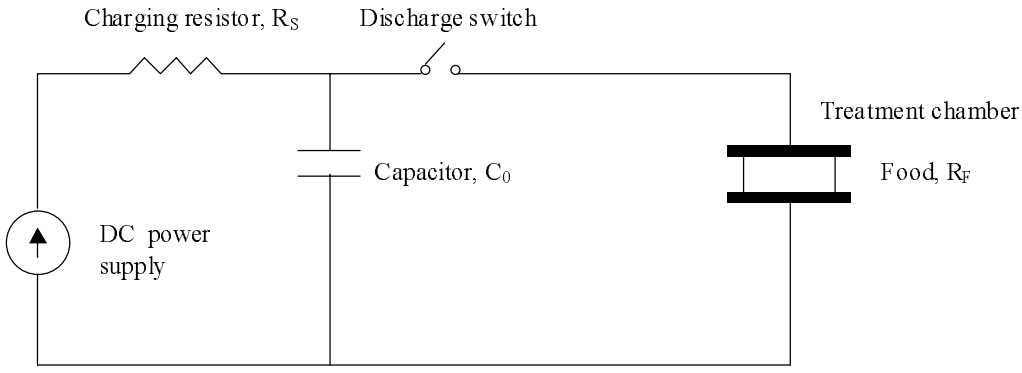

Figure 2. *Basic components of the PEF system for an exponential decay wave form pulse.*

The average electric field strength is defined as the electric potential difference in space between electrodes:

$$E = \frac{V}{d} \qquad \text{Eq. (2)}$$

Treatment time (t) of PEF is given as

$$t = n\tau \qquad \text{Eq. (3)}$$

where n is the number of pulses and τ is the pulse length which, for exponential pulses, can be approximated by:

$$\tau = R_F C_0 \qquad \text{Eq. (4)}$$

where R_F is the effective resistance of the food and C_0 is the capacitance of the energy storage capacitor.

The energy expenditure of PEF treatment is an important parameter in maintaining the non-thermal and cost characteristics of the treatment. Energy expenditure is largely determined by the electrical and medium variables employed. Energy calculation also takes pulse shape factor into account (Barbosa-Cánovas, Swanson & Zhang, 1995). The energy density (Q) for exponentially decaying pulses is approximated by:

$$Q = \frac{V_0^2 C_0 n}{2v} = \frac{V_0^2 t}{2R_F v} \qquad \text{Eq. (5)}$$

where V_0 is the initial charge voltage, and *v* is the volume of the treatment chamber.
Similarly, the energy input, Q, for square wave pulses is:

$$Q = \frac{VI\tau n}{v} = \frac{V^2 \tau n}{R_F v} = \frac{V^2 t}{R_F v} \qquad \text{Eq. (6)}$$

where V, I, τ are the voltage, current, and pulse width of the square waves, respectively.

By varying the design of the pulse generating circuit, as well as that of treatment chamber, the PEF system can offer a wide rage of electrical conditions. Pulses of different shape, exponentially decaying, square-wave, bipolar or oscillatory are generated by modification in the pulse generating circuit. The intensity of each individual electrical variable can be altered through pulse generating circuit. Optimum performance of the PEF system for permeabilization of the membranes in food depends on both electrical variables (such as the field strength, the number of pulses, their shape and length and the inter-pulse delay) and product variables (such as temperature, electrical conductivity, air entrapment and suspended particles), and flow rate in case of fluid food) (Barbosa-Cánovas, Swanson, & Zhang, 1995; Barbosa-Cánovas et al., 1997; Barsotti, Merle, & Cheftel, 1999).

The treatment of liquid food with high electric pulses typically in the range 10-50 kV/cm results in decrease in microbial load without a significant increase in temperature. Microbial cells are smaller than plant cells, and consequently the critical external electric field required to induce the breakdown voltage is higher. The external electric field required for the inactivation of population of microorganisms varies between different species and also depends on the electrical conditions employed and a number of external factors. Hamilton and Sale (1967) studied the survival rate of *E. coli*, and *Staph. aureous* after applying electrical field strength up to 30 kV/cm; 10 pulses, each of 20 μs at 1 Hz and concluded that the percentage of survival rate of *E. coli* decreased rapidly to 16 % at field strength of 11 kV/cm, and was only 1 % at 19.5 kV/cm. Similarly for *Staph. aureous*, the first decrease to 35 % was reported at about 15 kV/cm, and at 27.5 kV/ cm, the survival rate was 0.6 %. Various models have been suggested for the prediction of inactivation rate of microorganisms. A common model was:

$$\frac{N_{PEF}}{N_0} = \frac{t}{t_c}\exp(-c_n(E - E_c)) \qquad \text{Eq. (7)}$$

where N_0 and N_{PEF} are the concentration (CFU/ml) of viable microorganisms before and after PEF treatment, t is the PEF treatment time (μs), t_c is the constant threshold value for the treatment time(μs), c_n is the normalization constant (cm/kV) and E_c is the critical value below which no activation occurs to specific microorganism (Zhang et al., 1994). Although most

vegetative bacteria can be inactivated by PEF, the effect of PEF on the inactivation of spores are often less pronounced. The spores of *B. cereus* and *B. polymyxa* are among those resistant to PEF treatment, even at 30 kV/cm. Germinated spores are, however, reported to be more susceptible to PEF (Wouters and Smelt, 1997; Hamilton and Sale, 1967).

The process of membrane permeabilization is influenced by electrical variables of the PEF processing system. Number, duration and shape of pulses can be varied, as can the electric field strength. The interval between the pulses can also be varied. Information in the literature on how each of the variables interacts with the cell membrane is limited. They are often associated with charging process of the membrane. When studying the effect of each individual variable on membrane permeabilization, one common approach is the evaluation of increase in transmembrane potential with reference to breakdown voltage of about 1 V. The charging time was found inversely influenced by the magnitude of field strength (Angersbach, Heinz & Knorr, 1999). Most importantly, the breakdown of voltage is dependent on the exposure time, i.e. pulse length of the external electric field. Zimmerman and Benz (1980) investigated the pulse length dependence of the breakdown voltage in cells of *V. Utricularis* using charge pulse technique he developed and concluded that the breakdown voltage was constant, about 1 V for pulse length longer than 20 μs and toward shorter charging times the breakdown voltage increases by a factor of about 2. Possible role of the interpulse delay on membrane permeabilization is not clear. Electrical conductivity is also a measure of tissue permeabilization (*see also, section PEF treatment of plant tissue*). At our experiment to permeabilize the potato tissue with PEF, use of longer interpulse delay led to a higher increase in electrical conductivity than that of shorter interpulse delay. A similar effect was also reported by Lebovka, Bazhal and Vorobiev (2001). Pulse shape is also another important factor in membrane permeabilization. For microbial inactivation, oscillatory pulses are reported to be the least effective. Oscillatory pulses do not expose the cell to an electric field continuously. Square pulses are more lethal, more energy efficient and less heat generating than exponentially decaying pulses. Exponentially decaying pulses with long tails cause heat generation, decreasing the efficiency of PEF, whereas with square waves a peak voltage is applied for a longer time than with exponentially decaying pulses (Wouters and Smelt, 1997). Treatment of *S. cerevisiae* with square pulses resulted in 60 % higher death than the treatment corresponding with exponentially decaying pulses (Barbosa-Cánovas et al., 1997; Qin et al.,

1995). The effects of bipolar and monopolar pulses on microbial inactivation were also compared and bipolar pulses were found to be more efficient, minimizing the electrolysis of food and reducing the solid deposit on electrode surfaces. A bipolar pulse is characterized by a positive and a negative part. When the membrane is exposed to bipolar pulses, sudden reversal of the applied field will theoretically change the orientation of the charged group in the membrane. Similar to bipolar pulses, it has also been suggested that instant-charge-reversal pulses will also be more effective in the membrane permeabilization. In this form of pulse, the reversal of the charge at the end of the pulse is virtually instantaneous, with almost no time lag, whereas in the case of bipolar pulses, the pulse is reversed with some relaxation between the pulses. Application of a small number such was reported to result in high microbial inactivation at a low electric field (Barbosa-Cánovas et al., 1997; Wouters and Smelt, 1997; Barsotti, Merle, & Cheftel, 1999).

In addition to the effect of electrical variables, the efficiency of PEF treatment in inactivating microorganisms in liquid food is influenced by external, medium-related factors and the interaction of these factors with the electrical conditions. Vega-Mercado et al. (1996) studied the effect of pH and ionic strength on the inactivation of *E. coli* in simulated milk ultra-filtrate and reported that the inactivation rate increased at lower pH, about 5.7, when the number of pulses at 40 kV/cm or 55 kV/ cm was increased at temperatures of 15 and 10 ^{o}C. Similarly, Aronsson and Rönner (2001) investigated the effects of pH and water activity on *E. coli* and *S. cerevisiae* by treating a model medium with 25 or 30 kV/cm and concluded that a decrease in pH value from 7.0 to 4.0 resulted in the reduction of *E. coli* by four additional log units, whereas for *S. cerevisiae*, the pH effect was less pronounced. Concerning the effect of ionic strength, study on inactivation of *E. coli* in simulated milk ultra- filtrate at 40 kV/cm and 10 ^{o}C resulted in a more significant increase in the log reduction rate at 28 mM of KLC_than at 168 mM (Vega-Mercado et al., 1996). Ionic strength is linearly correlated with conductivity and fluid temperature (Zhang et al., 1995). Also, the conductivity properties of the medium may strongly interact with the electrical conditions of the PEF. In the capacitor type of PEF apparatus, for example, pulse duration was inversely correlated with conductivity (Eq.4) (Wouters and Smelt, 1997). At high conductivity, the charge of water dipole molecules is quickly discharged locally, thus the effect of narrow pulses disappears rapidly (Zhang et al, 1995). The PEF treatment of highly conductive media also promotes electrolysis between

electrode and liquid, and causes a temperature rise, mainly due to Joule heating (electrical energy dissipated as heat in a resistor. Sale and Hamilton (1967) reported a maximum temperature increase of 10 ^{o}C when *E. coli* was subjected to 10 pulses of 20 μs each at 4.9 to 18.5 kV/cm. The thermal effect in solution during PEF treatment in the range 2-7 kV/cm had little effect on the inactivation of *E. coli* (Zimmerman, Pilwat, & Riemann, 1974). Apart from the temperature increase due to PEF treatment of conductive solutions, effect of medium temperature on the inactivation of microorganisms has been investigated. The reports claim that inactivation rate increased with temperature increase. Aronsson and Rönner (2001) studied effect of the temperature increase on *E. coli* and *S. cerevisiae* and concluded that an increase in the inlet temperature from 10 to 30 ^{o}C enhanced significantly the killing effect of PEF for both. In another study the effect of medium temperature during PEF treatment was investigated measuring the breakdown voltage of cells at different temperatures and the breakdown voltage was found to be 1.2 V at 4 ^{o}C, and 0.6 V at 30 ^{o}C. (Zimmerman and Benz, 1980). The increasing effect of PEF on microbial inactivation at higher temperatures has been attributed to the susceptibility of the membrane in the liquid-crystalline state. The phase transition of membranes from gel to "liquid-crystalline" is dependent on temperature. At low temperature, the phospholipids are closely packed in a rigid gel structure, while at high temperature they are less ordered and in the form of liquid-crystalline structure. (Wouters and Smelt, 1997).

The appropriate design of the treatment chamber in PEF processing is also important for effective permeabilization, prevention of undesirable reactions and ease of process operation.
A common consideration in treatment chamber design is to provide a uniform electric field and to prevent field enhancement. Cell permeabilization may vary at different electrical field strengths. Also, field enhancement at high PEF strengths is claimed to cause the dielectric breakdown of liquid food. Dielectric breakdown of liquid food, observed as the passage of a spark through the liquid inside chamber, takes place when the applied electrical field strength becomes equals to the dielectric strength of the food (Barbosa-Cánovas, Swanson & Zhang, 1995). Dielectric breakdown of food is shown to be affected by the roughness of the electrodes and impurities. Impurities in a liquid, gas or solid can substantially enhance a local electric field due to differences in dielectric properties, and food material often contains gas bubbles or air. Measures to prevent dielectric breakdown of food during PEF include using

smooth electrodes to minimize electron emission, and rounding the edges of electrode to prevent field enhancement, degassing of the food prior to PEF to eliminate gas bubble formation, and avoidance of applying too high an electric field (Barbosa-Cánovas, Swanson & Zhang, 1995). Electrode material is also of concern in treatment chamber design. Electrochemically inert materials such as gold, platinum, carbon, stainless steel and metal oxides have been suggested for construction of electrodes or electrodes surfaces. In the past, various treatment chamber designs, static or continuous, have been proposed for optimization of the desired characteristics during PEF treatment (Zhang et al., 1996; Qin et al., 1995; Bushnell et al., 1991). Earlier studies were carried out with a static, parallel-electrode treatment chamber. Using optimized electrodes in the coaxial treatment chamber, a prescribed field distribution in liquid foods was achieved without electric field enhancement. Recently, several other both static and continuous chambers have been designed taking shape into consideration to provide uniform field strength and functional characteristics such insulation and cooling of the chamber to avoid temperature increase during PEF. Electrodes should also be designed so as to be easily washable since repeated pulse application to protein-containing liquid foods is reported to lead to deposits on electrodes, which can affect the uniformity of the electric field (Barbosa-Cánovas, Swanson, & Zhang, 1995). Such deposits could be related to electrolysis phenomena, protein aggregation and insolubilization (Barsott, Merle, & Cheftel, 1999).

The effect of PEF on the nutritive components and sensorial properties of food is one of the research topics still under investigation. The degree of destruction of most nutritive components and the deterioration in the taste of foodstuffs were generally found to be low. When subjected to PEF, charge-carrying molecules such as proteins, ionic sugars or polar lipids, may undergo chemical modification. Enzymes are probably most sensitive since their active site depends on a native conformation maintained by low-energy bonds and interactions. Reports concerning the effects of PEF on enzymes and nutritive components are not complete in the literature, however, some enzymes are reported to be inactivated while others remain unaffected by PEF treatment. Apple juice, milk (2 % fat), whole liquid egg and pea soup were subjected to PEF prior to processing. No significant sensorial difference was noted between these foods and those processed by thermal pasteurization, except apple juice concentrate. The sensorial quality of apple juice treated with PEF was preferable to that

treated thermally and is comparable to that of freshly squeezed apple juice. However, viscosity and color changes were observed in beaten whole liquid egg subjected to 10 pulses at 35 kV/cm (Barsott, Merle, & Cheftel, 1999). Although several reports claimed that PEF processing of food by repeated electric pulses of short duration (a few μs) does not induce significant sensorial changes, more research appears to be necessary under other processing conditions.

An overview of plant tissue

Structurally, intact plant tissue is a material in which living cells are packed vertically and horizontally inside an extracellular matrix surrounding them. The tissue, as a whole, is in the semi-solid state with the fluid contained. Most fluid is held inside the cells by a surrounding membrane; the rest is in the extracellular space. The fluid is mostly water, containing various active/inactive biological substances. The water content can be up to 90-95 %; there is also some air between cells. Cell size is usually not uniform, and can vary from 20 to 200 μm. The extracellular matrix surrounding the cells is an integral part of the tissue holding the cells together.

The cell wall

The cell wall is largely responsible for determining the size and the shape of the cell, in conjunction with the internal hydrostatic pressure. The cell walls may also affect transport of substances into or out of the cells as they surround the plasma membrane.

Cell walls consist of three types of layers, primary wall, secondary wall and middle lamella. The primary wall is an elastic part outside the plasma membrane in young, growing plant cells. The secondary wall is a much more rigid than the primary wall and is formed between the primary wall and plasmalemma after cell expansion ceases. These different regions vary in composition and structure.

The primary cell wall is thin, of the order of 1-3 μm, and consists of cellulose (9-25 %), hemicellulose (25-50 %), pectic substances (10-35 %) and proteins (10 %). Cellulose chains are held together by non-covalent forces in the form of long cylindrical fibers, so-called microfibrils, and these molecules are found embedded in a matrix of other materials. The main molecule is hemicellulose, which form a branching, a molecular network filled with water. Closely related are the pectic substances, which are highly hydrated and form a gel phase between hemicellulose and cellulose. Pectins are mainly polygalacturonic acids with negatively charged residues. The cell wall also contains proteins such as glycoproteins, extensins and several enzymes such as peroxidases, pectinases, cellulases, and expansins.

The structure of cellulose gives the cell wall one of its unique characteristics. Because of the parallel arrangement of the cellulose, the microfibrils behave like a crystal and have as much tensile strength, in relation to their weight, as steel wires in a cable. The microfibrils play an important role during cell expansion by counterbalancing the turgor pressure exerted on the wall and preventing the cell from expanding freely. As the protoplast absorbs water, expanding like a balloon and creating pressure against the cell wall, microfibrils both move apart and slide past each other in the water-filled matrix. When the cell is not growing, the primary wall resists stretching, due to the high tensile strength of its cellulose microfibrils and cross-linking with the matrix molecules and within the protein network. Yet the wall is porous enough to allow the free passage of water-soluble molecules (Park, 1999). The pores (openings between microfibrils) are about 3.5 to 5.2 nm in diameter. This can be compared with the size of a water molecule (0.3 nm) and that of sugar molecule (1 nm size including hydration).

The secondary walls are usually much thicker than primary walls and consist of about 41-45 % cellulose, 30 % hemicellulose, and in some cases 22-28 % lignin, which is much more rigid than cellulose, especially in combination with cellulose microfibrils. Food is often selected on the basis of a low amount of lignified secondary cell wall (Åman and Westerlund, 1996). The middle lamella (about 0.1-0.2 μm) makes up the outer wall of the cell and consists mainly of amorphous pectic substances forming a gel-like material which cements adjacent cells together (Mauseth, 1995; 1988).

Primary cell walls usually have thin areas called primary pit-fields. These have a high density of plasmodesmata, which have extremely thin strands of cytoplasm that extend through the walls of adjacent cells, connecting the protoplast of adjacent cells. They appear as a channel, 40-50 nm in diameter, and may make up 1 % of the area of the cell wall.

The structure of the primary and secondary cell wall varies between plant cells, and the differences are used to classify plant cells as parenchyma (those with only a thin primary cell wall), sclerenchyma (those with a thick secondary cell wall, usually lignified) and collenchyma (those with a thinner secondary cell wall). Most of the plant parts that are used for food: seeds, fruits, and tubers are composed primarily of storage parenchyma (Mauseth, 1995). In these tissues, plant cells usually store starch (cereal grains, potato), protein (beans, peas) or oil (avocados, safflower). Cells of this type are often full of their storage product.

The plasma membrane

The key function of plasma membranes, located just below the cell wall matrix, is to separate the cell interior from the external environment. Membranes surrounding the cell and cell organelles are very thin (6-10 nm), elastic and fluid-like structures which are composed of lipids and proteins. The basic membrane architecture is like an oil film of lipids in which proteins are embedded. Noncovalent interactions hold the proteins together.

The lipid part has the form of two leaflets (lipid bilayer) which are amphipathic in that each leaflet has a hydrophilic (globular) head pointing outward and a hydrophobic portion forming the core. The principal components of the lipid part are phospholipids and glycolipids together with cholesterol in animal membranes or phytosterol in plant membranes. Most membrane proteins are found embedded in the lipid bilayer (integralmembrane proteins), others may be bound to the cytoplasmic inner surface (peripheral membrane proteins). Due to its impermeability to ions, the plasma membrane can also be considered an electrical insulator with a dielectric constant of about 2 (of water, 80).

Plasma membranes are selectively permeable to molecules. The lipid layer and the proteins play different roles in the movement of molecules into and out of the cell. The lipid bilayer is highly impermeable to ions and most hydrophilic molecules (except water, CO_2, O_2) due to the hydrophobic core of the structure, which allows the diffusion of small hydrophobic solutes across the membrane, but inhibits the diffusion of charged and hydrophilic molecules. Water crosses plasma membranes by diffusion through the lipid bilayer, and through water channels called aquaporins. The incorporation of protein into the lipid bilayer provides the necessary pathways for all charged and hydrophilic molecules (Stryer, 1995).

Transport of ions across the plasma membrane and membrane potential

Substances essential for the function and metabolism of cell must both enter and leave the cell. As result of this, there is an electrical gradient and a chemical gradient between the outside and inside of a cell across the plasma membrane. In the resting state the cell interior is negative relative to the cell exterior about -120 mV. Maintenance of membrane potential is associated with the transport of molecules across the membrane, which occurs in both lipid and the protein domain (ion channels). Hydrophobic molecules can diffuse across the lipid bilayer, depending on their solubility in the lipid bilayer. Transport of ions and solutes across the plasma membrane is mediated by three classes of integralmembrane proteins: pumps, carriers and channels. In pumping system, primary active transport across the plasma membrane is driven by the energy obtained from ATP hydrolysis, and catalyzed by phosphorylated ATPase. A H^+ -ATPase pumps protons across the plasma membrane from cytosol to the apoplast, thereby forming a H^+ and electrical gradient across the membrane which provides the driving force the secondary active transport. The H^+ -ATPase also controls intra-and extracellular pH, as well as turgor, and is regulated by a number of endogenous and environmental factors. Also, a Ca^{2+}-ATPase pumps calcium from the cytosol to the apoplast, thereby maintaining the resting level of Ca^{2+} in the cytosol at about 0.1 μM against at least 1000-fold higher extracellular concentration. Carriers are transport proteins that bind the transported solutes at specific sites, similar to the active sites of enzymes. The bound solutes are then carried through the membrane. The carriers (symporters, antiporters and uniporters) are very specific. The symporters couple the transport of two solutes in the same direction,

whereas antiporters couple the transport of two solutes in opposite direction. The uniporters simply facilitate the passive diffusion of solutes across the membrane. The secondary active transport depends also upon the H^+-ATPase, which is used by symporters and antiporters. Channels function as selective pores in the membrane, simply opening to let an unlimited number of ions and solutes through, and then closing again. The opening and closing (gating) may be controlled by, e.g., the membrane potential set up by the H^+-ATPase (voltage-gated channels). Channels are less specific than carriers, but have instead a much higher transport capacity. K^{+2} channels are responsible for K^{+2} uptake into the plant inward. There are also anion channels, with selectivity for monovalent anions, preferentially chloride and nitrate. Voltage-gated anion and K^{+2} channels are important for regulation of cell turgor. Water channels (aquaporins) are important for water uptake in the roots and for the mass transport of water within the plant. Aquaporins are very abundant proteins and may constitute more than 20 % of total plasma membrane proteins (Stryer, 1995; Encylopedia of life science, 1998 Macmillan Reference Ltd, London).

Organization inside cells

The material inside the cell consists of fluidic cytoplasm in which cell organelles float. The integrity of the contents is maintained mainly by the plasma membrane. Most of these organelles have also endomembrane systems, such as the endoplasmic reticulum, the nuclear envelope, the vacuolar membrane or tonoplast. Numerous biologically active substances are also found both within the individual cell organelles as well as in the cytoplasm. Among these organelles is the vacuole, the largest organelle in cell.

The vacuole

The vacuole often constitutes more than 90 % of the total protoplast volume and leaves the rest of the protoplasm as a thin layer pressed against the cell wall. The simple structure of the vacuole is a membrane, i.e. the tonoplast, and the liquid that it encloses. The tonoplast resembles the plasma membrane but it differs in function and is often thinner (7.5 nm). It

transports solutes into and out of the vacuole and thus controls the water potential of the cell (it is highly permeable to water). The liquid inside the vacuole is mainly water, containing high concentrations of dissolved materials.

Due to the amount of water and the concentration of materials dissolved in it, the vacuole exerts pressure (turgor) on the wall, and significantly affects the shape and rigidity of the tissue. The vacuole is able to absorb water rapidly; swelling and thereby pressing the tonoplast and cytoplasm against the wall, exerting sufficient turgor pressure on it to stretch it to a larger size. Especially rigidity of tissues with a primary cell wall (for example, parenchyma cells in fruits) are determined by the turgor pressure.

The concentration of dissolved materials in the vacuole is as high as that of cytosol (commonly 0.4 to 0.6 M) and the substances include mainly salts, small organic molecules such as sugars and amino acids, some proteins and other molecules. Some vacuoles have a high concentration of pigments, which produce the color of plant, some materials toxic to the cytoplasm and degrading enzymes (hydrolases, such as proteases, exopeptidases, nucleases and other esterases). The vacuole is regarded as a kind of dumping reservoir for cellular waste products and excess mineral ions taken up by the plant. The vacuole also plays a role in maintaining various physiological parameters at a constant level (homeostasis). For example, in the maintenance of constant cytosolic pH, the excess hydrogen ions in the cytosol may be pumped into the vacuole. Most vacuoles are slightly acidic (pH 5-6) while the pH of the surrounding cytoplasm is close to neutral (pH =7-7.5). It has been experimentally shown that when the pH around various plant cells changes drastically, the change is reflected in the pH of the vacuole while the cytosol pH remains constant (Mauseth, 1995; 1988).

PEF treatment of plant tissue

Plant cells contain various commercially interesting products and considerable amounts of water surrounded by membranes. Cell membranes constitute a barrier in processes designed to remove intracellular substances from the tissue. In such processes, the transport is highly influenced by the degree of cell disintegration. The method used for disintegration of cell

membranes should also be energy saving, cost effective and not lead to the quality deterioration. The main aim in the treatment of plant tissue with PEF is the facilitation of the mass transport via permeabilization of the membrane, non-thermally and economically (Knorr, D., 1994; Mertens and Knorr, 1992; Knorr, & Angersbach, 1998).

Pretreatment of the tissue before PEF treatment

The properties of plant tissue at the time of PEF treatment can influence the tissue permeabilization. In one part of the investigations, plant tissue was used in the form of small pieces removed from the plant body (Bouzrara and Vorobiev, 2000; Bazhal, Lebovka, & Vorobiev, 2001; McLellan, Kime, & Lind, 1991). Removal of tissue from the body is usually accomplished by cutting. The cutting process inevitably causes damage to cells and results the release of conductive intracellular liquid. This conductive liquid is generally found as a film covering the surface of the cut sample. Additionally, in studies when PEF was combined with mechanical pressure, plant tissue used for PEF treatment was in a state of mash, thin strips or pressed cake with conductive liquid. The electrical conductivity of the medium is a factor influencing PEF treatment. PEF treatment of highly conducting medium results in a larger current passing through the sample causing an increase in temperature and electrolytic reactions between the sample and electrode surface whereas that of poorly conductive medium results in smaller current. Moreover, the low conductive medium promotes cell permeabilization, leading to the formation of larger cell membrane pores (Barbosa-Cánovas, Swanson, & Castro, 1993). The low conductivity of the medium gives also rise to the pulse length (Wouters and Smelt, 1997) and vice versa. In other studies, after cutting the plant tissue it was subjected to a washing step with a low-conductivity water before PEF treatment to remove the cellar debris from the surface, or it was suspended in a low-conductivity solution during the PEF treatment (Eshtiaghi, & Knorr, 2002; Fincan, & Dejmek, 2002; 2003). Pretreatment of plant tissue before PEF is also used in order to change cell properties, particularly pretreatment that can cause extra stress on the cell membrane as this can facilitate PEF-induced permeabilization process.

Choice of electrical variables

The primary concerns in selecting the electrical parameters are the achievement of the desired level of permeabilization using the least amount of energy, minimum temperature increase, avoidance or minimization of the unwanted reactions and, possibly the inactivation of some enzymes (Knorr, 1994). Permeabilization of plant cells requires relatively low-level PEF treatment compared with that in microbial treatment, as plant cells are large in size and have large vacuoles. The field strength is considered to be the dominant electrical parameter leading to the polarization across membrane. Values quoted in the literature range from 0.2 up to 4 kV/cm with various pulse numbers, pulse lengths and interpulse delay. Usually, effect of short pulse duration in combination with high field strength is more pronounced for effective tissue permeabilization (Knorr, personal communication). In addition to the energy and cost concern, longer pulse duration and higher numbers of pulses can also lead to undesirable reactions such as electrolysis of water. Field strengths in the range 400-800 V/cm are reported to be effective in achieving irreversible permeabilization in plant tissue (Angersbach, Heinz, & Knorr, 2000).

Changes inside the tissue during and after PEF treatment

The changes in the structure of plant food material during PEF treatment are continuous and involve different phases at different times. After the formation of pores on the membrane, dynamic changes in the cell environment take place and proceed towards a new equilibrium state. One of the most of important of these dynamic changes is the leakage or the diffusion of intracellular liquid. The transport phenomena are probably related to turgor, concentration differences between the intra- and extra-cellular regions and presence or absence of an electric field, particularly when the rich ionic intracellular liquid begins to be freed through the membrane after permeabilization, as the movement of ions and charged molecules is significantly influenced by the electric field. The transport phenomena during PEF treatment are primarily electrically driven (Prausnitz et al., 1994; Prausnitz, Corbet, & Gimm, 1995)
During the treatment, with each cell permeabilized, the concentration of leaked intracellular

liquid inside the tissue gradually increases, at the same time giving rise to increase in the overall conductivity until all the cells are permeabilized. From the electrical point of view, PEF treatment exerts an electric field across the tissue, which influences the movement of ions. An increase in the conductivity of the material after each cell is permeabilized during PEF treatment simultaneously results in an increase of the current between the electrodes (Angersbach, Heinz, & Knorr, 2000). The efficiency of PEF treatment can also be affected due to the continuos increase in the conductivity or the current during PEF treatment.

After the electric field is removed, the prominent change in the state of the plant tissue is the presence of pores in the membrane of each cell. Pores in the membrane are also the main cause of other changes, which will move towards a new equilibrium state. Due to loss of membrane integrity, intracellular liquid is not maintained inside the cell but moves through the pores in the membrane and the cell wall matrix, and eventually mixing of intracellular liquid and extracellular liquid takes place. The driving force for this transport is probably controlled by concentration, i.e. the difference in osmotic pressure inside and outside of cell. In our study, mixing of intracellular content with the extracellular liquid was visualized in the case of onion epidermis tissue. This process was completed within 2-3 min after permeabilization (***Paper I***).

The pores in the membranes are not constant in size and, in some cases they will reseal. Long-term conductivity measurements after PEF treatment of plant tissue have shown that the conductivity of tissue does not remain constant but decreases slowly, and this was attributed to diffusion process inside the tissue and resealing of pores (Lebovka, Bazhal, & Vorobiev, 2001).

Literature on fundamental changes in plant tissue brought about by PEF

At the present time, there is a lack of knowledge on process optimization preventing the commercial application of PEF in food processing. It has not yet been established whether the electrical characteristics of the PEF treatment should be matched to the characteristics of the

food, of whether the food material should be manipulated by pretreatment in order to achieve the desired degree of permeabilization.

To enable PEF to be introduced in plant food processing, there is e need for a better understanding of the changes in the structure of plant tissue brought about by PEF treatment. Some of the work aimed at elucidating and characterizing these changes at basic research level has been performed. Various methods have been investigated by researchers to detect, monitor and characterize the effects on plant tissue to elucidate the changes occurring in plant tissue structure after PEF.

Conductivity measurement of the whole tissue, before, during and after PEF treatment, has been one of the most common ways of relating the changes with PEF. Plant cells contain conductive liquid surrounded by non-conductive membranes, and the conductivity measurement of intact tissue is based on the amount of ions available in the extracellular region. The increase in conductivity observed after PEF was interpreted as being due to the release of ionic species into the extracellular region, suggesting that the ionic impermeability of the membrane had been impaired, or that the membrane had become permeable to ionic species (Angersbach, Heinz, & Knorr, 1999).

In measuring the electrical conductivity of material, a small alternating current (voltage), AC is applied across the material between the electrodes at a given frequency, and the ability of the material to conduct the current is measured. In principle, the conductivity related to the dielectric properties of a material is a measure of ions available. It is sensitive to the changes in the amount and mobility of the ions within the material. The mobility of the ions is also influenced by secondary external factors such as temperature (Barrow, 1973). As far as the electrical conductivity of cellular plant tissue is concerned, the conductivity measurement is reported to be indicative of the proportion of intact cells or ruptured cells. The availability of ions in the intracellular region depends on whether or not cell membranes are intact since cell membrane acts as an electric insulator separating two conducting-aqueous phases. The conductivity of intact tissue is, thus, a measure of the number of ions in the extracellular liquid outside the cell membrane.

Due to the dielectric properties of the cell membrane, the electrical conductivity of intact tissue is also frequency dependent. The resistance of cell membranes is highest at low frequencies, while it is negligible at high frequencies range, implying that the membrane does not constitute a hinder to alternating current. For biological tissue systems, the effect is most pronounced in the frequency range between 1kHz and 100 MHz. The frequency dependency of the conductivity of biological tissue is given by:

$$K(\omega) = \frac{l}{A|Z(j\omega)|} \qquad \text{Eq. (8)}$$

where l is the length of the sample, A is the area perpendicular to the electric field, j is complex number and Z (jω) is the total system impedance, where $\omega = 2\pi f$ is the angular frequency (Angersbach, Heinz, & Knorr, 1999; 2002).

The frequency-dependent response of tissue conductivity has been interpreted as the result of polarization of individual atoms or molecules in the membranes exposed to an alternating electric field (Russel, Saville, & Schowalter, 1989). The polarizability is a complex function of the frequency of the forcing field. In other words, the material is said to be dispersive owing to inertial effects on the atomic or molecular scale. More specifically, the frequency-dependent response of plant tissue systems was interpreted as being analogous to the case in Maxwell-Wagner theory which describes the behavior of the dielectric response of uncharged spheres suspended in a conducting medium to an alternating field in a frequency range of 10^3-10^7 Hz (Zimmerman, 1982). Assuming that both phases are homogeneous, the theory suggests that the interface polarization depends on the frequency of the imposed field and, at low frequencies, that the interface charge is that for a static interface, while at high frequencies, conduction processes are swamped and free charge disappears, leaving a residual polarization charge. (Russel, Saville, & Schowalter, 1989; Angersbach, Heinz, & Knorr,1999)

In reports dealing with the conductivity of tissue in connection with PEF, the tissue system was often likened to equivalent circuits for interpretation of the electrical behavior of tissue. The cell membrane was assumed to be a capacitor connected to one resistor (the membrane resistance itself) in parallel, and liquid phases on both sides of the membrane as two

additional series resistors (extra- and intracellular liquid). More complex equivalent circuits, considering different compartments of cells such as plasmalemma, cytoplasm, the tonoplast and vacuolar region, were also studied to express the effect of PEF in terms of conductivity. (Lebovka, Bazhal & Vorobiev, 2000; Angersbach, Heinz, & Knorr, 1999).

Various methods employing conductivity to indicate cell damage within the tissue have been suggested. Direct conductivity measurement is also relevant, and PEF-induced damage is typically characterized by an increase in conductivity at low frequencies. Knorr and Angersbach (1998) developed a cell disintegration index, Z_p which was based on the frequency dependence of conductivity of intact and permeabilized tissues, and used it in later reports to define the degree of damage caused to tissue after any PEF treatment. The cell disintegration index, Z_p is defined:

$$Z_p = 1 - b\frac{(K_h' - K_l')}{(K_h - K_l)} \quad b = \frac{K_h}{K_h'} \quad 0 \le Z_p \le 1 \qquad \text{Eq. (9)}$$

where K_l and K_l' are the electrical conductivity of untreated and treated materials in a low-frequency field (1-5 kHz), respectively; and K_h and K_h' the electrical conductivity of untreated and treated materials in a high-frequency field (3-50 MHz) respectively. For intact cells, Z_p=0 and for total cell disintegration, Z_p=1.

Having associated the conductivity of tissue with the degree of permeabilization, the PEF-induced conductivity change was also used to investigate the influence of electrical parameters on process optimization of tissue permeabilization (Ade-Omowaye et al., 2001; Tedjo et al, 2002).

In earlier studies on transmembrane voltage, it has been reported that the breakdown voltage reached by externally applied electric field strength is the main phenomenon leading to cell disintegration. Most studies dealing with the optimization of PEF therefore also aimed at attaining the breakdown voltage with the least amount of energy. The breakdown voltage was found to be approx. 1 V for many types of cell and artificial membranes (Ho and Mittal, 1996).

The electric field strength at which the transmembrane voltage reaches that the breakdown voltage is referred as critical electric field strength. Information on the critical electric field strength for different tissue types is limited in the literature, most of these data are also reported together with other electrical parameters. Angersbach, Heinz and Knorr (1999) studied that the breakdown voltage in terms of the electrical potential difference across the membrane using different tissues (potato, apple, fish tissues and potato cell suspension culture), and the conductivity, current density and field strength were measured versus time during a single discharge of 2-μF storage capacitor. The occurrence of membrane breakdown was first observed at field strength levels in the range of 150-200 V/cm, and significant membrane breakdown occurred in the range 400-800 V/cm. In other words, significant breakdown of the membrane was observed when the electric potential over the cell membrane reached 0.7-2.2 V, while no effect on tissue structure was reported at a value 0.3 V in potato tissue. When using a field strength of 800 V/cm, the conductivity of potato increased to 5.5 mS/cm as a result of permeabilization whereas 66 V/cm led to no further increase above the untreated value of 0.33 mS/cm. Additionally, they reported that charging time at breakdown was inversely influenced by the magnitude of field strength. The charging and the formation of a conductive membrane were completed within a few microseconds after the pulse started, however, the timescale of membrane rupture at supercritical electric field strength was within 1 μs.

Using the conductivity, Lebovka, Bazhal and Vorobiev (2002) investigated the effect of PEF in terms of total PEF treatment time, i.e. number of pulses × pulse duration, (with an interpulse delay between 1-3 s at different field strengths) on the cell disintegration index of potato. The cell disintegration index was defined slightly differently from that of Knorr and Angersbach (1998) and was used to characterize the PEF-induced damage after PEF treatment.

$$Z_p = \frac{K_l^{'} - K_l}{K_{ld}^{'} - K_l} \qquad \text{Eq. (10)}$$

where K_l , K_l' and K_{ld}' are the electrical conductivity of untreated, treated and totally destroyed materials, respectively, in a low-frequency field (1-5 kHz). The equation gives Z_p=0 for intact tissue and Z_p=1 for totally disintegrated tissue. The report suggested that the continuous application of pulses of relatively small electric field intensities (E <300-500 V/cm) did not lead to an increase in electric conductivity and hence cell disintegration index. After an initial increase, the conductivity stabilized and further increase was observed only after a relatively long time, whereas the cell disintegration index was reported to reach saturation, i.e. Z_p= 1 after the continuous application of pulses at field strength of 600 V/cm.

Lebovka, Bazhal and Vorobiev (2001), studied the long-term conductivity changes in apple tissue, using a long interpulse delay of 60 s and a short interpulse delay of 0.01 s at field strengths of 500 and 200 V/cm (pulse length: 1 ms, number of pulses: 1-15). The conductivity versus time increased asymptotically with each successive pulse of 200 V/cm for about 1000 s. When the long inter pulse length at 500 V/cm was employed, a sharp increase in the conductivity which occurred at the beginning was followed by a small decrease. They interpreted the continuous increase in conductivity during PEF treatment as transport inside the tissue after membrane breakdown due to diffusional motion, osmotic flow and redistribution of the moisture, and the decrease as evidence of the resealing process of the membrane. The results also interestingly suggest that the longer the interpulse length, the greater the conductivity increase.

In a further study on the kinetics of PEF-induced material damage, the relative conductivity of apple tissue versus pulse number up to 10^5 (field strength: 0.2-2 kV/cm, pulse length: 100 μs and interpulse delay: 10 ms) and the simultaneous temperature increase were measured (Lebovka, Bazhal and Vorobiev, 2000). Their results suggested that smaller numbers of pulses (up to 200) at higher field strength (2 kV/cm) led to a rapid and continuous increase in conductivity, while the ohmic temperature increase was much lower. In comparison, using up to 10^5 pulses at field strengths of 0.4-0.2 kV/cm did not lead to a continuous increase, but conductivity tended to remain constant before reaching the final value, and the ohmic temperature increase was up to 30 ^oC.

Studies attempting to employ PEF in plant-based food processing

The potential of using PEF in cellular food processing has been investigated in several studies. In these investigations, PEF has so far been associated with extraction and drying processes of fruits and vegetables as a non-thermal pretreatment alternative. Reports on extraction claimed that PEF pretreatment of plant tissue facilitated extraction, by increasing extraction efficiency and produced fruit juice of better quality. However, degree of efficiency obtained using PEF however varied in these reports as the choice of electrical variables as well as plant tissue handling before and during PEF treatment differed.

Extraction

Bazhal and Vorobiev (2000) studied the effect of pressing apple cossettes in combination with PEF treatment on juice extraction yield. In this study, apple cossettes were compressed in a pressure cell by moving one of the electrodes towards the other stationary one, from a distance of 2 cm. The following processing procedure was used: initial pressing of cossettes, followed by PEF treatment (1000 pulses of 1000 V with a duration of 100 μs and a period of 10 ms), and final pressing of the PEF-treated cake. They also tested juice yield by changing the pressure from 0 to 30 bar, both before and after the PEF treatment. The PEF treatment of the cossettes between two pressing stages resulted in a 12-13 % increase in juice yield at a pressure of 30 bar (before and after) and the total amount of juice obtained at this pressure was similar to that obtained at 2-3 bar, where PEF treatment increased the yield by about 40 %. Thus, they suggested that pressing the apple cake up to 3 bar before and after PEF treatment (1000 pulses of 1 kV/cm at 100μ) was a suitable method of extracting the juice with less energy.

In a further study (Bazhal, Lebovka & Vorobiev, 2001) to this finding, the choice of when to apply PEF during compression on juice yield was investigated. Finely cut apple slices, 3-5 mm in thickness, 10-40 mm in length, were compressed up to 3 bar for 90 min using the same set-up as above, and a pressure of 3 bar was reached in 2 min after the start of compression. PEF treatment intensity was 50 pulses of 0.1-0.5 kV/ cm with 100 μs pulses and a repetition

rate of 10 ms. The results suggested that when PEF is applied between 2 min and 10 min the contribution of PEF to juice yield increased more than that obtained applying in other times. When PEF (0.5 kV/cm) was applied at 10 min, i.e. the time at which the apple slices were in the form of a pressed cake under 3 bar, the juice yield was about 10 % higher after 90 min than in the control experiment.

The possibility of using PEF in sugar processing has also attracted attention. Sucrose is present in aqueous solution in the intracellular structure of the sugar beet, and conventional sugar extraction processes use thermal treatment (typically 70 ^{o}C for 10-20 min) to denature the membrane. The thermal treatment, however, reduces the purity of the juice due to the thermal destruction of cell wall components and transformation of high-molecular-weight substances. In the protocol used by Estiaghi and Knorr (2002) to investigate sugar extraction using PEF, sugar beet slices (V-shaped, 5-7 cm in length) suspended in tap water (conductivity 0.75 mS/cm) were treated with PEF (2.4 kV/cm field strength and 20 pulses), and the slices were then mechanically pressed to either 2 and 5 MPa for 5 min or to 30 MPa for 15 min, and resuspended in distilled water. After repeating this cycle, the pressed pulp and raw juice were analyzed and the results compared with the same treatment cycle without PEF. The results suggest that the three-cycle protocol using 2 or 5 MPa yielded 97 % sugar extraction (expressed as relative gain) and the speed of the extraction was 2-3 times faster than with conventional thermal process (70-90 min). It was also reported that effect of PEF was less pronounced with decreasing in size of the slices and PEF-treated pressed pulp had 10 % less sugar content than untreated.

A similar test was conducted using sugar beet cossettes to investigate the effect of pressure during compression in combination with PEF on juice yield. (Bouzrara & Vorobiev, 2000). The protocol consisted of initial pressing for 20 min, followed by PEF treatment (1000 pulses of 1000 V with a duration of 100 μs and a period of 10 ms) and final pressing for 30 min. The pressing of the cossettes up to 30 bar resulted in a juice yield of 36.3 % at the first pressing stage and 44 % at the second pressing stage, whereas the yields at 5 bar were 27.5 and 51 % at the same stages.

Dörnenburg and Knorr (1993) applied PEF treatment up to 1.6 kV/cm and up to 30 pulses to cell cultures suspended in solution, and studied the PEF-induced release of intracellular pigments, namely amaranthin and anthraquinones, from *Chenopodium rubrum* and *Morinda citrifolia* cells, respectively. They reported that 85 % of the total amaranthin and 5.7 % of the anthraquinones were released into the culture medium following electrical treatment up to 1.6 kV/cm and up to 30 pulses, while cell viability was lost at release rates higher than 16 % for *C. rubrum* and 2 % for *M . citrifoli.*

Drying

In several other studies, PEF pretreatment has been considered for facilitating the drying process of fruits and vegetables. In a study on the effect of PEF on the dehydration characteristics of potato, the potato cubes (1×1×1cm) were pretreated with a field strength of 0.35-3 kV/cm and 1 to 70 pulses. Centrifugation (700 × g) of the PEF-treated samples resulted 29 % higher cell liquid release than control. Also, the PEF treatment with optimum parameters (E = 0.9-2 kV/cm, number of pulses = 1-15) was reported to improve the mass transfer within the product and shorten the drying time during fluidized bed drying (Angersbach and Knorr, 1997).

In a recent investigation, (Ade-Omowaye et al., 2001), the effect of PEF on the dehydration characteristics of red bell pepper slices was studied, and the result was compared with other forms of pretreatment. Drying was accomplished in fluidized bed dryer (at 60 ^{o}C for 6 h and an air flow of 1 m/s), and the various forms of pretreatment employed before drying were water blanching (100 ^{o}C, 3 min), skin treatment, PEF (2.4 kV/cm, pulse length: 300 μs, number of pulses: 10 with at a frequency of 1 Hz) and high pressure (400 MPa for 10 min at 25 ^{o}C). Significant effects on drying time during the falling rate period were reported following PEF treatment. The drying time during this period was reported to be approx. 220 min for PEF-treated samples, while it was 360 min for untreated samples and 290-295 after blanching.

Osmotic dehydration

PEF treatment has also been suggested as a method of pretreatment to accelerate osmotic dehydration. Rastogi, Eshtiaghi, & Knorr (1999) studied the effect of PEF on the dehydration of carrot cubes and reported that PEF pretreatment (0.22 to 1.60 kV/cm) of carrot cubes resulted in a decrease in the moisture content during osmotic dehydration (immersion in a 50^{o} Brix sucrose solution at 40 ^{o}C for 5 h). The energy expenditure during the PEF treatment was in the range of 0.04 to 2.25 kJ/kg and the corresponding increase in cell disintegration index was in the range of 0.09 to 0.84 with < 1 °C rise in the product temperature. The effective diffusion coefficients of water and solute, which were determined using a Fickian diffusion model, were reported to increase exponentially with electric field strength. Similarly, in another report, water loss from PEF (1.4 kV/cm, 20 pulses of 800 μs) pretreated apple slices was found to be significantly higher than that of untreated apple during an immersion time of 6 h (Taiwo, et al., 2001). In a later report, (Taiwo, Angersbach, & D. Knorr, 2002) dealing with the influence of PEF and osmotic dehydration on the rehydration characteristics of apple slices, it was reported that the combination of PEF with osmotic dehydration (as above) improved the rehydration capacity of apple slices by decreasing the rehydration time at temperatures of 20, 45, 60 and 90 ^{o}C.

METHODOLOGICAL CONSIDERATIONS

The effects of a PEF on plant tissue were studied by visualizing different cell regions, and observing the PEF-induced changes under a stereomicroscope. (***Paper I***). The methodology followed to achieve this was based on staining of the cell regions using one cell thick plant tissue (onion epidermis). The pH of different cell regions, namely the cell wall, cytoplasm and vacuole differs, the vacuole being the most acidic (about pH of 5-6) (Mauseth, 1988). A pH sensitive stain, Neutral Red (0.02 g/ml) (NR) was used to stain these regions. NR changes color from red to yellow over the pH range 6.8-8.0. The pH is, however, a critical factor for the transfer of NR through the cell membrane as it is in the ionic form below and above this pH range (Singh, Pal, & Sapre, 1999). At around pH 7, NR is mostly neutral (not ionic). Membranes, i.e. the plasmalemma and tonoplast, do not constitute a barrier to NR in its neutral form, and allow the passage of NR into the cytoplasm and vacuole (Ehara, Noguchi, & Ueda, 1996). NR molecules are not particularly stable at pH 7, due to reactions resulting in a pH drop, precipitation occurs and hence a change in the form of NR from neutral to ionic (Baker, 1999). This difficulty was overcome in our experiments by preparation of the NR solution immediately before dye infusion. Freshly prepared NR solution was obtained by mixing NR stock (pH =6.8-7.0) and dilution solutions (pH=7.8) in equal proportions. NR was also dissolved in 0.33 M mannitol in order to avoid any osmotic-related changes in plant cell shape. Differences in hydrophobic properties between the upper and lower surface of the epidermis are also important for infusion of NR into the cells. The NR solution was applied to the cuticle-free surface as this surface allows the passage of hydrophilic NR into the tissue.

The amount of staining was influenced by:

-the pH of the NR solution (*at neutral pH, NR stained the intracellular region, at acidic pH the extracellular matrix*)

-the concentration of NR (*with a higher concentration of NR, the intensity of red color increased*)

-the application of the stain to the hydrophilic or the hydrophobic surface of the epidermis layer (*staining was unsuccessful through the surface covered with a cuticle layer due to hydrophobic nature of the cuticle*)

When placing the epidermis on electrodes, the stained surface was oriented so as to face against the top surface of electrode (Figure 3). A wetting solution was also used between the surfaces of the tissue and the electrodes in order to establish sufficient contact during PEF treatment and conductivity measurements. The wetting solution in our experiment was prepared from distilled water, and the pH was adjusted to 6.72 with HEPES buffer and the osmolarity to 0.33 M with mannitol.

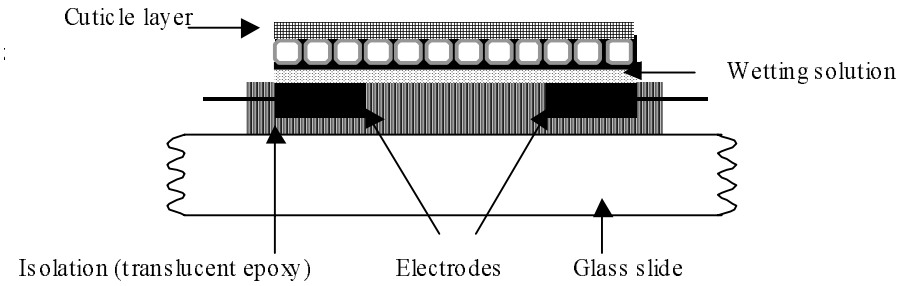

Figure 3. *Cross section of treatment chamber and onion epidermis sample, not to scale.*

Top view images of the tissue placed in the treatment chamber were captured every minute and the conductivity was measured for 20 min using the set-up described in the section, materials and methods in *Paper I*. PEF was applied after 5 min to give a sufficient time to observe the state of cells before and after PEF treatment. To observe the effect of PEF at different field strengths, from low to high, the field strength of the treatment was varied from 0.17 to 0.52 kV/cm using a single monopolar rectangular pulse of 100 μs duration. It is already well established that the effect of short pulses on permeabilization is more pronounced than that of longer pulses.

The effect of PEF on permeabilization appeared as a color shift from red to yellow and a sudden rise in conductivity (Figures 8 and 9).

The permeabilization and/or internal mixing using this method were also observed to be influenced by:

-the conductivity of the wetting solution under the tissue (*at the same electrical conditions, permeabilization was much more pronounced with a poorly conducting medium than a highly conducting medium*)

-pH of the wetting solution (*the intensity of the yellow color after PEF treatment was proportional to the pH of the wetting solution, see also the major findings in results and discussion*)

-the osmolarity of the wetting solution (*due to cell shrinkage upon exposure of the tissue to a hypertonic medium for a long time, the intensity of red color appeared to increase and vice versa*)

-the use of hydrophilic or hydrophobic surface of epidermis layer (*in the reported experiment, it was always cuticle free surface of the epidermis faced the electrode*)

Due to this characteristic color change, the images of the samples were also used in image analysis with the aim of obtaining information about internal mass transport after permeabilization. When the images before and after permeabilization were examined, it was shown that the green component of the RGB signal carried the most information, and was higher in the intracellular region of the permeabilized cell than in the intact region and was lower in the extracellular region of PEF-treated tissue than in intact tissue (Figure 4).

To study the kinetics of the events, differences between successive images were extracted using Matrix software. To separate the changes in the extracellular region from those in the intracellular region, the increase in intensity (intracellular region) and decrease in intensity (extracellular region) were evaluated separately. Difference images were formed by subtracting the image at the (n+1) th min from the image at n min or vice versa. For subtraction, " subtract with saturate " function in the Matrox software was used, which

automatically sets resulting negative values to zero. (*see also the section, materials and methods in Paper I*).

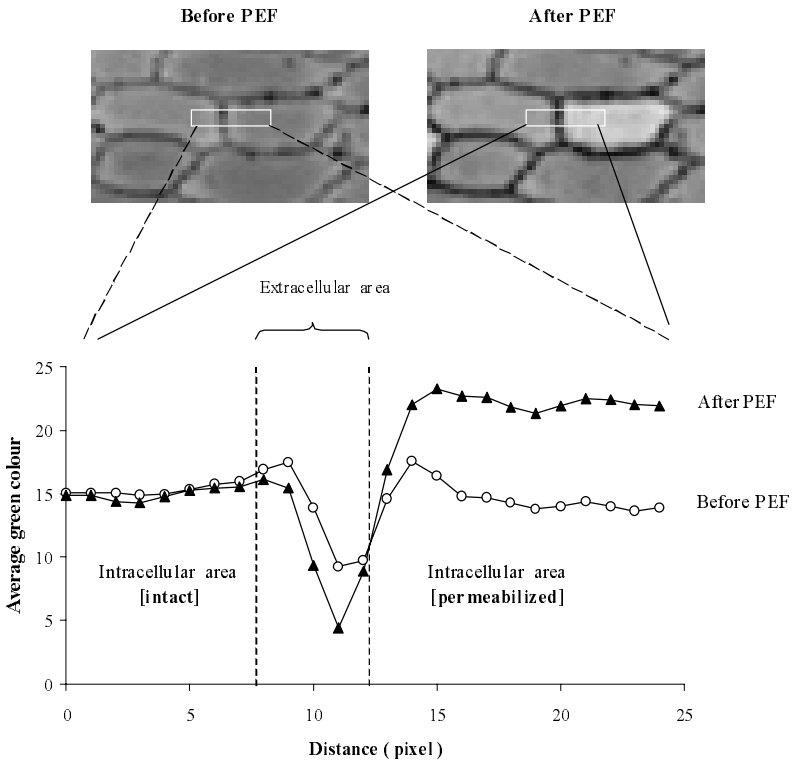

Figure 4. *Change in RGB green color in the cell images following PEF* (*0.35 kV/cm*).

The effects of PEF with and without osmotic treatment on the viscoelastic properties of potato tissue (*Solanum tuberosum*) were also studied (***Paper II***). The viscoelastic properties of tissues depend on turgor, and the properties of the cell wall and middle lamella (Warner, Thiel, & Donald, 2000; Scanlon, Pang, & Biliaderis, 1996). The time-dependent or viscoelastic behavior of soft tissue can be attributed to a variety of mechanisms:

-Flow of tissue into and out of the fibrous network in a response to applied loads or deformations.

-Time-dependent behavior of particular components (such as fibers, cell and wall matrix).

-Time-dependent response of the network as connections separate and re-attach.

Some rheological models represent the behavior of viscoelastic materials, as a combination of ideal elastic and viscous behavior. In these models, a spring and a dashpot represent the ideal elastic and viscous material behavior. Hook's law assumes that rate of loading of a spring does not affect the strain-stress relationship:

$$\sigma = E\varepsilon \qquad \text{Eq. (11)}$$

while a viscous device, such as a piston in an oil, provides a constant strain rate at a given stress:

$$\sigma = \mu\left(\frac{d\varepsilon}{dt}\right) \qquad \text{Eq. (12)}$$

where σ is the stress, E is the modulus of elasticity, ε is the strain and μ is the viscosity. Common viscoelastic models combine these two components in the best possible network to describe the viscoelastic behavior of the material or to predict the time-dependent behavior.

The relaxation test is one of the primary tests applied to biological tissues for determination of viscoelastic properties (Peleg and Calzada, 1976). The material is deformed to a fixed strain and the strain kept constant. The observed stress caused by this strain decreases with time. The Maxwell model can describe the complex time-dependent behavior of the material by a parallel arrangement of the springs and dashpots in the network (Rao and Steffe, 1992). The appropriate technique for solving the Maxwell equation for the stress versus strain relationship depends on the network formulations and the boundary conditions imposed.

In our experiment, potato tissue slices, 1 mm in thickness and 4 mm in diameter, taken from the cortex region, after a washing step to remove the conductive debris, were subjected to PEF with and without osmotic treatment. Before PEF treatment, some of the samples were exposed to hypo-, iso-and hyperosmotic solutions using distilled water and mannitol. The PEF treatment included a wide range of electrical conditions, i.e. a three-level factorial design of field strength: 0.5, 1.0, 1.5 kV/cm; the pulse length: 10, 100, 1000 μs: and pulse number: 1, 9, 90, with an interpulse delay of 10 ms. The pulse shape was rectangular monopolar throughout

the all experiments described in this thesis. The PEF treatment of all samples was conducted with a parallel stainless steel electrodes with a gap distance of 1 mm and the conductivity after PEF were measured with a vector impedance meter.

To evaluate the effect of PEF and osmotic treatment on viscoelastic behavior, the samples, were subjected to a uniaxial compression in an Instron, universal-testing machine (Model 4400, USA), after loading at a constant deformation rate. The compression was applied from the top cylindrical surface at 10 mm/min up to a depth of 0.3 mm and stress of 0.4-0.45 MPa. Stresses during the loading period and stress relaxation over 20 sec were recorded with 0.1 s resolution.

Typical data obtained during the stress relaxation period are shown in Figure 5 for different numbers of PEF pulses and for different osmotic treatments. The results for raw potato agree with the results of Peleg and Calzada (1976). The relaxation behavior was almost identical to the behavior in 0.25-0.30 M mannitol, which is an isotonic concentration for potato tuber tissue (Lin and Pitt, 1986).

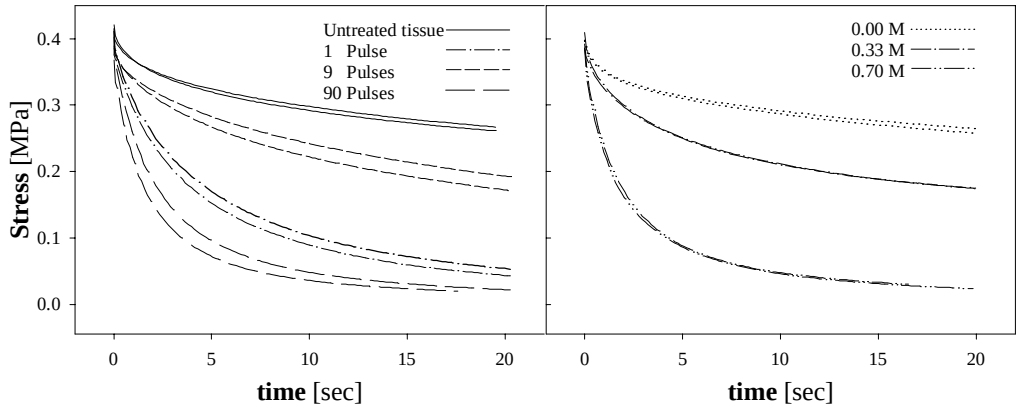

Figure 5. *Stress relaxation of a potato slice subjected to a) a different number of pulses at field strength of 1 kV/cm and pulse length of 100μs and b) different osmotic treatments. Two replicates are shown for each treatment.*

For loading at finite deformation rate, the generalized Maxwell model predicted by De Baerdemaeker & Segerlind (1976) can be expressed as follows.

$$\sigma(t) = E_0 \varepsilon_{max} + \dot{\varepsilon} \sum_{i=1}^{n} \frac{E_i}{\alpha_i} \left(1 - e^{-\varepsilon_{max}/\alpha_i \dot{\varepsilon}} \right) e^{-t/\alpha_i} \qquad \text{Eq. (13)}$$

A model with two Maxwell elements and a residual spring for a total of five parameters could represent our relaxation data with a correlation coefficient of 98 %.

$$\sigma(t) = E_0\, \varepsilon_{max} + \dot{\varepsilon} \left[E_1\, t_1 \left(1 - e^{-\varepsilon_{max}/\dot{\varepsilon} t_1} \right) e^{-t/t_1} + E_2\, t_2 \left(1 - e^{-\varepsilon_{max}/\dot{\varepsilon} t_2} \right) e^{-t/t_2} \right] \qquad \text{Eq. (14)}$$

where $\dot{\varepsilon}$ is deformation rate, ε_{max} is the maximum deformation, σ is the stress, t_1 and t_2 the short and long characteristic relaxation times and E_0, E_1 and E_2 residual, short and long relaxation moduli.

The calculations of these viscoelastic parameters (E_0, E_1, E_2, t_1, t_2) were performed with Table Curve software (Table Curve 2D Windows, v4.07, SPSS Inc., AISN Software Inc., Chicago, USA), and the correlations between the viscoelastic coefficients and PEF and osmotic treatment are presented and discussed in the results and discussion sections of this thesis and in *Paper II*.

In this part of the study, the extractability of a commercially interesting intracellular compound from plant tissue was tested with use of PEF treatment ***(Paper III)***. Extraction of the intracellular compounds and liquids from cellular plant tissue can be achieved using various solid-liquid extraction methods such as comminution, sometimes combined with mechanical expression, heating or freezing. The choice of method often takes into consideration the cost and extraction efficiency factor and whether or not the processing has a deleterious effect on the product of interest. One of the factors influencing the efficiency of the extraction process is the degree of cell membrane disintegration, which frees the intracellular compounds so that they can migrate into the external medium.

The main purpose of the study undertaken here was to investigate the PEF-induced extractability of red beetroot pigment in the solid-liquid extraction process. Red beetroot (*Beta vulgaris*) cells contain considerable amounts of red pigment, betanine, located mainly in

the vacuoles (Leigh et al., 1979). The red beet juice mainly takes up its color from this pigment (Kujala et al., 2000; 2001; 2002) Extraction of the beetroot juice is also of commercial interest due to its use as a universally accepted food colorant (E162) and antioxidant. Conventional methods of extracting the red beetroot concentrate generally employ mechanical pressing of the tissue or aqueous extraction of shredded beetroot.

In this investigation, discs of beetroot tissue were initially subjected to PEF treatment. The sample size, washing procedure, treatment chamber, pulse generator and vector impedance meter used were the same as in the study described in *Paper II*. In the application of PEF, the intensity of the treatment was gradually increased by varying the number of short pulses (10 μs) from 27 to 270 at a field strength of 1 kV/cm. The energy consumption of the treatment was calculated (*see the section, material and methods in Paper III)*. Before and after PEF, tissue conductivity was also measured using the vector impedance meter and PC function generator in the set-up (*Figure 1, Paper III*) in order to determine the degree of damage caused by the PEF treatment. Using the ohmic and complex conductivity data obtained, the degree of damages induced by PEF treatment was defined as:

$$\text{Relative complex conductivity} = \frac{KX'}{KX} \qquad \text{Eq. (15)}$$

$$\text{Relative increase in tissue conductivity} = \frac{(K'_{15} - K_{15})}{(K'_{15f} - K_{15})} \qquad \text{Eq. (16)}$$

where KX and KX' are the complex conductance (Ω^{-1}) before and after PEF treatment, respectively K_{15} and K'_{15} are the ohmic conductivity (mS/cm) of the samples at 5 kHz before and after PEF treatment and K'_{15f} is the average ohmic conductivity (mS/cm) of the samples obtained following the application of 270 pulses at 3.1 mS/cm. The degree of damage caused by PEF in terms of complex conductivity over a frequency range is shown in Figure 6.

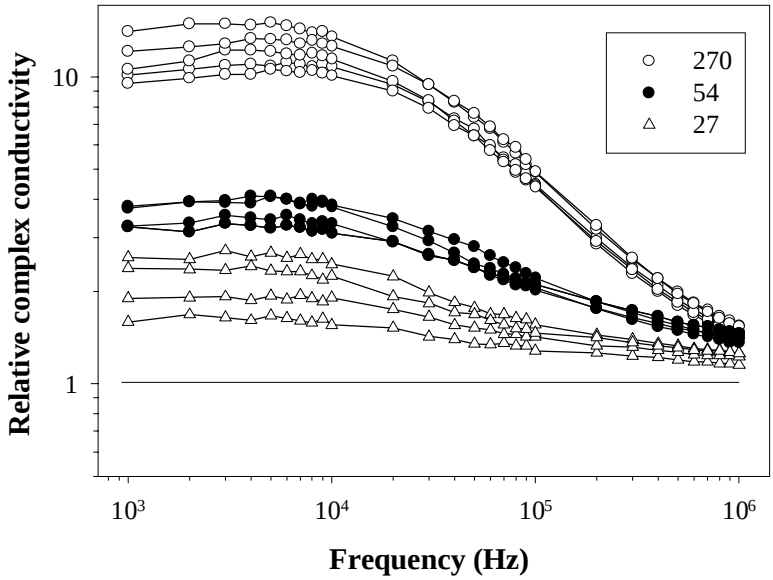

Figure 6. *Change in relative complex conductivity of the PEF-treated samples versus frequency* (*log scale*) *with the indicated number of pulses. Replicate experiments are shown.*

Another purpose of this study was to investigate the relation between PEF-induced ohmic tissue conductivity increase and the extraction efficiency.

After characterizing the tissue damage by PEF in terms of conductivity, the treated samples were used in the solid-liquid extraction process. The samples were introduced into an isotonic solution (0.25 M Mannitol) which was circulated in the set-up shown in Figure 7, and the release of red pigment and ionic species was recorded at every 10 s for 60 min using the conductivity meter and the spectrophotometer. The absorbance in the spectrophotometric measurements was measured at 530 nm, i.e. corresponding to the maximum wavelength of betanine. Following this on-line monitoring, the extraction continued in a shaker with data sampling every 10 min for about 3 h.

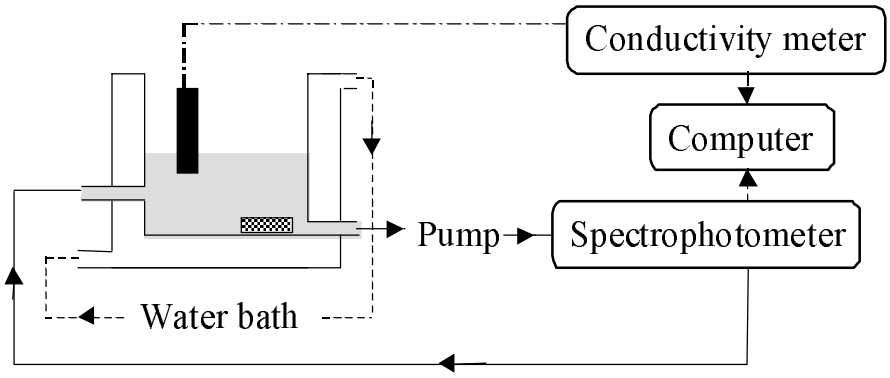

Figure 7. *Experimental set-up for extraction kinetics measurements.*

The kinetics of the release were described as follows:

$$\text{Relative solution absorbance} = \frac{ABS_t}{ABS_{AF}} \qquad \text{Eq. (17)}$$

$$\text{Relative solution conductivity} = \frac{K_t}{K_{AF}} \qquad \text{Eq. (18)}$$

where ABS_t and ABS_{AF} are the absorbance (nm) at time t and after freezing/thawing, and K_t and K_{AF} are the conductivity (mS/cm) at time t and after freezing/thawing, respectively (with the mannitol solution blank subtracted). During and after the extraction step, the cross-section of the samples was also examined for traces of red color, and images were recorded.

Freezing-thawing and mechanical pressing are well-established methods which can lead to full permeabilization. The degree of extraction achieved by PEF was thus compared with these methods using the untreated, frozen-thawed, mechanically pressed samples in the same procedure as described above. The freezing treatment was conducted by storing the samples at -24 ^{o}C for 4-5 days, and the mechanical pressing of the tissues was performed by placing the tissue under a finely corrugated, rolling cylinder. Traces of color were examined in the same way as above to ensure that the treatments had led to almost 100 % permeabilization.

As a continuation of previous work, the kinetics of the release of red pigment and conductive matter from PEF-treated red beet tissue slices into isotonic medium was studied using a two-dimensional bimodal Fickian diffusion model (***Paper IV***). The rate of mass transfer during extraction is of interest for process design. Diffusion of solutes from plant tissue into the extraction liquor also depends on the structure of the plant tissue. This type of diffusion, usually called apparent or effective diffusion, can often be modeled by Fick's second law of diffusion.

The purpose was to determine the diffusion coefficient of the solute in the solid by observing the change in solute concentration in the surrounding liquid with time. The mass transfer from the solid phase to the liquid phase is assumed to be dominated by the internal resistance, i.e. the resistance due to the liquid boundary layer adjacent to the surface was neglected.

For a finite cylindrical geometry, the analytical solution can be determined by making use of the superposition principle, as the product of the infinite slab solution and the infinite cylinder solution (Crank, 1975; Carslaw and Jaeger, 1959). The concentration of the solute (C) at any point in the cylinder can then be determined by solving Eq. (19):

$$\frac{C}{C_0} = \Phi(x,l)\Psi(r,a) \qquad \text{Eq. (19)}$$

where C_0 is the initial concentration of the solute, Φ is the solution of Fick's second law for an infinite slab with a thickness of 2 l assuming that diffusion takes place in the axial direction, and Ψ is the solution for a long cylinder with a diameter of 2a assuming that diffusion takes place in the radial direction. Solution of both equations, Φ and Ψ includes apparent diffusion coefficients (*see also the section, material and methods in paper IV*).

The concentration in the external solution can be evaluated by using mass balance:

$$C_0 V_{\text{int}} - C_{\text{int},ave} V_{\text{int}} = C_{ext} V_{ext} \qquad \text{Eq. (20)}$$

$$C_{ext} = A(1-f) \qquad \text{Eq. (21)}$$

where $C_{\text{int},ave}$ is the average concentration in the cylinder, C_{ext} is the concentration in the external solution, V_{int} is the volume of the cylinder, V_{ext} is the volume of the external solution, A is C_0 (V_{int} / V_{ext}) and f is the average superposed concentration in the cylinder. For $C_{\text{int},ave}$, the series analytical solution of the finite cylinder problem, Eq. (19) was evaluated on a radial $a/10$ and axial $l/10$ grid using a Matlab subroutine, and the average concentrations at each of the 100 grid points were calculated.

It was shown in our previous work (*Paper I*) that the PEF-induced permeabilization of tissue is not uniform. A simplified model taking this non-uniformity into account is bimodal diffusion (Jason and Peters, 1973). The model assumes the tissue to be an interconnected system of two tissues, each with its own concentration and corresponding diffusion coefficient, and transport occurs along parallel but separate paths, with no interaction. If both tissues have the same initial solute concentration, and H is denoted as the relative fraction of tissue type 1, it follows from mass balance that the observed concentration in Eq. (21) must be modified to:

$$C_{ext} = C(H(1-f_1)+(1-H)(1-f_2)) \qquad \text{Eq. (22)}$$

where f_1 and f_2 are the average calculated concentrations in tissues 1 and 2, corresponding to the diffusion coefficients $D_{app,1}$ and $D_{app,2}$, respectively.

To calculate the diffusion coefficients, least-squares fitting of Eq. (22) to the observed external solution concentrations as a function of time was performed. Four-parameter fitting was carried out in order to find C, H, $D_{app,\,1}$, corresponding to f_1, and $D_{spp,\,2}$, corresponding to f_2. Note that C is the maximum amount extracted for the individual samples and H is the fractional amount extracted corresponding to $D_{app,1}$.

RESULTS AND DISCUSSION

Visualization of the plant tissue permeabilization induced by PEF, and internal mass transport

In studies investigating the effect of a pulsed electric field on plant tissue, several methods are used to demonstrate the occurrence of cell permeabilization; most of these methods are indirect, providing only limited information on overall changes inside the tissue. In this work, a new method was developed to visualize permeabilization and the changes followed *in situ*. This was achieved by: 1) using a pH-sensitive stain, which could distinguish intra- and extra-cellular regions, and the vacuole, 2) using a one cell thick samples of plant tissue, onion epidermis, which avoided the problem of focus depth.

Under the standardized conditions described in section, *methodological considerations*, the permeabilization of the membrane in this method could be observed as a color shift of the vacuole as the vacuole contents mixed with the higher pH cytoplasm and the wetting solution through the pores resulting from PEF (Figures 8 and 9).

Detailed studies, including image analysis provided the following major findings:

-Permeabilization started to occur at a level of one pulse of 0.35 kV/cm (Figure 8).

-At one pulse of 0.5 kV/cm, full tissue permeabilization was observed (Figure 8).

-Permeabilization of cells, and the leakage of internal cell content appear to occur asymmetric i.e., from one side of the cell facing the electrodes (Figure 9) (*see also the discussion in section, Future perspectives*).

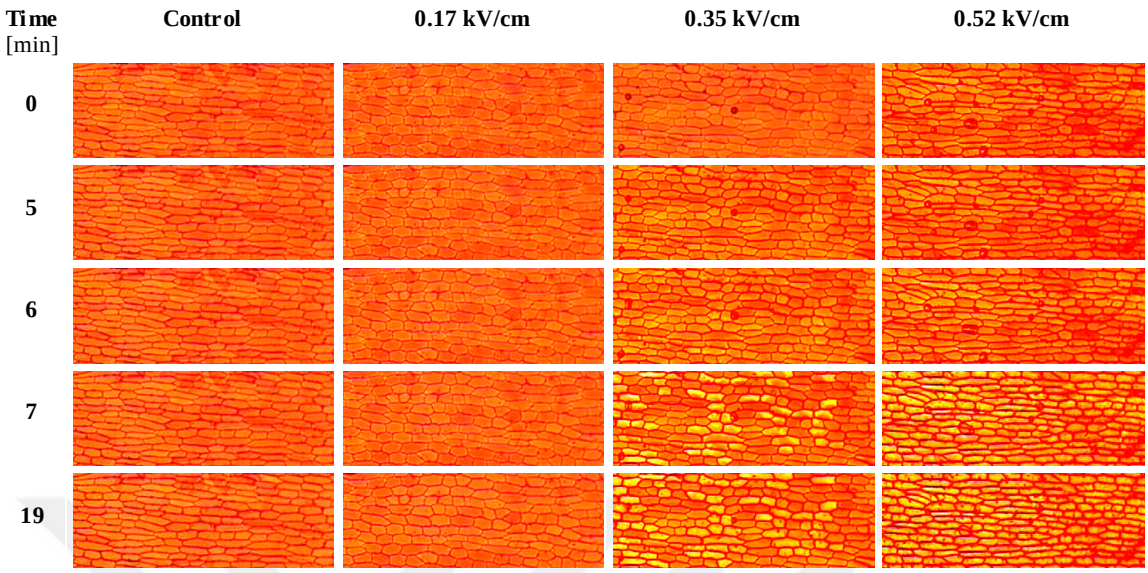

Figure 8. *Raw images of onion epidermis at 0, 5, 6, and 19 min. The image labeled 5 min corresponds to 5 s after PEF treatment* (*Top view, the positive electrode is just above, the negative just below the observed region*).

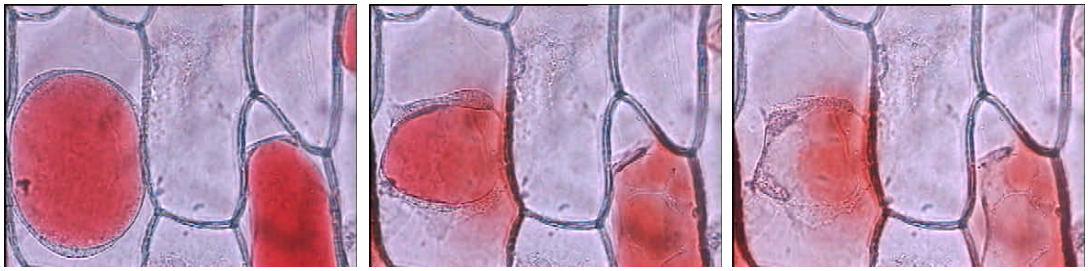

Figure 9. *Permeabilization of a single NR-stained cell with PEF, and the leakage of internal content. a) Before PEF treatment b) & c) 17 s and 77 s after the PEF treatment. Unpublished data, ã 2003, Mustafa Fincan.*

-The timescale of internal mass transport after permeabilization was found to be 2 min (Figure 10), (the rate of PEF-induced changes in samples subjected to 0.35 and 0.52 kV/cm was low nearly 60 s after PEF (*images labeled as "6-5"*), but more pronounced between 60 and 120 s (*images "7-6"*)).

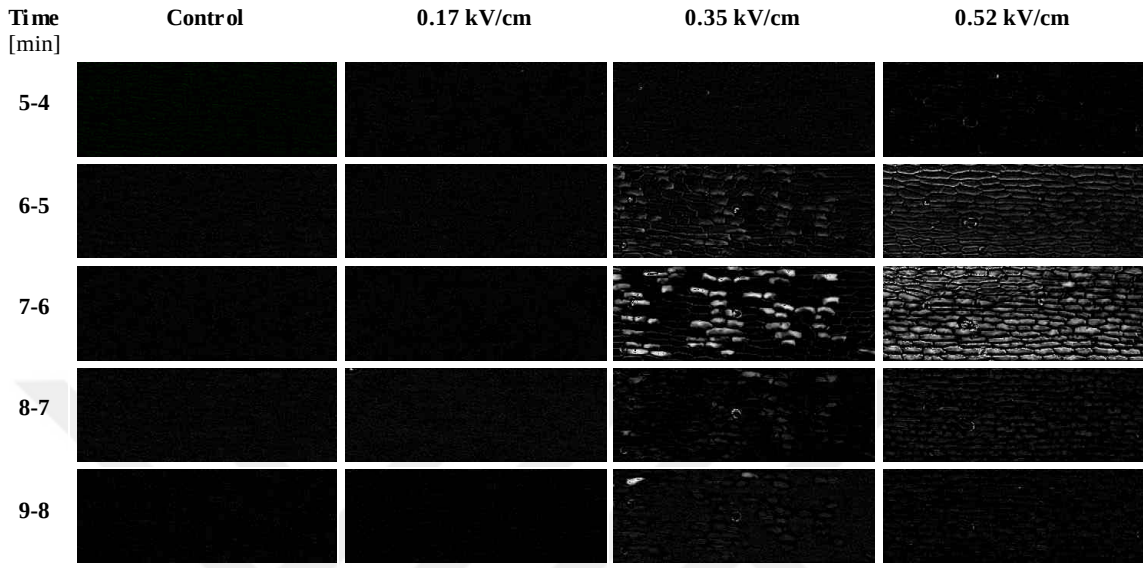

Figure 10. *Difference images of the green color component showing the rate of change in the intracellular region.*

Effect of PEF with and without osmotic pretreatment on the viscoelastic properties of plant tissue

The stress relaxation behavior of potato subjected to uniaxial compression at constant deformation rate could be represented by a model with two Maxwell elements and a residual spring for a total of five parameters with a correlation coefficient of 98 % (*see methodological consideration*). Pulsed electric field treatment led to significant changes in the coefficients of the model, which are used to interpret the viscoelastic behavior of a material. In Figure 11, the changes in viscoelastic coefficients are shown versus the change in conductivity, which reflects the intensities of PEF treatment.

Electric conductivity is a widely used indicator of PEF-induced damage to cellular tissue. When changes in the viscoelastic coefficients were compared with those in electrical conductivity, the most pronounced change was the decrease in residual modulus, which

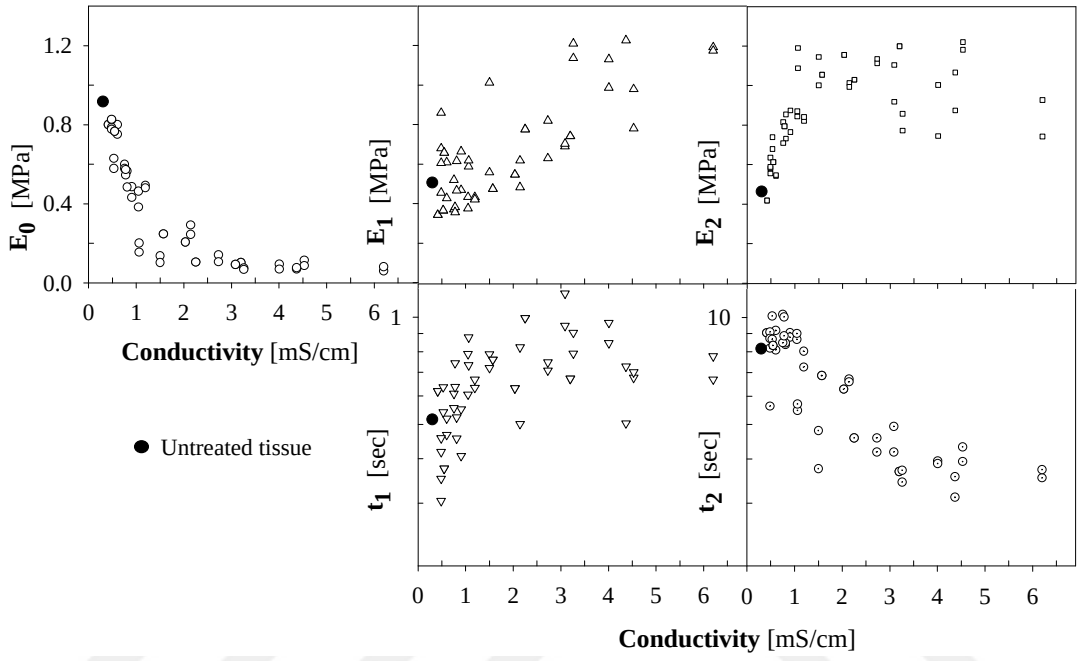

Figure 11. *Relationship between viscoelastic coefficients and conductivity in response to different intensities of the electrical treatment.*

occurred at relatively low treatment levels and was paralleled, by a decrease in the long-term relaxation modulus. PEF treatment may cause loss of cell liquid (turgor) which is the primary cause of plant tissue stiffness (Warner, Thiel, & Donald, 2000), therefore, residual stiffness E_0, decreases with the level of treatment. More intense PEF treatment causes larger pores in the cell membrane and thus allows a faster escape of cell liquid content and thus faster relaxation. The time constants gradually converge over the range of treatment intensities to a level of 1-2 s; i.e., the longer timescale component of relaxation, t_2, virtually disappears. We assume that the short timescale behavior is not related to the PEF treatment, but is caused by cell damage induced by compression.

All of the field gradients used were well beyond the accepted permeabilization threshold of 0.15-0.2 kV/cm membrane (Angersbach, Heinz, & Knorr, 2000); but in spite of this, effects

increased with increasing voltage gradient. A plot of the viscoelastic coefficients vs. the total exposure time of the tissue to the electric field is given in *Paper II*. Treatment effects correlated fairly well with the total length of the train of pulses at each level of electrical field. Multiple short pulses tended to be more efficient than a smaller number of longer pulses of the same total length in affecting the residual modulus (P=0.002) and the long characteristic relaxation time (P=0.000).

Exposing potato tissue to hypoosmotic or hyperosmotic pretreatment before electric field treatment did not appear to lead to significant interaction effects (Figure 12).

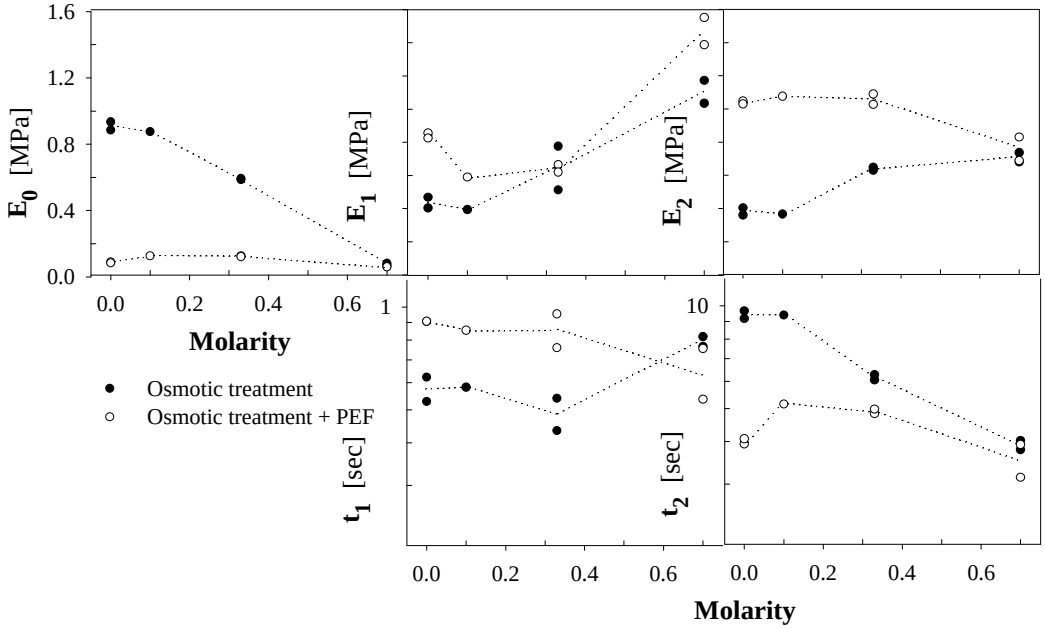

Figure 12. *Changes in viscoelastic constants of osmotically treated potato before and after PEF vs. mannitol concentration* (*PEF: 1 kV/cm, 9 pulses of 100 ms pulse length*). *The dotted lines are drawn to guide the eye.*

At treatment solution molarities up to 0.4 M, the effect was dominated by the PEF treatment. For a 0.7 M osmotic pretreatment and the intensity of the PEF treatment applied, there was no significant difference between the viscoelastic properties of tissue treated osmotically only and tissue treated osmotically in combination with PEF.

Effect of PEF on plant tissue during solid-liquid extraction

After treatment of red beetroot tissue with PEF, a significant increase in the release of red pigment and ionic species during solid-liquid extraction process was observed (Figure 13). In contrast to untreated sample and frozen and thawed tissue, the increase in the extraction efficiency was paralleled by the intensity of PEF treatment. The increase in the release of pigment also suggests the disintegration of the vacuolar membrane, the tonoplast, which freed the betanine out of the cell, making it able to diffuse into the external medium.

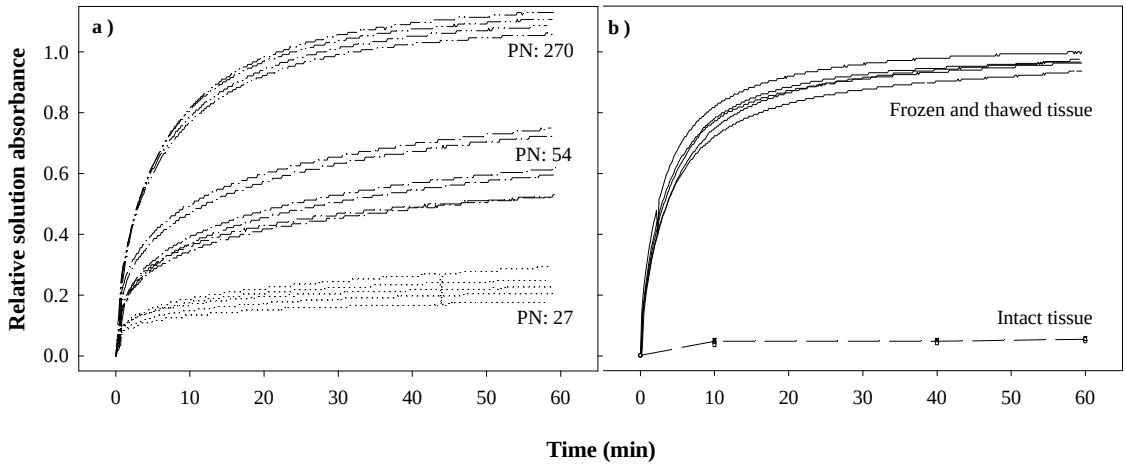

Figure 13. *Kinetics of red pigment extraction. a) PEF treatment with the indicated number of pulses and b) freezing/thawing. Replicate experiments are shown.*

The extent of extraction

When the extent of extraction achieved by pretreating the tissue with PEF, PEF+freezing/thawing and mechanical pressing prior to extraction were compared, the treatment of mechanical pressing led to highest release of red pigment (absorbance = 0.156 ± 0.011 (mean ± SD)) (Figure 14). Examination of the mechanically pressed sample flakes under a light microscope indicated that no traces of red color remained, confirming that the degree of cell disintegration by mechanical pressing was nearby 100 %.

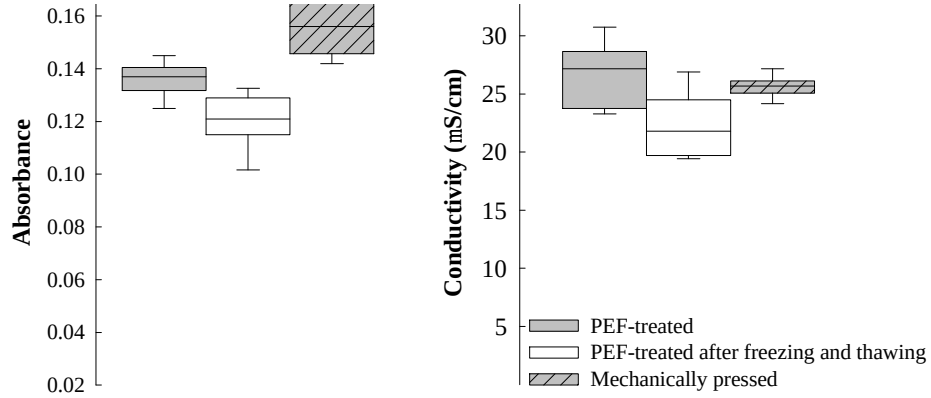

Figure 14. *The absorbance and conductivity after 1 hour of aqueous extraction following PEF treatment* (*270 pulses*), *PEF treatment followed by freezing/thawing and mechanical pressing*.

When the extent of extraction following different PEF treatments were compared with that by mechanical pressing, it was found that PEF treatment at maximum intensity, i.e. 270 pulses of 10 μ at 1 kV/cm extracted almost 90 % of the red pigment (ABS= 0.138±0.008) during 1 h of solid-liquid extraction. This was qualitatively confirmed by microscopy analysis (Figure 15). Comparing the size of the colored domains in the right panel with those in the left panel, it appears that, in agreement with theory (Zimmermann, 1986; Ho & Mittal, 1996), the smaller cells were not permeabilized at the PEF conditions employed.

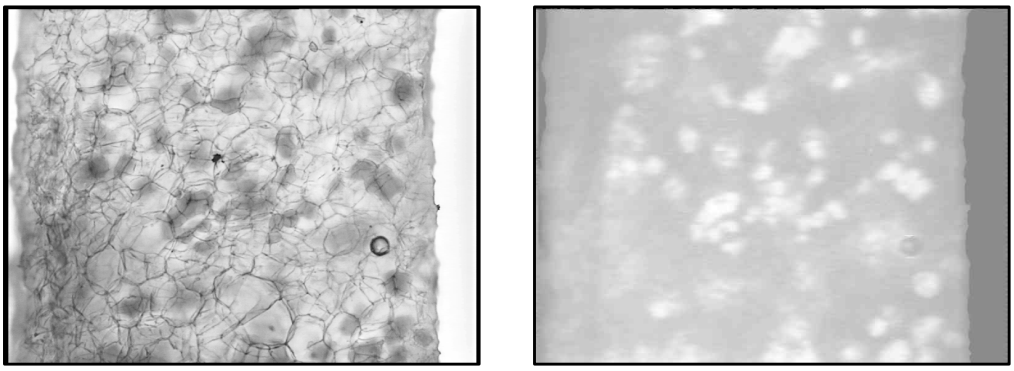

Figure 15. *Cross-section of a PEF-treated sample* (*270 pulses*) *after extraction. Left panel lightness component; right panel hue component; matching dark areas in the right panel and light areas in the left panel correspond to intact cells containing red beetroot color* (*Cell walls were stained with tryptophan blue*).

Our supplementary experiments on freezing of beetroot juice indicted a 10 % loss of color and conductivity, on average, due to the freezing/thawing cycle. Thus, the values exceed 1 in Figures 13, 16 and 17 because absorbance and conductivity of the solution were expressed relative to the values obtained after freezing/thawing. The (non-significant) trend of higher conductivity of PEF-treated samples, and the significantly larger variance (P=0.54) may be related to the electrolysis between electrode and tissue surface at the relatively high total charges transferred.

The relationship between pigment and ionic release as a function of treatment intensity is shown in Figure 16. The fitted linear relationship did not pass through zero. This could be interpreted as extraction of ionic species from the apoplast with no concomitant extraction of the intercellular betanine. Beyond this initial level, the amount of pigment extracted was found to be directly proportional to the release of ionic species, indicating that at the conditions studied there was no differential permeabilization of any of the intracellular compartments. The higher degree of variation of the extractability of pigment and the conductivity observed with intermediate PEF treatment was interpreted as being related to the non-homogenous permeabilization of the cells in tissue due, for example, to variations in cell size.

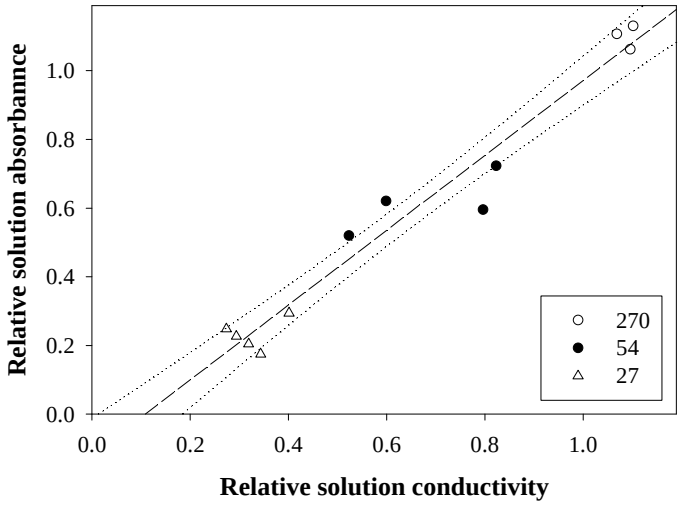

Figure 16. *Absorbance of the solution versus conductivity of the solution after 1 h aqueous extraction following PEF treatment with the indicated number of pulses* (*dashed line: linear fit, dotted line: 95 % confidence interval*).

Conductivity-based tissue damage index as a predictor of the extent of extraction

The changes in beetroot tissue conductivity after PEF were found to be correlated with the changes in both pigment and ionic release in aqueous extraction (Figure 17). However, the relationship was not linear over the whole tissue conductivity range. The data suggest that the degree of extraction is not markedly increased for index values higher than 0.4-0.5. Why this should be so is not obvious. Theoretically, additional electrical pulses will not significantly affect cells or organelles that have already been ruptured, because no potential gradient is developed over their membranes, and therefore if PEF treatment increases tissue conductivity it should lead to concomitant increase of extractability.

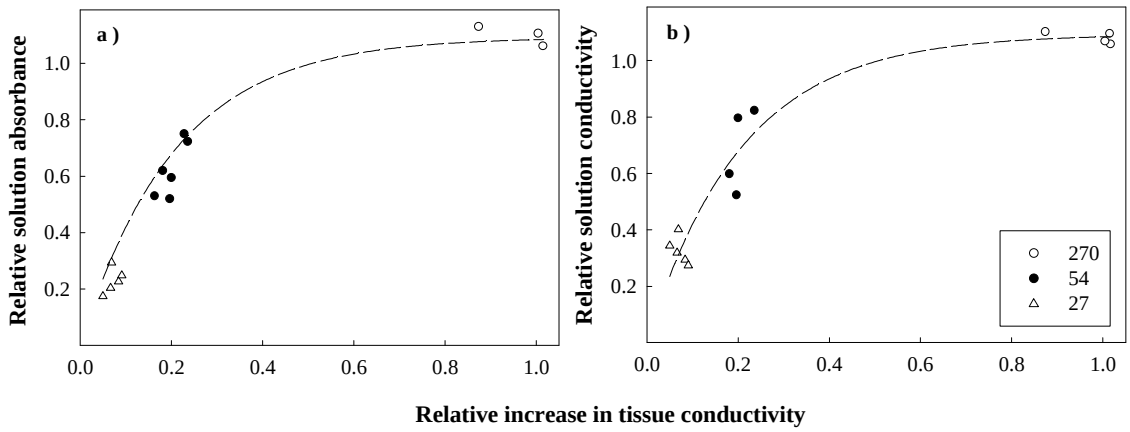

Figure 17. *a) Absorbance and b) conductivity of the solution at the end of 1 hour of aqueous extraction versus relative change in tissue conductivity following PEF treatment with the indicated number of pulses* (*dashed line: exponential fit to guide the eye*).

Energy cost of effective PEF treatment

The energy consumption (Knorr & Angersbach, 1998; Barbosa-Cánovas, Swanson, & Zhang, 1995). of the treatments applied, based on the tissue resistance in Figures 1a and 2 in Paper III is calculated in Table 2. At the maximum treatment of 270 pulses, the energy corresponds to 7 kJ/kg, i.e. energy corresponding to heating of the tissue by 2 ^{o}C.

Field strength (kV/cm)	**PN**	**PL** (μs)	**Energy** (kJ/kg)
1.0	1	10	0.02
	27		0.67
	270		7.20

Table 2. *Approximate energy consumption during PEF treatment.*

Effects of a PEF on mass transfer: mathematical modeling of mass transfer

In our previous work (*Paper III*), the PEF-induced conductivity increase was defined as relative conductivity. In Figure 18 the diffusion coefficients ($D_{app,\,1}$ and $D_{app,\,2}$) calculated as described in Paper IV are plotted against the relative increase in tissue conductivity.

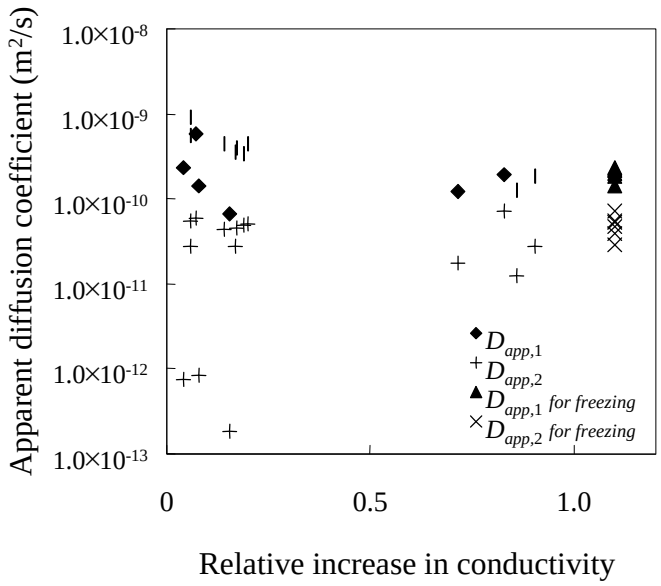

Figure 18. *The distribution of the apparent diffusion coefficients of red pigment over the range of PEF treatment applied in terms of relative increase in conductivity.*

It can be seen in Figure 18 that the increase in the intensity of PEF treatment does not affect the rate of mass transfer. This was observed in both the diffusion of red pigment and ionic species, whereas in the previous study (*Paper II*), the extraction yield increased with the intensity of the PEF treatment. The results of the simulation, however, gave us the fractional amounts of the species extracted by the fast and slow processes of diffusion (Figure 19).

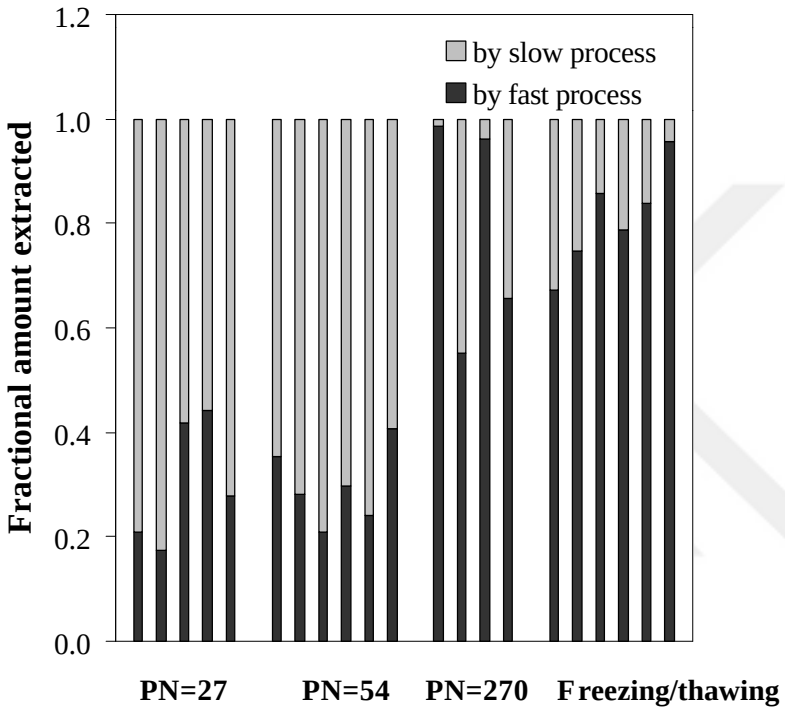

Figure 19. *The extractability of red pigment by the two different diffusion processes, fast and slow.*

These results indicated that total observed concentration in the external solution is the result of the two kinds of diffusion. Following short and moderate treatment (PN=27 and PN=54), most of the components were extracted by the slow process while the fast process played the major role in extraction following more intense treatment (PN=270 and when the tissue was frozen and then thawed.

Our results suggest that the level of PEF treatment determined the fraction of tissue that was permeabilized, and thus determined the yield. When the kinetics of extraction was described by bimodal diffusion, the fastest transport mode, corresponding to fully permeabilized tissue,

was often, but not always, observed following low treatment levels corresponding to a 5-15% increase in relative conductivity. The fraction of the total yield resulting from the fast transport increased with treatment duration. At maximum treatment duration and in frozen and thawed samples, only this fast mode was observed and the extraction was satisfactorily described by a single diffusion coefficient, with a hindrance factor of 2-5.

CONCLUSIONS

Pulsed electric field treatment was shown to be effective for permeabilization of plant tissue. By visualizing the PEF-induced changes at cell level, the method introduced in this study laid foundation to further investigations of the detailed consequences of PEF treatment in plant tissue. The method yielded these major findings. At partial tissue permeabilization, cells along the path connecting the electrodes were preferentially permeabilized. The timescale for completion of internal transport and mixing in permeabilized tissue was about 2 min.

In macroscale experiments, the PEF effects were studied on more complex plant tissue. It was first found that pulsed electric field treatment of potato tissue resulted in changes in the viscoelastic properties of the tissue. As these changes were shown to be of the same nature as those brought about by hyperosmotic treatment, PEF-induced mechanical property changes were interpreted as being due to loss of turgor. At lower treatment levels, the effect was seen as a lower residual modulus, while higher treatment levels further shortened the relaxation time. This could be interpreted as increase in cell membrane damage allowing faster escape of cell contents under stress. At all field strengths, the effects of treatment correlated reasonably well with the cumulative pulse time, but a large number of short pulses was found to be more efficient than few longer pulses of the same total on-time. Increasing field gradients led to significantly greater effects even at gradients far beyond the critical permeabilization level. Contrary to expectations, no synergistic effects were found between osmotic pretreatment and pulsed electric field treatment.

When the effect of PEF pretreatment on solid-liquid extraction of pigment from red beetroots was studied, it was demonstrated that PEF treatment level of 270 pulses at 1 kV/cm permeabilized the tissue effectively with a low energy consumption of about 7 kJ/kg, and increased the extraction efficiency to about 90 %. The treatment made both the pigment and ions in the tissue equally extractable, so electrical conductivity is a good measure of the progress of pigment extraction. The tissue damage index based on post-PEF tissue conductivity was linearly correlated with the extractability of the tissue only at relatively low levels of tissue damage.

Modification of the diffusive properties of the red beetroot tissue after permeabilization was also studied using mathematical modeling. The mass transfer from red beetroot permeabilized by a PEF prior to solid-liquid extraction can be described satisfactorily by bimodal Fickian diffusion. At high levels of permeabilization by PEF or freezing/thawing, a single mode is sufficient. Two processes of diffusion due to partial permeabilization allowed us to determined diffusion coefficients by fitting the model to experimental data. The diffusion coefficients were approximately constant at all levels of electrical treatment, but the contribution of the slower one decreased.

FUTURE PERSPECTIVES

The method presented in Paper I was found to be a useful tool. By *in situ* visualization of the plant cell and its components it can further be used to explore the role of electrical and medium-related conditions on permeabilization and internal mixing phenomena during the post permeabilization period.

Another indication of the method was that the PEF treatment of plant tissue in a poorly conducive leads to an asymmetric permeabilization (Figure 9). Asymmetric character of the permeabilization appears to contrast the mechanism claimed to act in dielectric breakdown model. Similar results have also been obtained by other researchers (Hibino, ltoh, & Knosita, 1993; Chang et al., 1992; Zimmerman, 1986). Neither factors inducing asymmetric permeabilization nor its mechanism are fully clear. In an earlier study (Zimmerman, 1986), uptake of fluorescence dye into plant protoplast was reported to be symmetric at a given conductivity medium but asymmetric below and above a conductivity range. On the other hand, when Knosita et al, (1992), and Hibino, ltoh and Knosita (1993) studied asymmetric behavior of membrane permeabilization by incorporating a voltage sensitive fluorescence dye into the membrane, he concluded that asymmetric permeabilization occurred when pulse length was varied from 0.5 μs to 1000 μs. The conductivity of the treatment medium (Ca^{+} .free sea water) was constant in this study,. This aspect of electropermeabilization calls clearly for further investigation.

It is also seen in Figure 9 that the red color intensity on side or cell wall imposed to this asymmetry increases. The increase in red intensity, reflected by the pH of neutral red, can partly be interpreted as being due to diffusion of acidic vacuole into less acidic cell wall region. The intensity of the red color in the cell wall region following permeabilization, however appeared to be higher than that of the color of vacuolar pH.

About studies attempting to employ PEF in processing, there is presently no common measure of the degree of permeabilization as a function of electrical and medium parameters. The effect of the medium conductivity on permeabilization or electrical parameters is often a less considered factor. Experiments on different conductive media in this study showed that lowering the conductivity of the treatment medium decreases the magnitude of the electrical treatment needed for tissue permeabilization. PEF treatment of a highly conductive medium is characterized by the passage of a large current across the electrodes, compared with less current in a low-conductive medium, consequently membrane permeabilization can be differently influenced under these two conditions. It was also observed in this study that the treatment of a highly conductive medium enhances undesirable electrolytic reactions between tissue and electrode. Decreasing in the tissue conductivity by washing, suspending it in a low-conductive medium or treatment to lower the cell wall matrix conductivity may facilitate the tissue permeabilization, lower the energy consumption and decrease the risk of electrolysis.

Reversible permeabilization of plant tissue with PEF has not yet been explored. To impregnate the plant tissue with substances, such as sugars, antioxidants, antifreezing agents etc via reversible permeabilization seems to be a promising research subject to develop new ghost fruits or products. Methods that can accelerate the mass transfer and slow down the resealing of pores can possibly be utilized in the achievement of this.

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NOTATION

Abbreviations

ABS_t , ABS_{AF}	absorbance at time t and after freezing/thawing
CFU	colony forming unit

Arabic symbols

A	area of sample perpendicular to electric field
C_{ext}	concentration of solute in external solution
$C_{int,ave}$	average solute concentration in cylinder
C_O	capacitance of capacitor
C_0	initial concentration of solute
C	maximum amount extracted for the individual samples
c_n	normalization constant
d	distance between electrodes
$D_{app,1}$, $D_{app,2}$	diffusion coefficients
E	electric field strength
E	modulus of elasticity
E_c	critical value below which no activation occurs to specific microorganism
E_0, E_1 , E_2	residual, short and long relaxation moduli
f	average superposed concentration in the cylinder
f	frequency
f_1 , f_2	average calculated concentrations in tissues 1 and 2
g	relative electric permeability of cell membrane (dimensionless)
H	relative fraction of tissue type 1
I	current
k	shape factor (dimensionless)
K_t , K_{AF}	electrical conductivity of solution at time t and after freezing/thawing
K_l' K_{ld}'	electrical conductivity of untreated and totally disintegrated tissue at 1-5 kHz
K_l	electrical conductivity of untreated and treated tissue at 1-5 kHz
K_h K_h'	electrical conductivity of untreated and treated tissue at 3-50 MHz
K_{l5}, K_{l5}'	electrical conductivity of untreated and treated tissue at 5 kHZ
K_{l5f}'	average electrical conductivity of the tissue after 270 pulses of 1 kV/cm at 5 kHZ
KX,	complex conductance before and after PEF treatment
l	length of the sample
M	point of interest on cell membrane
n	number of pulses
N_0 , N_{PEF}	concentration of viable microorganisms before and after PEF
Q	energy density
r_c	cell radius

R_F	effective resistance of food
t_1 , t_2	short and long characteristic relaxation times
t	PEF treatment time
t_a	time after the field was applied
t_c	constant threshold value for treatment time
V	voltage
V_{ext}	volume of external solution
V_{int}	volume of cylinder
V_0	initial charge voltage
Z	total system impedance
Zp	cell disintegration index

Greek Symbols

θ	angle between direction of E and M
ω	angular frequency
$\dot{\varepsilon}$	deformation rate
ε_{max}	maximum deformation
τ	pulse length
ε	strain
σ	stress
μ	viscosity
α	characteristic time (μs) constant/relaxation time of the membrane
v	volume of treatment chamber

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In situ visualization of the effect of a pulsed electric field on plant tissue

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Abstract

A new method was developed for in situ visualization of changes related to electropermeabilization of plant tissue. Onion epidermis stained with neutral red was subjected to a pulsed electric field (PEF), and serial images were captured by a camera connected to a stereomicroscope. Sample resistance was recorded simultaneously.

Above a threshold level of a field strength of 0.35 kV/cm, it was possible to distinguish the individual permeabilized cells by their colour. Over 90–95% of the PEF-induced colour changes occurred during the first 2–3 min after the electric pulse and the rest after some 20 min. The size and rate of the observed changes were correlated with the severity of PEF, and were further influenced by the pH and conductivity of the solution used in mounting the epidermis, the sampling location on the onion epidermis, and cell size and number. The final conductivity increase was directly proportional to the number of permeabilized cells. Permeabilization was not randomly distributed but occurred along preferential paths connecting the electrodes.

Keywords: Visualization; Cell permeablization; Pulsed electric field; Onion epidermis; Neutral red

1. Introduction

The application of high voltage electric pulses to cells can form transient or permanent pores on the cell membrane. The mechanism of electropermeabilization is believed to be related to the accumulation of opposite charges on the two sides of electrically non-conducting cell membranes and the possible movement of these charges through the membrane under the influence of the electric field. Previously, the process, known as electroporation, has been used successfully in biotechnology for the incorporation of genetic material into living cells. During recent years, many researchers have made efforts to employ it in food processing. Most of these efforts have been focused on the possibility of pasteurization or sterilization of liquid foods with a pulsed electric field, PEF. Studies on the effects of PEFs on plant tissue are few, and most of them have been concerned with investigations of whether PEF could be combined with other processes. Knorr and Angersbach (1998) studied the effect of PEF on the dehydration characteristics of potato tissue, and reported that PEF treatment resulted in a reduction in drying time. A similar report (Ade-Omowaye, Rastogi, Angersbach, & Knorr, 2001) indicated that PEF treatment increased the drying rate of red paprika. Several others have also reported that moderate electric fields resulted in a significant increase in juice yield of apple cossettes and a decrease in discoloration (Bouzrara & Vorobiev, 2000). A new process consisting of pressing combined with PEF application was also proposed to increase the efficiency of juice extraction from sugar beet cossettes (Bazhal & Vorobiev, 2000).

Plant tissue can be regarded as a semi-solid, multiphase, heterogeneous material. The treatment of heterogeneous semi-solid material with PEF may require modifications, either to the material, or of the PEF treatment characteristics to achieve the desired level of cell permeabilization. Cell disintegration in heterogeneous food material using PEF has not been fully optimized and, in particular, the mass transport and mixing of the vacuole content, the cytoplasm and the liquid of apoplastic space, and their interaction with the cell wall during the post-permeabilization period are not fully understood. Using the electrical conductivity response of plant tissue subjected to PEF, cell disintegration was studied in a model circuit which took into account the resistance of the vacuole interior, tonoplast,

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cytoplasm, plasmalemma and extracelullar compartment (Angersbach, Heinz, & Knorr, 1999). Recently, an experimental investigation of the PEF-induced breakage of cellular tissue based on conductivity data and numeric analysis of a two-dimensional model was reported (Lebovka, Bazhal, & Vorobiev, 2001). The breakage kinetics of heterogeneous food material during PEF treatment was simulated and correlated with experimental data (Lebovka, Bazhal, & Vorobiev, 2000). So far, most of the methods used to elucidate mass transport in tissue following PEF have been on the theoretical interpretation of conductivity data. Conductivity is a relevant measure from the electropermeabilization point of view, but supplies no information on the spatial distribution of the permeablized cells in the tissue. Together with conductivity, real-time visualization of PEF-related changes in 2 or 3 dimensions in tissue can be informative in understanding both the pattern of permeabilization and post-permeabilization and the mass transport thereafter in tissue.

In this study, a method was developed for in situ visualization of changes in simple plant tissue during and after treatment with PEF. A one-cell-thick layer of onion epidermis was stained with neutral red (NR) and observed under a stereo-microscope before, during and after PEF treatment. Simultaneous image recording and conductivity measurement were automatically performed using an integrated experimental set-up which consisted of a video camera, a computer, a data logger, an impedance meter, a pulse generator and a stereo-microscope.

2. Materials and methods

2.1. Sampling

Red onion bulbs, *Allium cepa*, were bought at a retail store. Abaxial epidermal tissue was manually stripped from the concave surface of the second outermost leaf of the bulb. Debris of onion flesh on the upper surface of the abaxial epidermis was partially removed by washing with deionized water and gently rubbing between the fingers for 1 min. Small sections (5 mm × 5 mm) were cut out using a sharp blade on cover glass, and the rest was discarded.

2.2. Staining

Five μl of freshly prepared NR dye (3-amino-7-dimethylamino-2-methylphenazine, Merck, Germany) solution was applied to a small area on the cuticle free surface of the epidermis and allowed to remain in contact with the surface for 1 min. The solution was then removed from the surface with a paper tissue and 10 μl of wetting solution was added to provide sufficient contact between the electrodes during PEF treatment.

Fresh NR solution was used to avoid problems associated with precipitation and pH changes, and this was prepared from the solutions described below, by mixing stock NR solution with dilution solution at a ratio of 1 to 1.

(a) *Stock solution:* 4 g of NR was dissolved and mixed in 100 ml distilled water. After the addition of mannitol (0.33 M) (Eastman Kodak Company, Rochester, NY USA), the solution was filtered using filter paper (IF, Munktell Filter AB, 9 cm, Grycksbo, Sweden). HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid], Sigma) was added to 0.1 M, and the pH of the solution was adjusted to 6.8–7.0 by the addition of 0.2 M NaOH (Merck).

(b) *Dilution solution:* 0.33 M mannitol solution was prepared from deionized water and the pH was adjusted to 7.8 using HEPES buffer solution as in (a).

(c) *Wetting solution:* A 0.33 M mannitol solution was prepared from deionized water, with a pH of 6.72.

2.3. Treatment chamber, experimental set-up and electrical treatment

PEF treatment of the epidermis was performed in a chamber consisting of two parallel stainless steel electrodes separated by a distance of 1.15 mm, and a width of 7 mm. The top surface, i.e. the contact area of each electrode was 1 mm × 7 mm (Fig. 1). At the time of PEF treatment, the stained epidermis surface was wetted with the wetting solution and placed carefully on the top surface of the electrodes. The sample was examined under microscope to make sure that no air bubble was entrapped. It was oriented so that the long axes of the cells were parallel to the electrodes. A cover glass was placed on top of the chamber.

Having placed the sample in the chamber, monitoring of the impedance and phase angle was started, and top view images of the sample consisting of a single cell layer were recorded in situ each minute for 20 min using the integrated experimental set-up shown in Fig. 2. At 4 min 55 s a single monopolar rectangular pulse of 100 μs duration and magnitude of 20, 40, or 60 V was applied. All experiments were replicated once.

The sample was observed in transmitted light using a stereo-microscope (SZ6045 CTV, at a magnification of 1.5×, observation tube: trinocular w/TV adapter, Olympus, Japan) and images were recorded with a video camera (Sony Video Camera SSC-DC38P, Japan), connected to a single-slot PCI frame grabber (Matrox Meteor Board, Matrox Ltd., Dorval, Canada) in the computer. Images of the control sample and of the samples subjected to PEF at field strengths of 0.17, 0.35 and 0.52 kV/cm were captured each minute for 20 min.

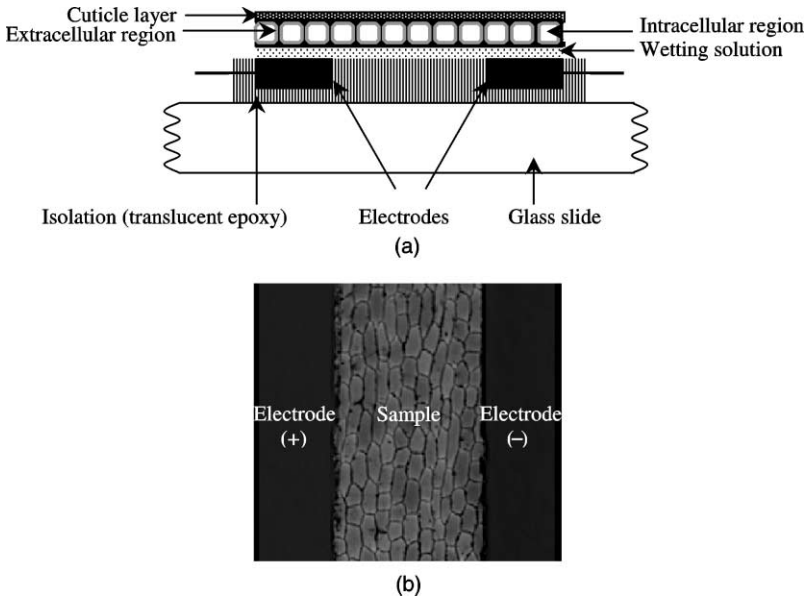

Fig. 1. (a) Cross-section of treatment chamber and onion epidermis sample, not to scale, (b) top view, stereo-microscopic image in transmitted light.

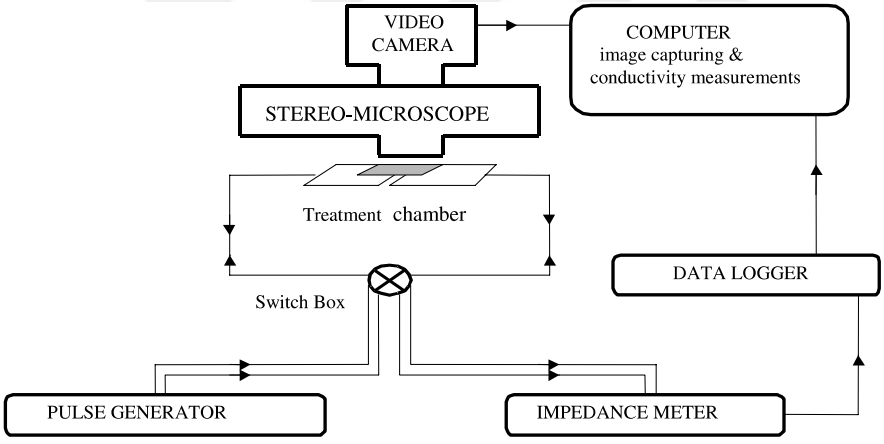

Fig. 2. Experimental set-up for simultaneous in situ image capture and conductivity measurement before and after PEF.

Care was taken to adjust the exposure so as to avoid saturation of any of the colour signal components.

The impedance and phase angle measurements were carried out with a vector impedance meter (Model 4800A, Hewlett Packard) at a constant frequency of 5 kHz. The analog signals from the vector impedance meter were sampled at 1 Hz, using a data logger (Wave Book/512™, 12 bit, 1 MHz, Data Acquisition System, IQtech, Inc., Cleveland, Ohio, USA), and software (Wave Book 7.7, DagX version 7,07, IQtech, Inc., Cleveland, Ohio, USA) in a computer, and transferred to another computer where they were converted to conductivity. The signals were locally smoothed using a one-step lowess routine over 36 data points with the software, Minitab, Release 12.2 (Minitab Inc., State College, PA, USA).

The application of electric pulses was performed by a Cellect electromanipulation instrument, BioFusion SCI AB, Sweden (320 V, 3 A).

2.4. Image analysis

After permeabilization, the permeabilized cells' intracellular region changed from red to yellow and the extracellular region from white to dark red. The images captured were processed and analyzed using image-processing software (Matrox Inspector, Version 2.1). The green component (G) of the RGB signal carried most information, and was higher in the intracellular region of the permeabilized cell than in the intact region and was lower in the extracellular region of PEF-treated tissue than in intact tissue (Fig. 3). To study in detail the

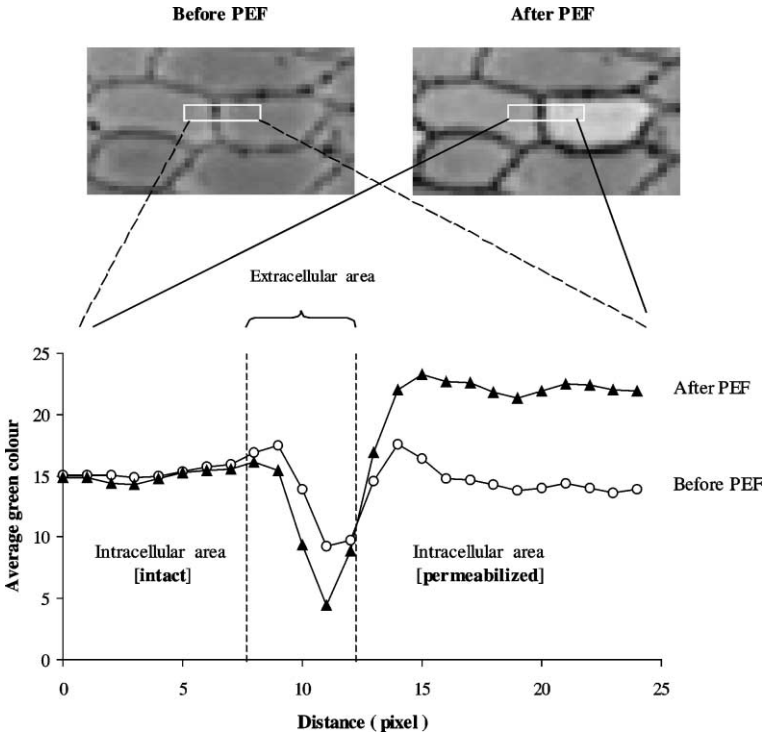

Fig. 3. Change in RGB green colour in the cell images before and after PEF (0.35 kV/cm).

kinetics of the events, differences between the successive images were extracted using the Matrox software. To separate the changes in the extracellular and in the intracellular region, increases in intensity (= the intracellular region, Fig. 5) and decreases in intensity (= the extracellular region, Fig. 7) were evaluated separately. Difference images were formed by subtracting the image at the $(n+1)$th min from the image at n min or vice versa. For subtraction, Matrox "subtract with saturate" routine was used, which automatically sets resulting negative values to zeros.

3. Results and discussion

3.1. Raw images

In Fig. 4, selected images of onion epidermis are presented at min 0, 5, 6, 7 and 19. The image at 5 min corresponds to 5 s after PEF treatment. In these images, the colour of certain individual cells shifted from red to white at a field strength above 0.35 kV/cm.

Concerning the colour shift in Fig. 4, the 7 min images (the 1st s of the 7th min) at 0.35 and 0.52 kV/cm were quite white, while 5 and 6 min images remained red.

The colour in the cells is largely determined by the NR dye, whose spectrum varies with pH. NR is a membrane-permeable dye, which easily diffuses through the plasmalemma and tonoplast into the vacuole, where it accumulates and ionizes due to the low pH of 5–5.5 (Ehara, Noguchi, & Ueda, 1996). The ionic form of NR in the vacuole is not membrane-permeable and is dark red in colour at pH 5–5.5 (Taiz, 1992). It is thus an intracellular pH indicator due to its colour change near neutral pH (Singh, Pal, & Sapre, 1999). A colour shift is likely to take place when the contents of the vacuole, cytoplasm and extracellular solution mix. In preliminary experiments it was found that if alkaline buffer was used as a wetting solution, the colour change was more pronounced.

The observed distribution of the permeabilized cells at partial tissue permeabilization (0.35 kV/cm) was non-homogeneous. It can be clearly seen in the top view images in Fig. 4 that stacks of cells along a straight path connecting the electrodes were preferentially permeabilized. This is not unexpected. During the pulse, the effective field strength increases with each cell permeabilized along the path. If the distance between electrodes corresponds to N cells then, when a cell is permeabilized, the cells along the path connecting the permeabilized cell to the electrodes are exposed to an electric field which is to a first approximation $N/N-1$ higher than the original field.

3.2. Processed images

Difference image analysis at times when the colour shift was most pronounced are shown in Figs. 5 and 7. Fig. 5 provides information on the rate of PEF-induced

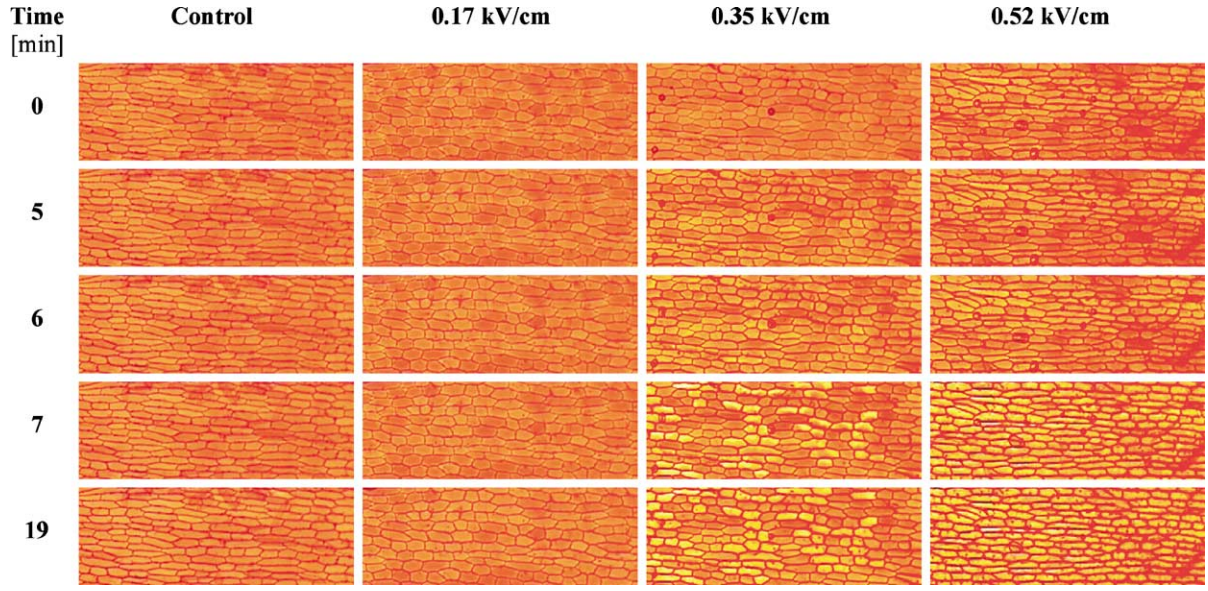

Fig. 4. Raw images of onion epidermis at 0, 5, 6, 7 and 19 min (Top view, the positive electrode is just above, the negative just below the observed region, image area 1.2×3.5 mm).

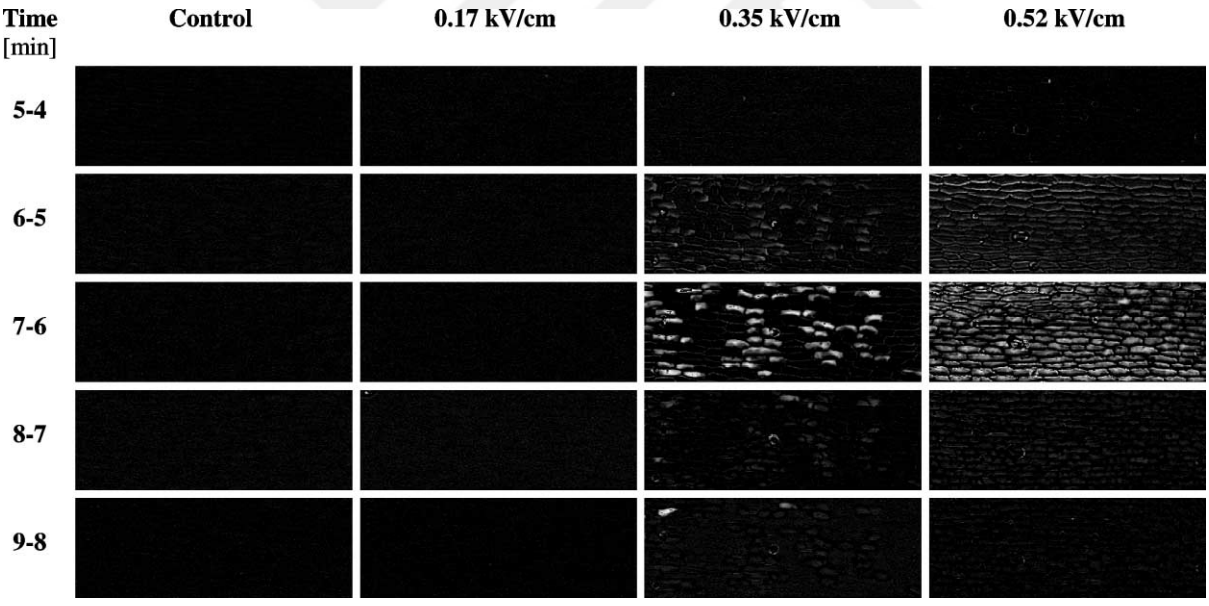

Fig. 5. Difference images of the green colour component showing the rate of change in the intracellular region.

changes from pH spectra of NR in the intracellular region.

No significant difference was observed between the control and other samples at 0.17 kV/cm, whereas in samples subjected to 0.35 and 0.52 kV/cm, a small difference started to appear nearly 60 s after PEF, in the subtracted images labelled "6-5" in Fig. 5 and continued to increase between 60 and 120 s (image "7-6"). The difference began to decrease again after the 120th s. Quantitatively, cumulative sum of the average pixel values of the successive images is shown in Fig. 6 for the whole observation period.

Similarly, the changes in the extracellular region in the subtracted images are shown in Fig. 7. Quantitative information, i.e. the average green value of the cumulative sum of the difference images is given in Fig. 8.

The baseline shift seen in Fig. 6 is identical to that in Fig. 8, confirming that it was an artifact of the summation of the random variation between images. Random variation between successive images is expected to be of the same magnitude in both "brighter" and "darker" images and to be constant in time. The time at which significant changes in extracellular space appeared was similar to that of the appearance of

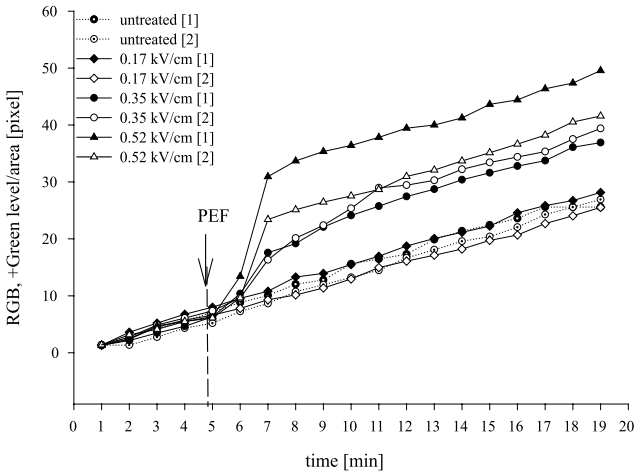

Fig. 6. The average value of the cumulative sum of the difference images reflecting the changes in the intracellular region during 19 min.

intracellular changes (Figs. 5 and 7) for PEF treatment at 0.35 and 0.52 kV/cm, but the time course appeared to be different, with a more gradual change in the extracellular space. This supports the hypothesis that change in the intracellular region due to PEF started first, and that changes in the extracellular region followed. The cell membrane would be expected to become permeabilized before the vacuolar membrane, because no significant voltage gradient can develop in the cytoplasm as long as the plasmalemma is intact. However, both the permeabilization events must occur here within the time frame of the single applied pulse of 100 μs, indistinguishable in our measurements. However, the mixing of the vacuole content with the extracellular content could be slower than the intracellular mixing due to the effect of the relatively high transport resistance of the cell wall.

3.3. Conductivity

In Fig. 9, the change in conductivity (mS/cm) as a function of the applied field strength is shown.

The application of a PEF at field strengths of 0.35 and 0.52 kV/cm, carried out at 4 min 55 s resulted in a steep increase in conductivity which took about 60 s, whereas the treatment at 0.17 kV/cm gave no increase, and was indistinguishable from that of the control samples.

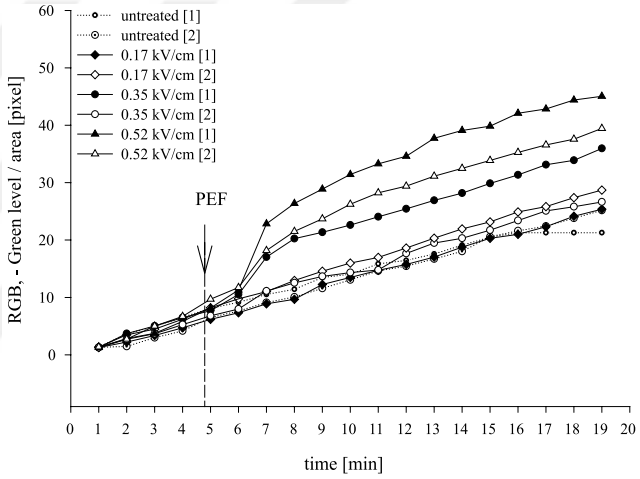

Fig. 8. The average value of the cumulative sum of the difference images reflecting the changes in the extracellular region during 19 min.

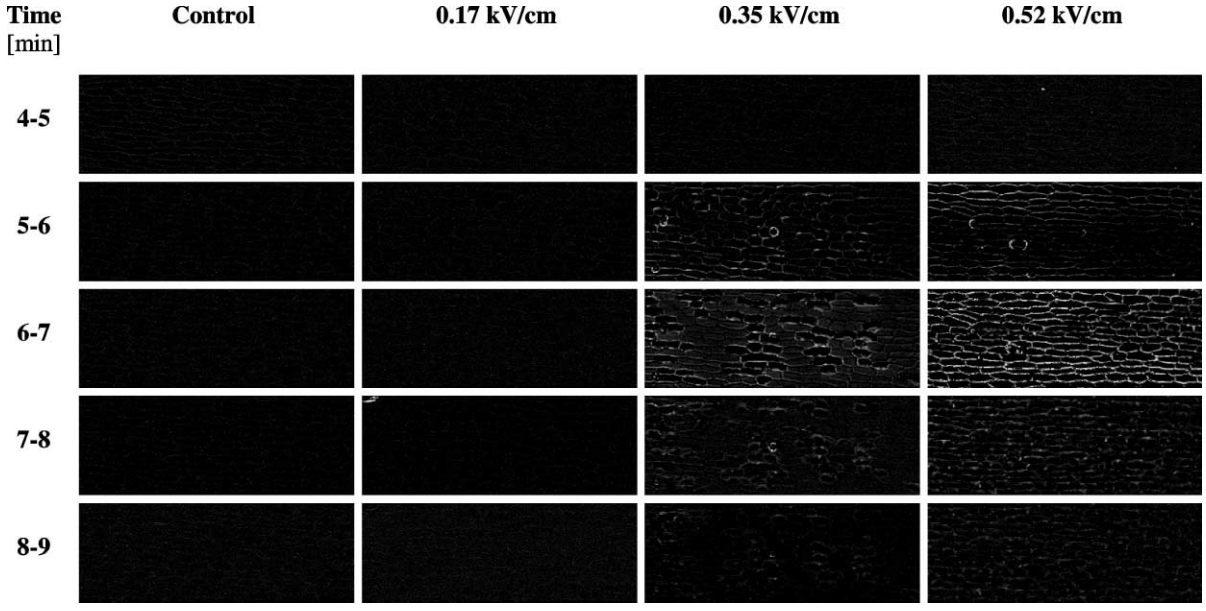

Fig. 7. Difference images of the green colour component showing the rate of change in the extracellular region.

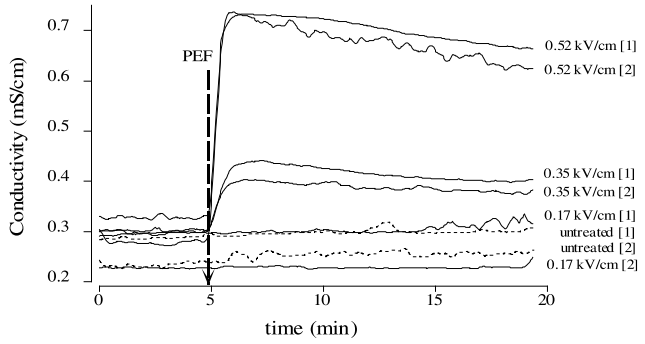

Fig. 9. Apparent conductivity of inner onion epidermis subjected to one rectangular pulse of 100 μs.

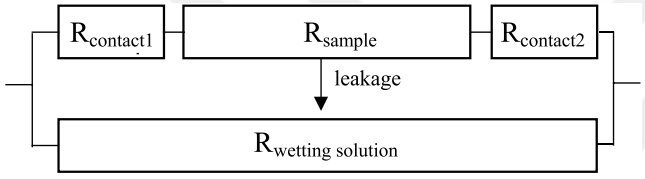

Fig. 10. A simplified equivalent circuit of the treatment cell and the sample.

Thus, the conductivity behaviour mirrored, in general, the observed changes in the microscope images. It is a well-established fact that the conductivity of PEF permeabilized tissue increases immediately during the pulse on a microsecond scale (Angersbach, Heinz, & Knorr, 2000; Ho & Mittal, 1996), rather than on the tens of seconds scale, as observed here. Our interpretation hinges on the geometry of the treatment cell used and the anatomy of the epidermis layer (Mauseth, 1988; Ng et al., 2000).

A simplified equivalent circuit of the treatment cell and the sample for interpreting the changes in resistances is shown in Fig. 10. R_{sample} is the total resistance of the epidermis between the electrodes, $R_{contact1}$ and $R_{contact2}$ are the contact resistances between the epidermis and the electrodes, and $R_{wetting\ solution}$ is the resistance of the wetting solution beneath the epidermis. We hypothesized that the contact resistances between the electrodes and the internal extracellular spaces of the tissue, $R_{contact1}$ and $R_{contact2}$ are high compared to the parallel resistance in the moisture path along the outside of the sample, $R_{wetting\ solution}$. Therefore, even if the tissue resistance R_{sample} changes dramatically with PEF, no sudden change in conductivity is observable in Fig. 7 at that time. Gradually, however, ionic species from the permeabilized intracellular space spread through the extracellular spaces to the electrodes and/or the "outside path" giving rise to the observed conductivity increase. We have confirmed this interpretation in supplementary experiments, in which we punctured the epidermis on top of the electrodes with a pin to decrease the contact resistance. In those experiments (data not shown), the conductivity increase was immediate.

The small decrease in conductivity observed over longer periods in Fig. 9 could be related to the resealing of the membrane (Ho & Mittal, 1996; Lebovka et al., 2001).

4. Conclusions

Direct observation of NR stained tissue corroborated information on permeabilization derived from conductivity measurements and allowed the detection of the individual permeabilized cells. It also allowed the assessment of the time scale of internal transport and mixing in permeabilized tissue.

Acknowledgements

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Effect of osmotic pretreatment and pulsed electric field on the viscoelastic properties of potato tissue

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Abstract

Compression stress relaxation of potato tissue exposed to pulsed electric field (PEF) treatment, with or without osmotic pretreatment, was measured and modelled with five parameter generalised Maxwell model. The changes in viscoelastic model coefficients were quantified as a response to applied field strength, pulse length, and pulse number and were correlated with conductivity changes. Using the same approach, additional effects of different osmotic treatments along with constant PEF treatment were also studied.

As measured by the post-PEF conductivity, the residual elasticity was the parameter most affected at lower levels of PEF treatment. At high PEF levels, the longer of the relaxation times dropped from 10 to 2–3 s range. Maximal PEF treatment had a similar effect to 0.7 M hyper-osmotic treatment, implying that the effect of PEF on relaxation behaviour was dominated by loss of turgor. Neither hypo-osmotic nor hyper-osmotic pretreatment appeared to interact with the PEF treatment.

Keywords: Potato; Pulsed electric field; Stress relaxation; Relaxation time; Maxwell model; Osmotic treatment

1. Introduction

In recent years, there has been an increasing interest in the possible use of pulsed electric field (PEF) in research areas other than those involving DNA transfer. The use of PEF and the resultant changes on the cell membrane have been interpreted in a model theory suggesting that a PEF polarises the ions across the electrically nonconductive membrane, and increase of the field beyond a critical membrane potential of about 1 V results in pore formation. After a low-magnitude electric field treatment, re-sealing of pores takes place on a microsecond-scale without having any great impact on the functioning of the cell, while pore formation is permanent at higher magnitudes, with a resultant loss of cell viability. Pore formation of a transient nature has mostly found applications in biotechnology, whereas irreversible permeabilization has been considered to have potential uses in food processing. The promising aspect of irreversible permeabilization of microorganisms is pasteurisation or sterilisation with less loss of nutritional value.

Application of electric pulses to semi-solid food is a new concept and has not been studied in detail. Subjection of cellular plant material to PEF has attracted attention as a potential combination or pretreatment stage in various food processes. One area of investigation has been on improvement of the dehydration characteristics of fruit and vegetables after PEF pretreatment. It has been suggested that PEF treatment accelerated the drying rate of cellular liquid (Ade-Omowaye, Angersbach, Taiwo, & Knorr, 2001; Ade-Omowaye, Rastogi, Angersbach, & Knorr, 2001). The majority of investigations of PEF pretreatment have been devoted to facilitation of expression of fruit juice. Several reports have claimed that liquid expression during mechanical compression is enhanced at a moderate level of electric field treatment (Bazhal, Lebovka, & Vorobiev, 2001; Bazhal & Vorobiev, 2000; Bouzrara & Vorobiev, 2000, in press; McLellan, Kime, & Lind, 1991). Similarly, a recent report has dealt with the potential use of high electric field pulses in sugar beet processing (Eshtiaghi & Knorr, 2002).

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To better understand how PEF or combinations of PEF with any other treatment could affect a potential food process, it is necessary to elucidate overall changes caused by PEF in the structure of plant tissue at the cellular level. PEF-induced changes at the cellular level have been studied only partially. Conductivity changes have been the most studied aspect, and have been related to permeabilization of the membrane as a result of fast release and transport of ionic spices in the vacuole or cytoplasm through the pores formed on the membrane (Angersbach, Heinz, & Knorr, 1999, 2000; Knorr & Angersbach, 1998; Lebovka, Bazhal, & Vorobiev, 2000, 2001, 2002). The understanding of permeabilization in heterogeneous tissue as well as internal mixing or mass transport thereafter, includes consideration of the anatomical structure of plant tissue and compositional differences in extracellular and intracellular space. Diffusion of intracellular content into the extracellular space has been visualised in onion epidermis, and was complete within 2–3 min after permeabilization (Fincan & Dejmek, 2002).

The viscoelastic properties of tissues depend on turgor, and properties of cell wall and middle lamella (Scanlon, Pang, & Biliaderis, 1996; Warner, Thiel, & Donald, 2000). In this paper, we report the effect of PEF on the viscoelastic properties of potato tissue alone and in combination with osmotic treatment. A cylindrical piece of potato tissue before and after PEF was subjected to a uniaxial compression test in an Instron universal testing machine. The relaxation data were processed using the generalised Maxwell model with a residual spring element and the changes in viscoelastic constants were determined as response to the applied field strength, pulse length and pulse number. After suspending potato slices in hypo-osmotic and hyper-osmotic mannitol solutions, the same approach was applied to evaluate the combined effect of osmotic and PEF treatment on the viscoelastic constants at a defined level of electrical treatment.

2. Materials and methods

2.1. Sampling

Mature potato tubers, *Solanum tuberosum,* were obtained from a retail store. Cylindrical potato tissue slices, 1 mm in thickness × 4 mm in diameter, were sampled from the cortex (parenchyma cells) using cylindrical cork borer and parallel sharp blades. Conductive debris of potato flesh was removed from all surfaces by washing with deionized water for 1 min. Samples of one group were allocated to be treated with electric pulses directly after the sampling stage in order to characterise PEF-related viscoelastic changes as a function of pulse length, pulse number and field strength. The other group was to be used in osmotic treatment prior to electrical treatment, in order to evaluate the combined effect of osmotic treatment with a given level of electrical treatment on viscoelastic properties.

2.2. Electrical and osmotic treatment

Application of PEF to both groups of samples was achieved in a parallel stainless steel electrode with a gap distance of 1 mm. Electrical pulses were applied using a Cellect electromanipulation instrument, BioFusion SCI AB, Sweden.

2.2.1. Electrical treatment of raw potato tissue

Raw potato slices at room temperature were subjected to electrical treatment according to a three-level factorial design, namely field strength: 0.5, 1.0, 1.5 kV/cm; pulse length: 10, 100, 1000 μs; pulse number: 1, 9, 90, and a pre-/postpulse length of 10 ms. For 90 pulses the 9 pulse sequence was triggered manually 10 times. The pulses were monopolar rectangular, with rise and fall times shorter than 1 μs. Fig. 2 shows the screen dump of the trace of treatment pulses at extreme ranges recorded in PC (circuit analyser, Software for Velleman PC oscilloscope PCS500, Belgium) from oscilloscope (PC Scope 1GS/s, PCS500, Velleman Instruments, Belgium) in the setup of Fig. 1.

Energy (J) distributed per pulse (E_{PP}) was approximated using in Eqs. (2) and (1)

$$I = \frac{V_R}{R_R} \tag{1}$$

$$E_{PP} = VI\mathrm{PL}_{SP} \tag{2}$$

where V_R and I are the average voltage (V) and average current (A) across the constant resistant, R_R (Ω) over the duration of a pulse (Fig. 1), V is the constant voltage (V) imposed by the pulse generator, PL_{SP} is the exposure time (s) corresponding to a single pulse. E_{PP} corresponding to the 1st and 90th pulse at the minimum and

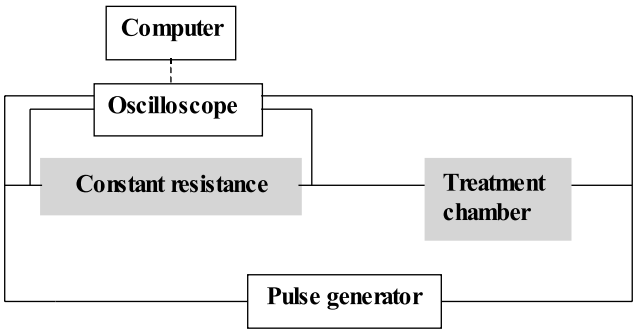

Fig. 1. Experimental setup of the PEF treatment during pulse current measurement.

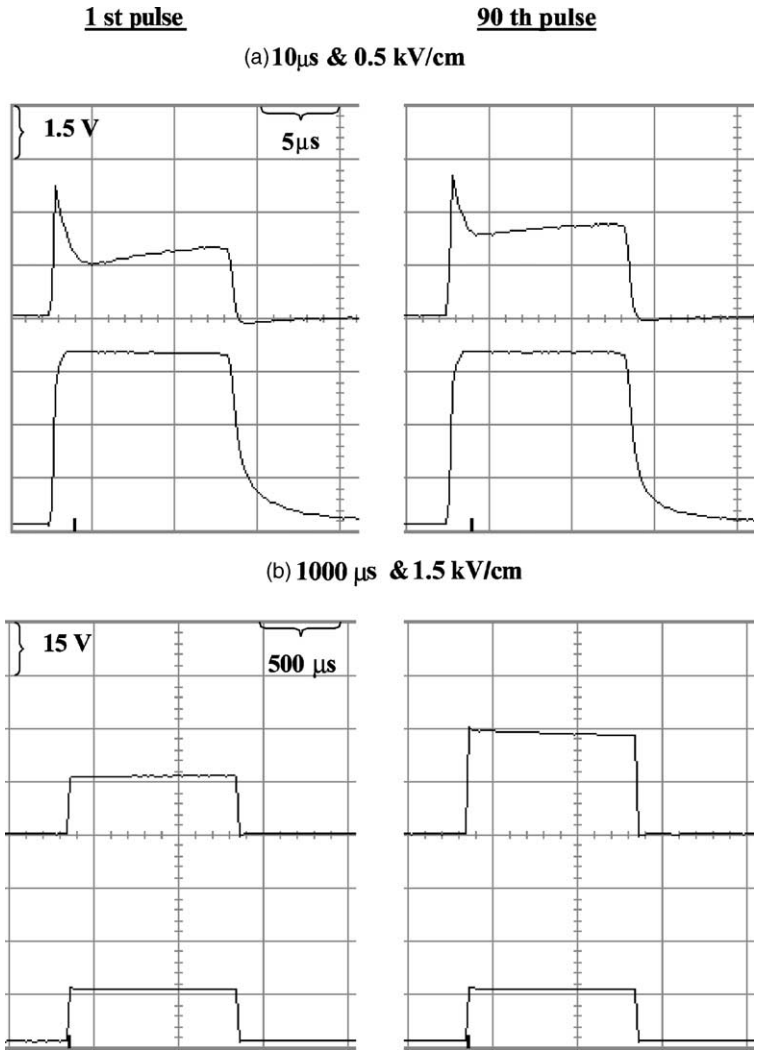

Fig. 2. Pulse shape at our minimum and maximum PEF treatment levels. Upper trace corresponds to the current across constant 41 Ω resistance; lower trace imposed voltage (attenuated 10×).

Table 1
Energy distributed per pulse (E_{PP}) at extremes of the PEF treatment

Field strength (kV/cm)	(nth) pulse	Pulse length (μs)	Energy (J/kg pulse)
0.5	1	10	2.2
	90		2.8
1.5	1	1000	5300
	90		8400

maximum level of field strength and pulse length employed was summarised in Table 1.

2.2.2. Conductivity measurement

In a separate series of experiments, the impedance and phase angle of samples were measured in place before and after the same magnitude of electrical treatments as in Section 2.2.1. The data were collected after 20–30 min of measurement and converted to conductivity. The measurements were done with a vector impedance meter (Model 4800A, Hewlett Packard) at a constant frequency of 5 kHz.

2.2.3. Electrical treatment of osmotically treated potato tissue

Osmotic treatment of the samples was done in dip solutions of distilled water and mannitol (Eastman Kodak Company, Rochester, NY, USA) at concentrations of 0.1, 0.33, 0.7 M. The samples were vertically positioned in the solutions to ensure all surfaces to come in contact with the solution for 4 h. Samples were then removed from the dip solution and drained prior to being placed into electrodes for PEF treatment. Electrical variables for this group of samples during PEF treatment were a field strength of 1 kV/cm, pulse length of 100 μs, pulse number of 9 and a pre-/postpulse length of 10 ms.

2.3. Textural measurement

All samples were exposed to a uniaxial compression in an Instron (Model 4400, USA). The compression was from the top cylindrical surface at 10 mm/min up to a depth of 0.3 mm and stress of 0.4–0.45 MPa. Stress during the loading period and stress relaxation over 20 s was recorded with 0.1 s resolution.

2.3.1. Data evaluation and processing

Experimental stress relaxation data vs. time were evaluated in Table Curve software (Table Curve 2D Windows, v4.07, SPSS Inc., AISN Software Inc., Chicago, USA), using a generalised Maxwell model in Eq. (3), with allowance for the finite rate of compression during loading (De Baerdemaeker & Segerlind, 1976; Rao & Steffe, 1992). A model using two Maxwell elements and a residual spring for a total of five parameters was used and the viscoelastic coefficients (E_0, E_1, E_2, t_1, t_2) of Eq. (3) were then determined

$$\sigma(t) = E_0\varepsilon_{\max} + \dot{\varepsilon}[E_1 t_1(1 - e^{-\varepsilon_{\max}/\dot{\varepsilon}t_1})e^{-t/t_1} + E_2 t_2(1 - e^{-\varepsilon_{\max}/\dot{\varepsilon}t_2})e^{-t/t_2}] \quad (3)$$

where $\dot{\varepsilon}$ is the deformation rate, $\varepsilon_{\max}$ is the maximum deformation, σ is the stress, t_1 and t_2 are the short and long characteristic relaxation times and E_0, E_1 and E_2 are the residual, short time and long time relaxation moduli.

For all experiments with equal total on-time, i.e. with an equal product of pulse number and pulse length, the additional influence of pulse length/pulse number on residual modulus and long characteristic relaxation time was statistically evaluated by two-way fixed factor Anova testing the null hypothesis that for any on-time

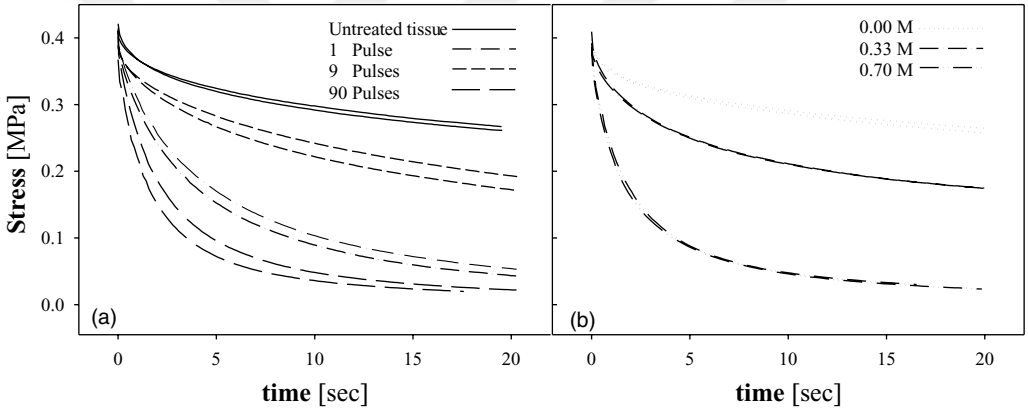

Fig. 3. Stress relaxation of potato slice subjected to (a) different number of pulses at a field strength of 1 kV/cm and pulse length of 100 μs, (b) different osmotic treatments. Two replicates shown for each treatment.

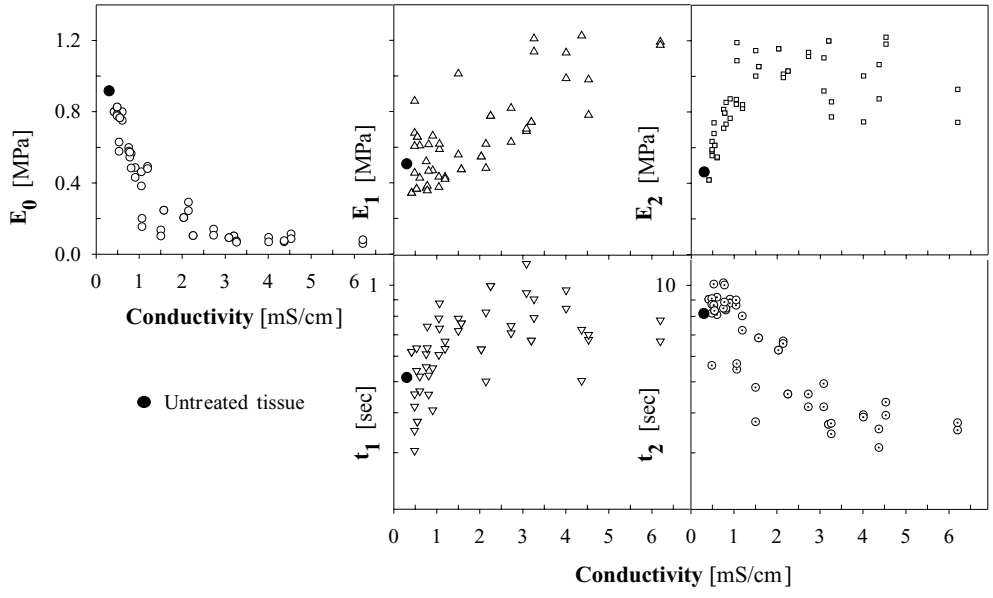

Fig. 4. Relationship between viscoelastic constants and conductivity at different levels of electrical treatment.

combination, fewer longer pulses were equivalent to a larger number of shorter pulses (Minitab, Release 12.2, Philadelphia, USA).

3. Results and discussion

The PEF treatment caused only minor changes in maximum stress or compression modulus (data not shown). The major effect was observed in relaxation behaviour. Examples of the stress relaxation of raw potato slices subjected to different numbers of pulses at a field strength of 1 kV/cm and pulse length of 100 μs, or subjected to different osmotic treatments, are given in Fig. 3(a) and (b). For raw potato, the relaxation behaviour agrees with the result of Peleg and Calzada (1976). The effects increased with the intensity of treatment and were qualitatively similar. Lin and Pitt (1986) used mannitol to adjust the turgor in potato tissue and reported that 0.25–0.30 M was an osmotically neutral

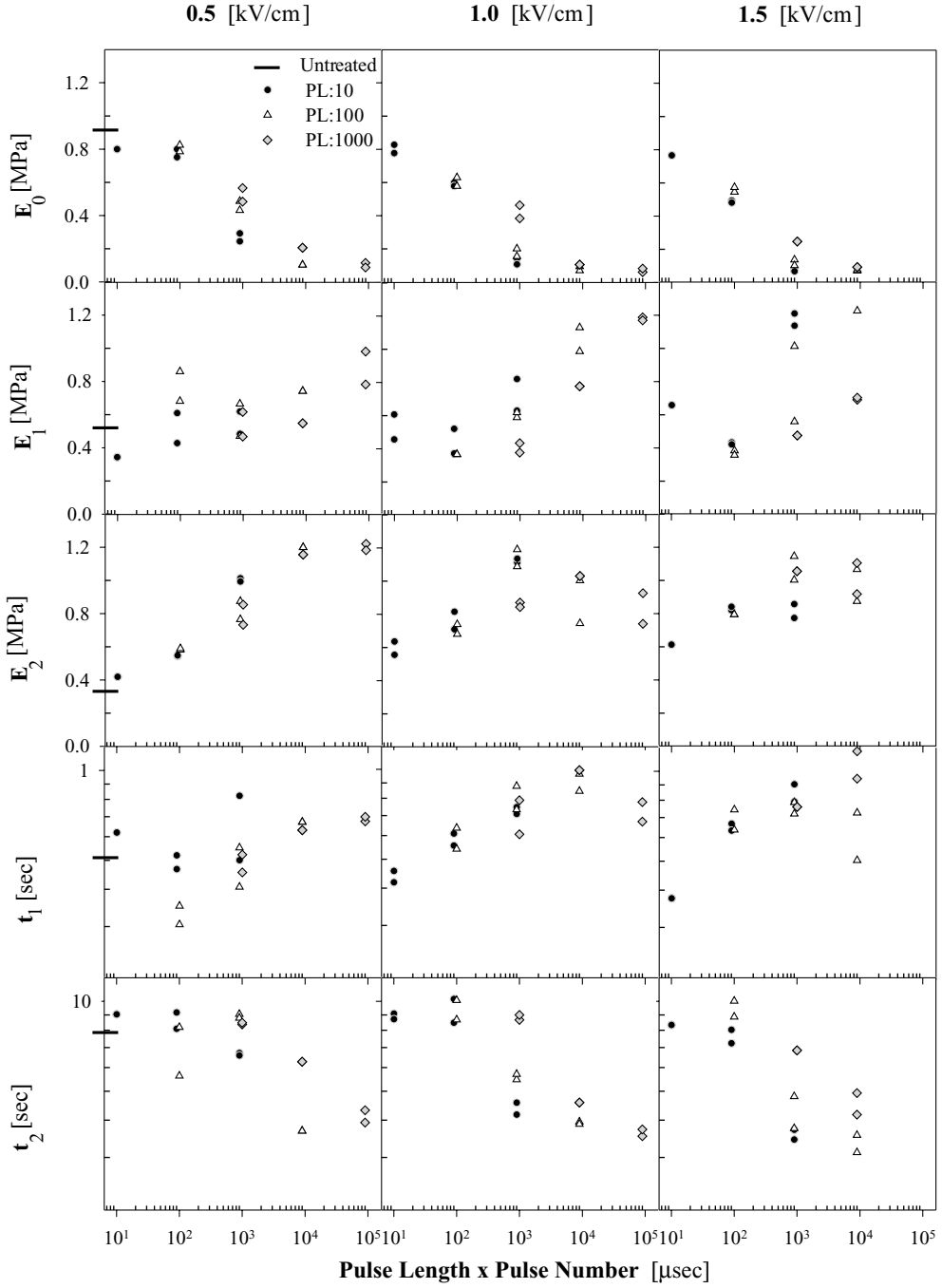

Fig. 5. Changes in viscoelastic constants at field strenghts of 0.5, 1.0 and 1.5 kV/cm vs. total pulse exposure.

(isotonic) concentration for potato tuber tissue and no plasmoptysis occurred at concentration between 0 and 0.1 M, which agrees with the similarity between the stress relaxation of samples soaked in water and fresh tissue in Fig. 3(a) and (b).

For a more detailed study, we have fitted the relaxation behaviour to a generalised Maxwell solid using Eq. (3). To achieve fits with correlations (r^2) better than 0.99 we had to use in addition to the residual spring two Maxwell elements. The viscoelastic constants of the model are interdependent, in particular the E_1 and E_2 moduli are highly negatively correlated. Due to time resolution of the experiment, the properties at short time scale (E_1, t_1) show more variability. The long characteristic time t_2 is a robust parameter, and so is the residual modulus E_0. From replicate we could estimate the pooled standard deviation of t_2 to be 0.35 s and that of E_0 to be 0.025 Pa.

Electric conductivity is a well-accepted indicator of PEF-induced damage to cellular tissue. In Fig. 4, relationship between viscoelastic constants and conductivity at different levels of electrical treatment are shown. The most pronounced change was the decrease in residual modulus, which already occurred at relatively low treatment levels and was paralleled by the decrease in the long-term relaxation modulus. A possible interpretation would be that PEF treatment leads to loss of cell liquid which is the primary cause of plant tissue stiffness (Warner et al., 2000) therefore residual stiffness E_0 decreases with the level of treatment. A more intensive PEF treatment causes larger pores in the cell membrane and thus allows a faster escape of cell liquid content and thereby faster relaxation. The time constants gradually converge over the whole range of treatment intensities to a level of 1–2 s, i.e. the longer timescale component of relaxation virtually disappears. We assume that the short timescale behaviour is not related to the treatment, but is caused by the cell damage induced in compression.

A more detailed view of the effect of the individual parameters of the electric treatment is given in Fig. 5. The viscoelastic parameters are plotted vs. the total exposure time of the tissue to the electric field, the total on-time. It is apparent that treatment effect correlated fairly well with the total length of the train of pulses at each level of electrical field. However, on closer inspection it can be seen that multiple short pulses tended to be more efficient in affecting residual modulus ($P = 0.002$) and long characteristic relaxation time ($P = 0.000$), in our conditions, multiple shorter individual pulses led to larger effects than a smaller number of longer pulses of the same total length, but whether the causal relationship is with the higher number of field changes or with the shorter individual pulse on-time cannot be resolved. All of the field gradients used were well beyond the accepted permeabilization threshold of 0.15–0.2 kV/cm membrane (Angersbach et al., 2000); but in spite of that, effects increased with increasing voltage gradient. A threefold increase of the gradient, from 0.5 to 1.5 kV/cm corresponded approximately to a tenfold increase in treatment time for the same level of tissue property change.

Exposing potato tissue to a hypo-osmotic or hyper-osmotic pretreatment before electric field treatment did not appear to lead to significant interaction effects (see Fig. 6). At treatment solution molarities up to 0.4 M, the effect was dominated by the PEF treatment. For a 0.7 M osmotic pretreatment and the applied intensity of the PEF treatment, there was no significant difference be-

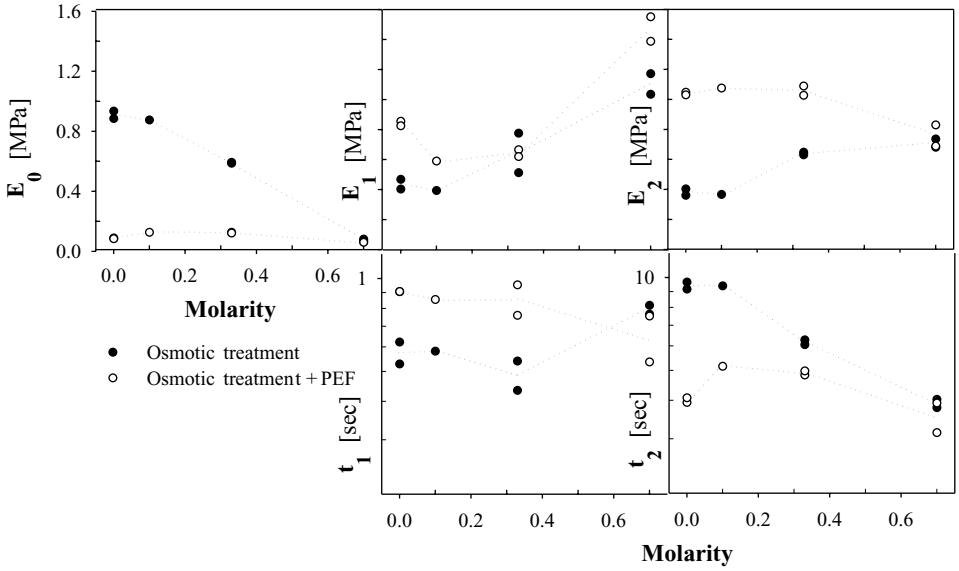

Fig. 6. Changes in viscoelastic constants of osmotically treated potato slice before and after PEF vs. mannitol concentration (PEF: 1 kV/cm, 9 pulses of 100 μs pulse length). Dotted lines to guide the eye.

tween viscoelastic properties of tissue treated osmotically only and tissue treated osmotically in combination with PEF.

4. Conclusions

PEF treatment of potato tissue led to changes in viscoelastic properties of the same nature as the changes brought about by hyper-osmotic treatment. The effects are consistent with the hypothesis that PEF-induced mechanical property changes are caused by loss of turgor. At lower treatment levels, the effect was seen as lowered residual modulus, higher treatment levels in addition further shortened the relaxation time. This could be interpreted as increased cell membrane damage allowing faster escape of cell contents under stress. At all filed strengths, the treatment effects correlated approximately with the cumulative pulse on time, but large number of short pulses was found to be more efficient for the same on-time. Increasing field gradients led to significantly greater effects even at gradients far beyond the critical permeabilization level. No synergistic effects between osmotic pretreatment and PEF treatment were found.

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Pulsed Electric Field Treatment for Solid-Liquid Extraction of Red Beetroot Pigment

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Abstract

Extractability of red pigment from red beetroot was investigated using pulsed electric field (PEF) treated tissue in solid-liquid extraction process. Thin disks of the tissue were subjected to PEF at different intensities and, 3-4 min after the treatment, the release of red pigment and ionic species into an isotonic solution was measured *in situ* using a spectrophotometer and a conductivity meter. The highest degree of extraction achieved with PEF was compared with that of freezing and mechanical pressing.

Subjected to 270 rectangular pulses of 10 μs at 1kV/cm field strength, with the energy consumption of 7 kJ/kg, the samples released about 90 % of total red coloring and ionic content following 1 h aqueous extraction. The increase in tissue conductivity after PEF treatment correlated linearly with the extraction yield of red-pigment and ionic species only up to an extraction level of 60-70 %. No differential permeabilization of the intracellular components was found. Higher degree of variation of the extractability of pigment and ionic species at intermediate PEF treatments was interpreted as related to non-homogenous permeabilization of the tissue due to cell size.

Key words: Red beetroot; Extraction; Betanin; Pulsed electric field; Permeabilization; Conductivity;

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1. Introduction

Commercially interesting intracellular compounds and liquids from cellular plant tissue can be extracted using various solid-liquid extraction methods such as comminution, sometimes combined with mechanical expression, heating or freezing. One of the factors influencing the efficiency of the extraction process is the degree of cell membrane disintegration, which frees the intracellular compounds so that they can migrate into the external medium. The degree of cell membrane disintegration achieved by each of the methods varies, and limitations may be imposed by product sensitivity or high cost. The degree of cell wall rupture may also be an important parameter influencing the process.

In addition to the methods mentioned above, disintegration of the cell membrane in plant tissue via application of electric pulses has been suggested as a non-thermal pretreatment stage to increase the extraction efficiency of the intracellular liquid (Bazhal, Lebovka, & Vorobiev, 2001; McLellan, Kime, & Lind, 1991; Bouzrara, & Vorobiev, 2000; Bazhal, & Vorobiev, 2000; Bouzrara, & Vorobiev, 2002) and to facilitate the drying of fruits and vegetables (Ade-Omowaye, Rastogi, Angersbach, & Knorr, 2001; Ade-Omowaye, Angersbach, Taiwo, & Knorr, 2001). Application of a pulsed electric field (PEF) to cells has been shown to result in pore formation on the membrane above a transmembrane potential of about 1 V. This pore formation has been theoretically interpreted as the result of polarization of ions across the membrane, which compresses the electrically non-conductive membrane to the degree of rupture (Zimmermann, 1986; Ho & Mittal, 1996). Electric treatment of plant tissue at the levels of 0.4-0.8 kV/cm was found to be permeabilizing a plant cell membrane significantly (Angersbach, Heinz, & Knorr, 2000). In most of the studies investigating the effect of PEFs on the extraction efficiency of fruit juice, PEF treatment of the plant material was applied either before or during mechanical pressing of cossettes or mash. In most reports it was claimed that the combination of pressure and PEF treatment increased the juice yield with a lower energy consumption, while McLellan, Kime, and Lind (1991) reported limited effect on yield with more rapid release, and lighter color. However, studies on pure solid-liquid extraction of PEF-treated tissue are limited in the literature.

Dörnenburg and Knorr (1993) studied the PEF-induced release of intracellular pigments, namely amaranthin and anthraquinones, from *Chenopodium rubrum* and *Morinda citrifolia*

cells, respectively. They reported that 85 % of the total amaranthin and 5.7 % of the anthraquinones were released into the culture medium following electrical treatment up to 1.6 kV/cm and up to 30 pulses while cell viability was lost at release rates higher than 16 % and 2 %. Additionally, Jemai and Vorobiev (2002) investigated the effect of moderate electric field pulses on the diffusion coefficient of soluble substances from apple slices and found that electric pretreatment combined with a low thermal treatment significantly increased the diffusion coefficient. Although plant tissue contains many other metabolites of commercial interest, PEF-induced extraction studies so far, concentrated on mainly on fruit juice and sugar.

Red beetroot (*Beta vulgaris*) juice concentrate is one of the approved food additives as a food colorant (E 162) and antioxidant. Production of the concentrate is generally based on pressing or aqueous extraction of shredded beetroots. The main constituent of the red coloring of beetroots are betalains that accumulate in the vacuoles (Kujala, Vienola, Klika, Loponen, Pihlaja, 2002; Leigh, Rees, Fuller, & Banfield, 1979). Betalains are categorized as red-violet betacyanines of which betanin accounts for 75-95 %. There is also a minor amount of yellow betaxanthine (Patkai, & Barta, 1996; Simon, Drdak, & Altamirano, 1993; Kujala, Loponen, & Pihlaja, 2001; Kujala, Loponen, Klika, & Pihlaja, 2000).

In the study reported here, thin disks of red beetroot were subjected to pulsed electric field at different intensities and, 3-4 min after the treatment, the kinetics of both the pigment and the ionic species released from the samples into isotonic solution was measured *in situ* using a spectrophotometer and a conductivity meter. The kinetics of the release was recorded, and the relationship between changes in tissue conductivity after PEF treatment and corresponding release of color and ionic species were studied. The final extraction values were also compared with those obtained using mechanical pressing and freezing.

2. Materials and methods

2.1 Sampling

Fresh red beetroot tubers (*Beta vulgaris*) were bought from a local store. Disks of tissue slices, 1 mm in thickness and 4 mm in diameter, were sampled from the homogeneously red upper third ring region (parenchyma cells) using a cylindrical cork borer and parallel sharp blades. Conductive debris was removed from all surfaces by washing with deionized water (5 μS/cm) at room temperature for 1 minute. Following the washing step, samples were placed in a treatment chamber consisting of parallel stainless steel electrodes with a gap of 1 mm.

2.2 Sample treatment

Each set of treatment conditions was replicated at least five times.

2.2.1 *Pulsed electric field treatment*

Trains of nine electric pulses at a constant field strength of 1 kV/cm, with a pulse length of 10 μs and an interpulse delay of 20 ms were applied to the tissue in the treatment chamber (Cellect electromanipulation instrument, BioFusion SCI AB, Sweden) (Fig. 1a). Pulse trains were manually triggered in rapid succession to achieve treatment with a total of 27, 54 or 270 pulses. The pulses were monopolar rectangular, with rise and fall times shorter than 1 μs. Fig 2 shows the screen dump of the trace of a treatment pulse observed on an oscilloscope (PC Scope 1GS/s, PCS500, Velleman Instruments, Belgium) in the set-up of Fig.1a.

2.2.2 *Freezing*

Reference samples were prepared as described above and placed in a drop of deionized water. They were then kept in freezer at –24 ^{o}C for 4-5 days, and thawed at room temperature for 3 h. PEF-treated samples were similarly frozen together with their extraction bath liquid (see below).

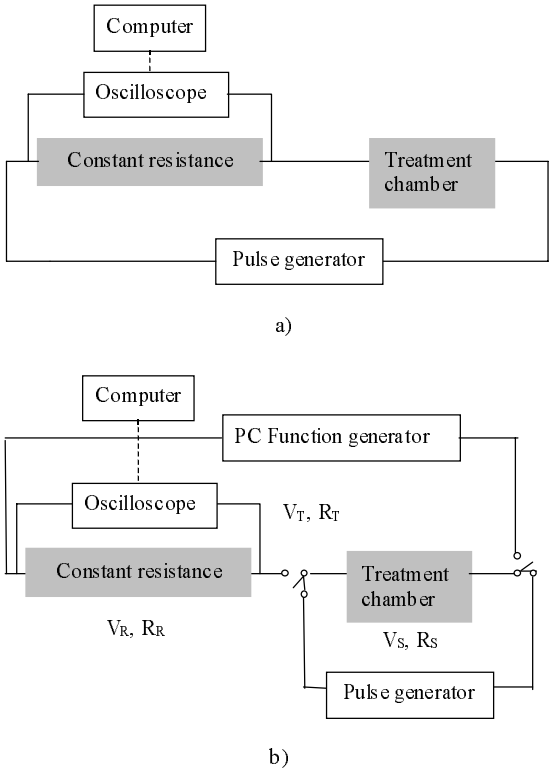

Fig. 1. *Experimental set-up for a) pulse current measurement during PEF treatment b) PEF treatment and conductivity measurements of the tissue samples.*

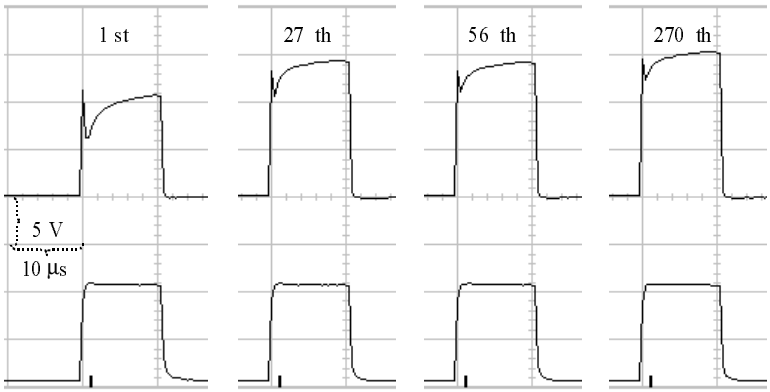

Fig. 2. *Shape of electric pulses with an increment in pulse number. Upper trace corresponds to the current across the constant resistance; lower trace imposed voltage (attenuated 10 ×).*

2.3 Measurements

2.3.1 *Tissue conductivity*

Tissue conductivity was measured prior to PEF treatment and 4 min after PEF treatment with a sinusoidal voltage of 0.565 V (rms) over the range of 1 KHz-1 MHz using a function generator (PC Function generator, K8016, Velleman instruments, Belgium) (see Fig.1b) and the rms voltage across the 41-Ω ohmic resistor was recorded using a circuit analyzer and Software for the Velleman PC oscilloscope. The complex tissue conductance was then calculated in Eq. (3) using Eqs. (1) & (2)

$$V_T = I(R_R + R_S) \qquad \text{Eq. (1)}$$

$$V_R = I R_R \qquad \text{Eq. (2)}$$

$$KX = \frac{1}{R_R} \times \frac{V_R}{(V_R - V_S)} \qquad \text{Eq. (3)}$$

where I is the current (A), R_R and R_S are the resistor and sample resistances (Ω), V_R and V_S are the voltages (V) across R_R and R_S, V_T = the voltage (V) across the complete circuit, and *KX* is the complex conductance of the sample (Ω^{-1}).

In addition, the ohmic conductivity of each sample was determined at 5 kHz pre-and post-treatment using a Vector Impedance Meter (Model 4800A, Hewlett Packard, USA). In preliminary tests at 1 kV/cm field strength, the number of pulses was increased cumulatively and the tissue conductivity monitored. Under these experimental conditions, the highest conductivity was achieved (asymptotically) at about 270 pulses. The tissue conductivity of the treated samples increased to 3.1 ± 0.2 mS/cm, from the untreated value of 0.27 ±0.06 mS/cm at 5 kHz. The conductivity of red beetroot juice was 6.90 mS/cm at room temperature. The effects of treatment are reported as follows:

$$\text{Relative complex conductivity} = \frac{KX'}{KX} \qquad \text{Eq. (4)}$$

$$\text{Relative increase in tissue conductivity} = \frac{(K'_{l5} - K_{l5})}{(K'_{l5f} - K_{l5})} \qquad \text{Eq. (5)}$$

where KX and KX' are the complex conductance (Ω^{-1}) before and after PEF-treatment, respectively. K_{l5} and K'_{l5} are the ohmic conductivity (mS/cm) of the samples before and after PEF treatment and K'_{l5f} is the average ohmic conductivity (mS/cm) of the samples obtained following the application of 270 pulses at 3.1 mS/cm. This measure of tissue damage is similar to the tissue damage index of Angersbach et al. (1999).

2.3.2 *Release of conducting species and color*

In all experiments, the individual samples were suspended in 7 ml 0.25 M mannitol (Eastman Kodak Company, Rochester, N.Y., USA) and the release of ions and red coloring matter was measured in the mannitol solution.

Kinetics of the release from PEF-treated and frozen samples

For on-line monitoring of both the red color and ionic release from PEF-treated tissue into isotonic solution, an integrated system consisting of a temperature-controlled container, a spectrophotometer (Hitachi, U-1500, Japan) and a conductivity meter (Radiometer, CDM210, Copenhagen, Denmark) connected to a computer was set up (Fig. 3).

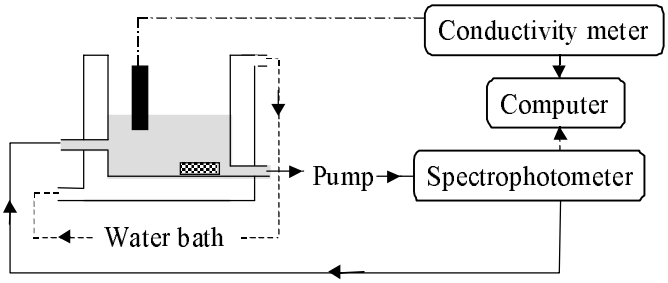

Fig. 3. *Experimental set-up for the extraction kinetics measurements.*

After filling the container with the mannitol solution and immersing the conductivity probe (Radiometer, CDC641T, K=0.75, Copenhagen, Denmark) in the solution, the system was operated so as to circulate the solution through the flow-through cuvette in the spectrophotometer. The absorbance was measured at 530 nm, corresponding to the maximum wavelength of betanin.

Three to four minutes post-treatment, a single slice of PEF-treated or frozen/thawed tissue was introduced into the mannitol solution, and the change in conductivity of the solution in the container and the absorbance of the solution in the flow-through cuvette were measured for 60 min (Fig. 3). The data were recorded in computer every 10 seconds using various programs.

The release after freezing of PEF-treated samples and mechanical pressing of raw samples

Following the 1-hour kinetics measurements described above, all the samples together with their solutions were frozen at –24 ^{o}C for 4-5 days. The samples were then placed in shaker (GFL, 3005, Germany), and shaken at 250 rev/min at room temperature until the absorbance and conductivity reached constant values.

For comparison with the release from PEF-treated and frozen samples, raw samples were compressed under a rolling finely corrugated metal rod to the degree of thin flake. The absorbance and conductivity were measured in the shaker as described above. Following extraction, the flake was observed under a light microscope for traces of red color.

Changes in the absorbance and conductivity of the mannitol solution were calculated according to Eq. (6) and Eq. (7):

$$\text{Relative solution absorbance} = \frac{ABS_t}{ABS_{AF}} \qquad \text{Eq. (6)}$$

$$\text{Relative solution conductivity} = \frac{K_t}{K_{AF}} \qquad \text{Eq. (7)}$$

where ABS_t and ABS_{AF} are the absorbance (nm) at time t and after freezing/thawing, and K_t and K_{AF} are the conductivity (mS/cm) at time t and after freezing/thawing, respectively (with the mannitol solution blank subtracted).

After 3 h extraction, the cross section of the samples was observed in transmission light microscope (Olympus BX50, magnification 5 × / 0.15, Olympus, Japan) and images were recorded with a video camera (Sony Video Camera SSC-DC38P, Japan), connected to a single-slot PCI frame grabber (Matrox Meteor Board, Canada) in the computer.

3. Results and discussion

PEF treatment caused significant changes in the electric properties of the tissue, Fig. 4, and the release of ionic species and red pigments.

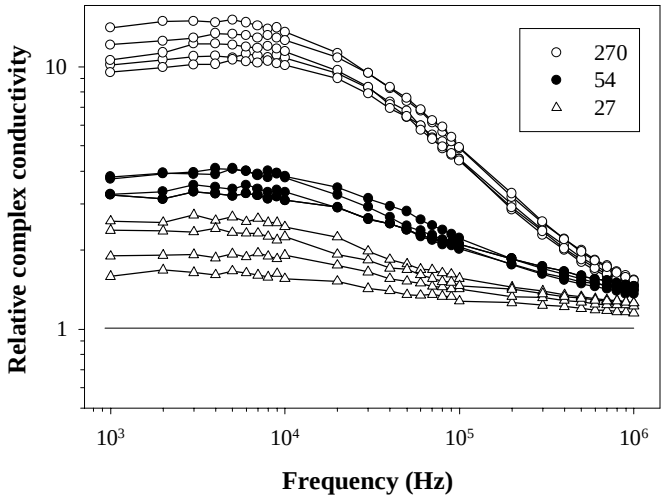

Fig. 4. *Change in relative complex conductivity of the PEF-treated samples versus frequency (log scale) with the indicated number of pulses. Replicate experiments shown.*

The diffusion kinetics of red pigments and ions from PEF-treated tissues into the isotonic solution are given in Figs. 5 & 6. As expected, the kinetics of ionic species was 3-4 times faster than that of pigments.

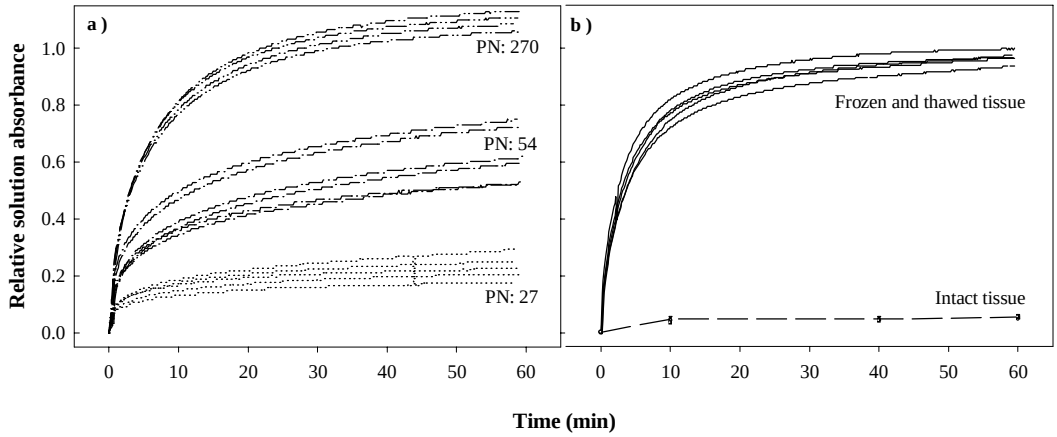

Fig. 5. *Kinetics of color extraction. a) PEF treatment with the indicated number of pulses and b) freezing/thawing. Replicate experiments shown.*

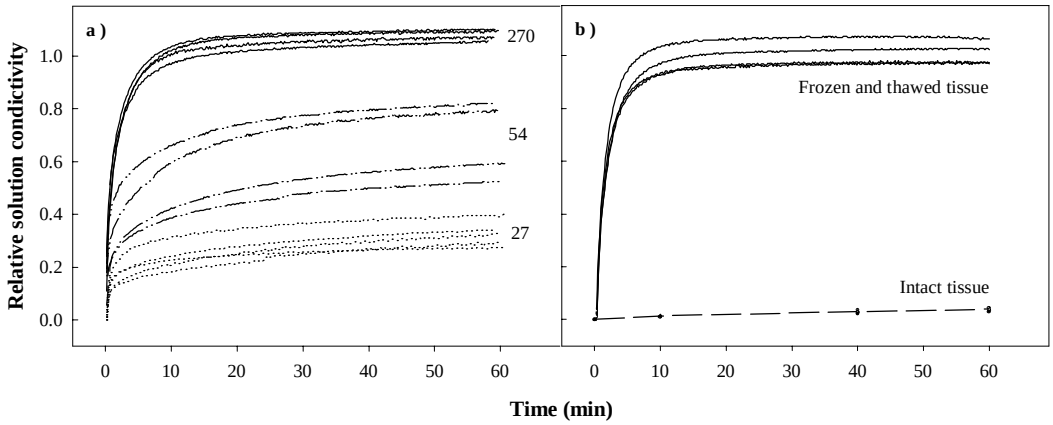

Fig. 6. *Kinetics of ionic species extraction. a) PEF treatment with the indicated number of pulses and b) freezing/thawing. Replicate experiments shown.*

3.1. Extent of extraction

The extraction values of the samples subjected to 270 pulses after 60 min, re-extraction values of all the PEF-treated samples after freezing and thawing and those of mechanically pressed samples are presented in Fig. 7. Freezing/thawing stage of PEF-treated samples resulted in lower color and conductivity values than before freezing (Fig. 7). In supplementary experiments on freezing of beetroot juice, it was shown that there was a 10% loss of color and

conductivity, on average, due to the freezing /thawing cycle. Thus, the values exceeding 1 in Figs. 5 & 6 were attributed to these losses.

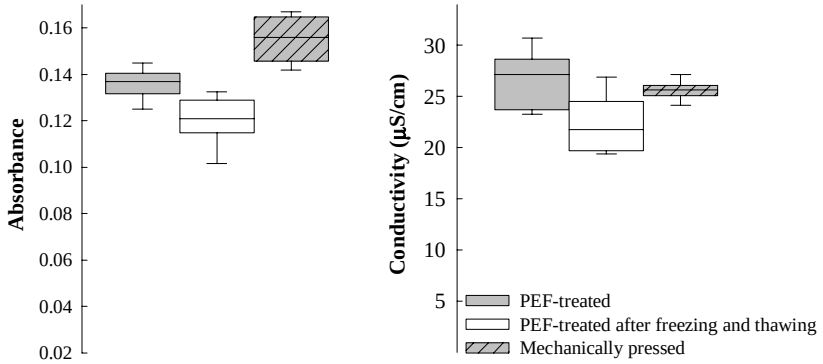

Fig.7. *The absorbance and conductivity after 1 hour of aqueous extraction following PEF treatment (270 pulses), PEF treatment followed by freezing/thawing and mechanical pressing.*

The extraction levels after the mechanical pressing and freezing, which disintegrated the tissue almost completely, also indicated the magnitude of the variations in the pigment and ionic composition of the tissue sampling region. The mechanically pressed samples yielded an absorbance increase of 0.156 ± 0.011 (mean ± SD) and a conductivity increase of 25.6 ±1.1, mS/cm. The color extractions from the PEF-treated samples (A= 0.138±0.008 and 26.60 ± 3.07) were found to be significantly different from mechanical pressing (P=0.025). The (non-significant) trend of higher conductivity of PEF-treated samples, and the significantly larger variance (P=0.54) may be related to the electrolysis between electrode and tissue surface at the relatively high total charges transferred. In total, the samples treated with 270 pulses resulted in an extraction level of about 90 % of the coloring matter, compared with mechanical pressing. This was qualitatively confirmed by microscopy analysis Fig. 8. Comparing the size of the colored domains in the right panel and the cell sizes in the left panel, it appears that, in agreement with theory (Zimmermann, 1986; Ho & Mittal, 1996), the smaller cells were not permeabilized at the PEF conditions employed.

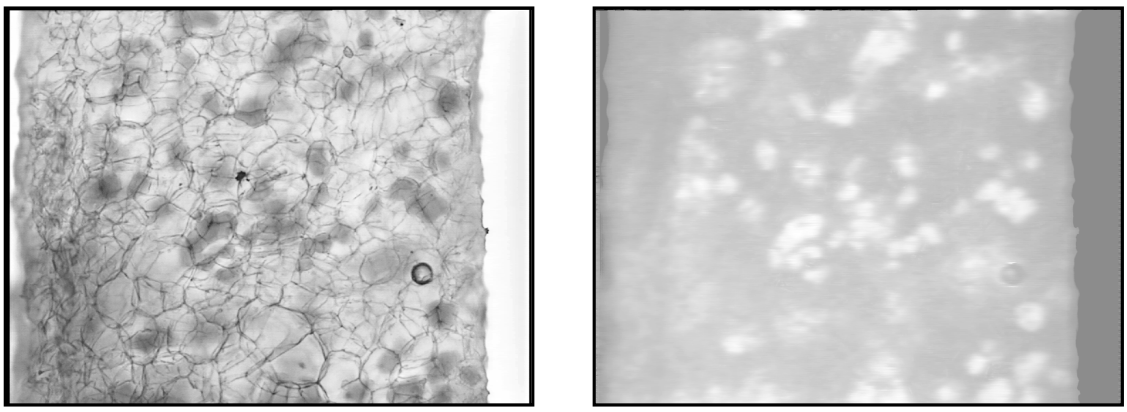

Fig. 8. *Cross section of a PEF-treated sample (270 pulses) after extraction. Left panel lightness component; right panel hue component; matching dark areas in the right panel and light areas in the left panel correspond to the intact cells containing red beetroot color (Cell walls were stained with tryptophan blue).*

The relationship between pigment and ionic release as a function of treatment intensity is given in Fig. 9. The fitted linear relationship did not pass through zero. This could be interpreted as extraction of ionic species from the apoplast with no concomitant extraction of the intercellular betanins. Beyond this initial level, the amount of pigment extracted was found to be directly proportional to the release of ionic species, indicating that

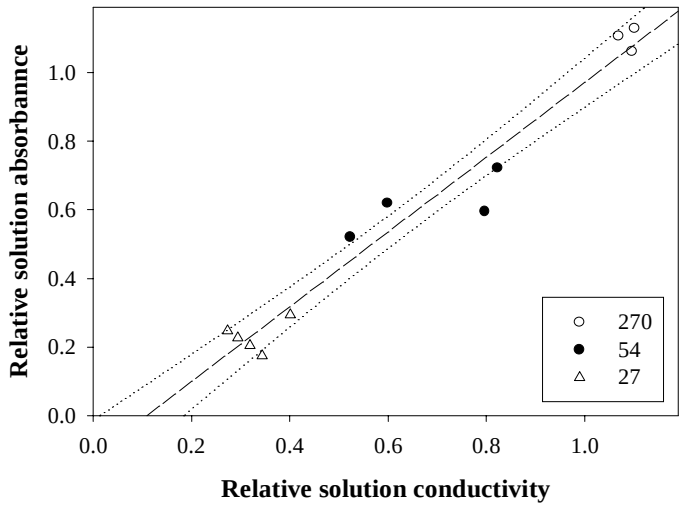

Fig. 9. *Absorbance of the solution versus conductivity of the solution at the end of 1 hr aqueous extraction following PEF treatment with the indicated number of pulses. (dashed line: linear fit, dotted line: 95 % confidence interval)*

at the conditions studied there was no differential permeabilization of any of the intracellular compartments. The higher degree of variation of the extractability of color and the conductivity observed with intermediate PEF treatment was interpreted as being related to the non-homogenous permeabilization of the cells in tissue due, for example, to variations in cell size.

3.2 Conductivity-based tissue damage index as a predictor of the extent of extraction

The relative increase in tissue conductivity resulting from three different levels of PEF treatment versus corresponding relative pigment and ionic release at the end of 1-hour of extraction is presented in Fig. 10.

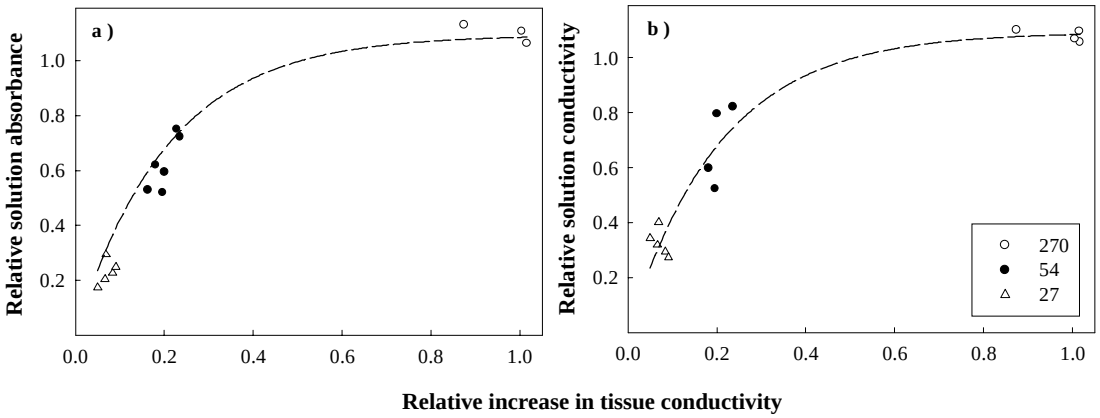

Fig. 10. *a) Absorbance and b) conductivity of the solution at the end of 1 hour of aqueous extraction versus relative change in tissue conductivity following PEF treatment with the indicated number of pulses. (dashed line: exponential fit to guide the eye)*

The changes in beetroot tissue conductivity after PEF were correlated with the changes in both pigment and ionic release in aqueous extraction. However, the relationship was not linear over the whole tissue conductivity range. The data suggest that the degree of extraction is not markedly increased for the index values higher than 0.4- 0.5. Why this should be so is not obvious. Theoretically, additional electrical pulses will not significantly affect cells or organelles that have already been ruptured, because no potential gradient is developed over their membranes, and therefore if a PEF treatment increases tissue conductivity it should lead

to concomitant increase of extractability. A possible explanation of the observed effect is that for low permeabilization levels, tissue conductivity measurements at 3-4 min post-permeabilization underestimate the equilibrium tissue conductivity by a factor of 2-3.

3.3 Energy cost of effective PEF treatment

The energy consumption (Knorr & Angersbach, 1998; Zhang, Barbosa-Cánovas, & Swanson, 1995) of the used treatments based on the tissue resistance in Fig. 2 is calculated in Table 1. Even at the maximum treatment of 270 pulses, the energy consumption for permeabilization corresponds to some 7 kJ/kg, i.e. energy corresponding to heating of the tissue by some 2 ^{o}C.

Field strength (kV/cm)	**PN**	**PL** (μs)	**Energy** (kJ/kg)
1.0	1	10	0.02
	27		0.67
	270		7.20

Table 1. *Approximate energy consumption during PEF treatment.*

4. Conclusions

PEF treatment at 1 kV/cm was shown to be an effective method of permeabilization for the extraction of pigment from beetroots, with a low energy consumption of about 7 kJ/kg. The treatment made both the pigment and ions of the tissue equally extractable, so extraction conductivity is a good measure of the progress of pigment extraction. The tissue damage index based on post-PEF tissue conductivity, was linearly correlated with extractability of the tissue only at relatively low levels of tissue damage.

Acknowledgement

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Pulsed Electric Field Treatment for Solid-Liquid Extraction of Red Beetroot Pigment: Mathematical Modelling of Mass Transfer

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Abstract

The extraction of red pigment and conductive matters from individual red beet tissue slices following pulsed electric field pretreatment of different durations was compared with a two dimensional bimodal Fickian diffusion model. The process appeared to be governed by two apparent diffusion coefficients, one slow and one fast. The values of these coefficients were independent of the duration of treatment, but the yield associated with each coefficient varied. Following the treatment of longest duration and treatment by freezing and thawing of the tissue, the yield from the faster diffusion coefficient accounted completely for the mass transport. The faster apparent diffusion coefficient was 2 to 5 times smaller than the diffusion coefficients in dilute solution.

Key words : Fickian diffusion; Bimodal; Red beet; Diffusion coefficient; Pulsed electric field.

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1. Introduction

One of the traditional methods of extracting some interesting intracellular components from plant tissues is based on solid-liquid extraction. The amount of the compounds released into the external liquid is characterized by the degree of cell disintegration, which influences the efficiency of the extraction process. There are various methods of achieving a high level of disintegration of the cell such as mechanical compression, comminution, thermal treatment and chemical treatment.

The rate of mass transfer during extraction is of interest for process design. In the case of plant tissues the structurally dependent diffusion, which is usually called apparent or effective diffusion, can often be modeled by Fick's second law of diffusion. This approach has been used with regard to both the translocation of the solutes and water from the tissues into the extraction liquor (Vukov, 1977), and the washing out of the sap from cells injured along the cutting surface.

High-and moderate-intensity pulsed electric fields may offer a way of disintegrating the cell membrane and have been considered as an alternative, non-thermal pretreatment (Fincan, DeVito & Dejmek, 2003). A number of studies have focused on enhancing the rate of mass transfer during fruit and vegetable processing (Taiwo, Angersbach & Knorr, 2002; Ade-Omowaye, Rastogi, Angerbach & Knorr, 2002; Bazhal & Vorobiev, 2000). Mild pretreatment with a pulsed electric field during osmotic dehydration to accelerate the loss of water from the plant tissues has been studied (Rastogi, Raghavarao, Niranjan & Knorr, 2002; Rastogi, Eshtiaghi & Knorr, 1999). Juice extraction coupled with electrical treatment in the sugar industry showed its high performance to obtain the highest possible content of sucrose in the juice from sugar beet at a low cost processing (Eshtiaghi & Knorr, 2002).

In the first part of our work (Fincan, DeVito & Dejmek, 2003), we studied the effect of electrical treatment in plasmolyzing the red beet tissues on the yield and kinetics of the release of red pigment and ionic components. In this, the second part, efforts were made to quantify the mass transfer phenomenon influenced by the electroplasmolysis in the red beet tissues. Early it became obvious that the mass transport could not be described by a single diffusion

coefficient. The calculations based on Fickian diffusion equations were thus performed with the modification of bimodal diffusion approach (Jason & Peters, 1973).

2. Materials and methods

2.1 Materials and sampling

Fresh red beetroot tubers (*Beta vulgaris*) were bought from the local market. Disks of tissues, 1 mm in thickness and 4 mm in diameter, were sampled from the homogeneously red outer third ring region (parenchyma cells) using a cylindrical cork borer and parallel sharp blades. Conductive debris was removed from all surfaces by washing with deionized water at room temperature for 1 minute. Following the washing step, the sample was placed in a treatment chamber consisting of parallel stainless steel electrodes with a gap of 1 mm.

2.2 Pulsed electric field treatment

Nine monopolar, rectangular electric pulses at a constant field strength of 1 kV/cm, with a pulse length of 10 μs and an interpulse delay of 20 ms, were applied to the tissue in the treatment chamber (Cellect electromanipulation instrument, BioFusion SCI AB, Sweden). Pulse trains were manually triggered in rapid succession to achieve treatments with a total number of pulses (PN) of 27, 54 and 270.

2.3 Release of conducting species and red pigment

The release of ions and red pigment was measured immediately after submitting the samples in 7 ml 0.25 M mannitol solution (Eastman Kodak Company, Rochester, N.Y., USA). An integrated system consisting of a temperature-controlled container, a spectrophotometer

(Hitachi, U-1500, Japan) and a conductivity meter (Radiometer, CDM210, Copenhagen, Denmark) connected to a computer was used to measure the values of conductivity and absorbance of the solution every 10 seconds for 60 minutes.

2.4 Theoretical considerations

A homogeneous solid cylinder, initially with a uniform concentration, is suddenly immersed in a well-stirred liquid. We wish to determine the diffusion coefficient of the solute in the solid by observing the change in its concentration in the surrounding liquid with time. The mass transfer from the solid phase to the liquid phase is assumed to be dominated by the internal resistance, i.e. the resistance due to the liquid boundary layer adjacent to the surface is neglected.

For a finite cylindrical geometry, the analytical solution can be determined by making use of the superposition principle, as the product of the infinite slab solution and the infinite cylinder solution (Crank, 1975; Carslaw & Jaeger, 1959).

For an infinite slab with a thickness of 2 l, the diffusion equation can be written as (Crank, 1975):

$$\frac{\partial C}{\partial t} = D_{app} \frac{\partial^2 C}{\partial x^2} \qquad \text{Eq. (1)}$$

with the following initial and boundary conditions, for the well-agitated unlimited volume of the bulk solution:

for $t = 0$ $C = C_0$

for $t > 0$ $C = 0$ at $-l < x < l$

The solution of Eq. (1) is:

$$\Phi = \frac{C}{C_0} = \frac{4}{\pi}\sum_{n=0}^{\infty}\frac{(-1)^n}{(2n+1)}e^{-Dapp(2n+1)^2\pi^2 t/4l^2}\cos\frac{(2n+1)\pi x}{2l} \qquad \text{Eq. (2)}$$

where C_0 is the initial concentration of the solute, l is the half-thickness of the slab, t is the diffusion time and D_{app} is the apparent diffusion coefficient of the solute.

For a long cylinder with a diameter 2a, assuming that diffusion takes place in the radial direction, Fick's second law can be written as:

$$\frac{\partial C}{\partial t} = \frac{1}{r}\left[\frac{\partial}{\partial r}\left(rD_{app}\frac{\partial C}{\partial r}\right)\right] \qquad \text{Eq. (3)}$$

with the following initial and boundary conditions :

for $t = 0$ $\quad C = C_0$

for $t > 0$ $\quad C = 0$ at $r = a$

The solution of Eq. (3) is:

$$\Psi = \frac{C}{C_0} = \frac{2}{a}\sum_{n=1}^{\infty}e^{-Dapp\alpha_n^2 t}\frac{J_0(r\alpha_n)}{\alpha_n J_1(a\alpha_n)} \qquad \text{Eq. (4)}$$

where r is the radius of the cylinder, J_0 is the Bessel function of the first kind of zero order, J_1 is the Bessel function of the second kind of first order and α_ns are the positive roots of

$$J_0(a\alpha) = 0 \qquad \text{Eq. (5)}$$

The solution for a finite cylinder with length, l and radius, a can be written as the product of the two functions Φ and Ψ given by Eqs. (2) and (4) (Carslaw & Jaeger, 1959):

$$\frac{C}{C_0} = \Phi(x,l)\Psi(r,a) \qquad \text{Eq. (6)}$$

As a consequence of superposition, the concentration of the solute at any point in the cylinder can be determined by Eq. (6).

Determination of the concentration in the external solution

The concentration in the external solution can be evaluated by using mass balance:

$$C_0 V_{\text{int}} - C_{\text{int},ave} V_{\text{int}} = C_{ext} V_{ext} \qquad \text{Eq. (7)}$$

$$C_{ext} = A(1-f) \qquad \text{Eq. (8)}$$

where $C_{\text{int},ave}$ is the average concentration in the cylinder, C_{ext} is the concentration in the external solution, V_{int} is the volume of the cylinder, V_{ext} is the volume of the external solution, A is $C_0(V_{\text{int}} / V_{ext})$ and f is the average superposed concentration in the cylinder.

Bimodal diffusion

We have shown previously (Fincan & Dejmek, 2002) that the pulsed electric field (PEF) permeabilization of tissue is not uniform. A simplified model accounting for the non-uniformity would, following Jason & Peters, be bimodal diffusion. In this model the tissue is

seen as an interconnected system of two tissues, each with its own concentration and corresponding diffusion coefficient, and transport occurs along parallel but separate paths, with no interaction. If both tissues have the same initial solute concentration, and H is denoted as the relative fraction of tissue type 1, it follows from mass balance that the observed concentration in Eq. (8) must be modified as:

$$C_{ext} = C(H(1-f_1)+(1-H)(1-f_2)) \qquad \text{Eq. (9)}$$

where f_1 and f_2 are the average calculated concentrations in tissues 1 and 2, corresponding to the diffusion coefficients $D_{app,1}$ and $D_{app,2}$, respectively.

Evaluation of the diffusion coefficients

A Matlab subroutine was written to evaluate the series analytical solution of the finite cylinder problem, Eq. (6), on a radial $a/10$ and axial $l/10$ grid and to calculate the average concentration at each of the 100 grid points. Least-squares fitting of Eq. (9) to the observed external solution concentrations was performed using FMINSEARCH routine for multidimensional unconstrained nonlinear minimization (Matlab version 6.1.0.450 Release 12.1, MathWork, Inc., MA, USA) with the default values of the parameters. The four-parameter fitting was carried out in order to find C, H, $D_{app,1}$, corresponding to f_1 and $D_{spp,2}$, corresponding to f_2. Note that C is the maximum amount extracted for the individual samples and H is the fractional amount extracted corresponding to $D_{app,1}$.

3. Results and discussion

Examples of plots of the fitted results versus experimental data are shown in Figs 1 and 2.

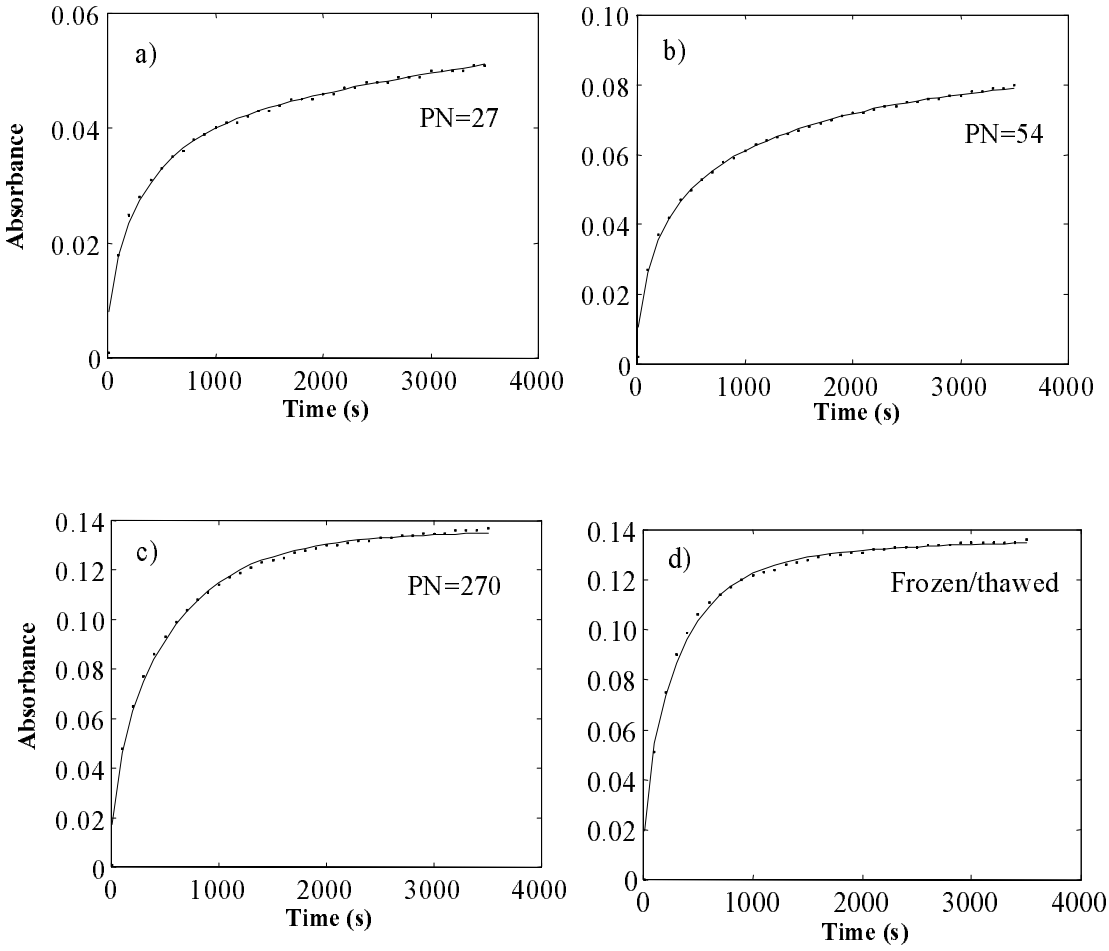

Fig. 1. *The typical plots of experimental data and fitted results for the release of red pigment following different duration of PEF treatment. a) 27 pulses, b) 54 pulses, c) 270 pulses and d) frozen/thawed. The points represent the experimental data and the solid lines represent the fit by the model.*

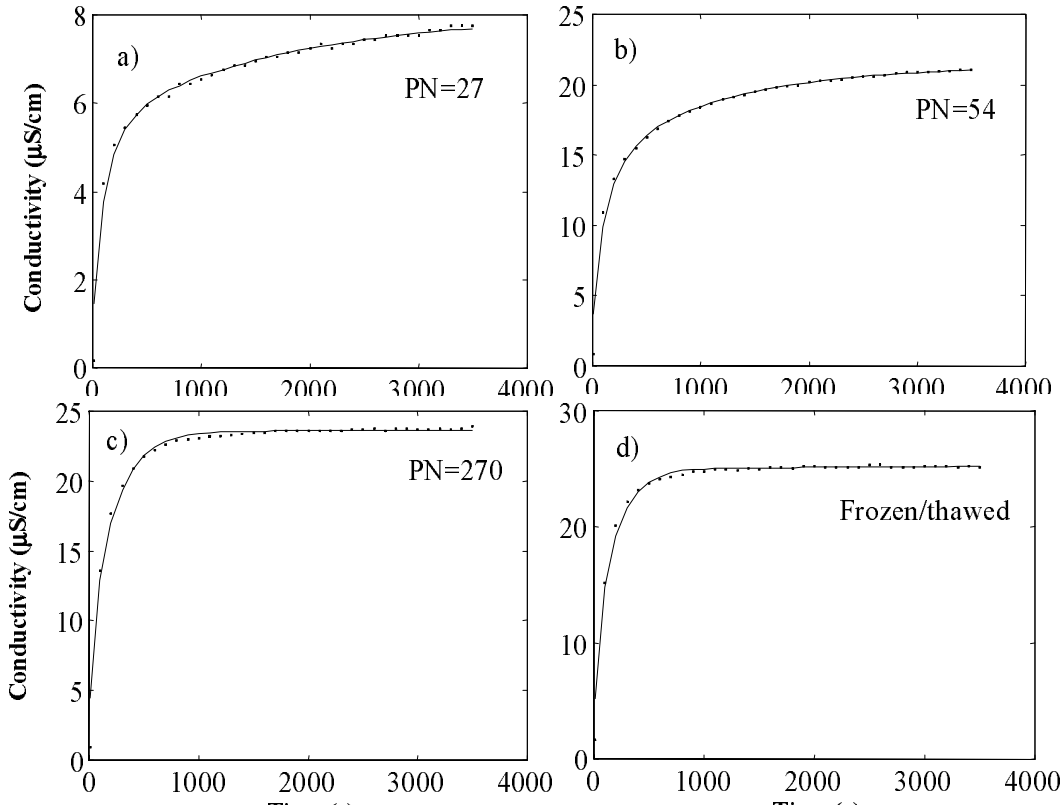

Fig. 2. *The typical plots of experimental data and fitted results for the release of ionic species following different duration of PEF treatment. a) 27 pulses, b) 54 pulses, c) 270 pulses and d) frozen/thawed. The points represent the experimental data and the solid lines represent the fit by the model.*

3.1 Effects of a PEF on mass transfer

In the first part of our work (Fincan, DeVito & Dejmek, 2003), the tissue conductivity was measured prior to PEF treatment and 4 minutes after PEF treatment. The magnitude of the damage in the tissue was characterized by the change in conductivity of the tissue. The relative increase in tissue conductivity was defined as (Fincan, DeVito & Dejmek, 2003):

$$\text{Relative increase in tissue conductivity} = \frac{(K'_{l5} - K_{l5})}{(K'_{l5f} - K_{l5})} \qquad \text{Eq. (10)}$$

where and are the conductivity of the samples at 5 kHz before and after treatment, respectively, and K'_{l5f} is the average conductivity of the samples obtained following the application of the maximum level of treatment that could be reached. The diffusion coefficients ($D_{app,1}$ and $D_{app,2}$) were then plotted against relative increase in tissue conductivity, and can be seen in Figs. 3 and 4.

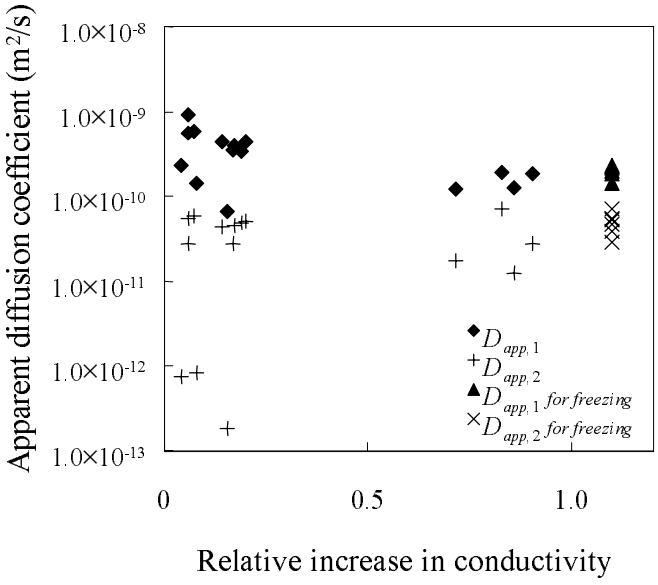

Fig. 3. *The distribution of the apparent diffusion coefficients of red pigment over the range of PEF treatment in terms of relative increase in conductivity.*

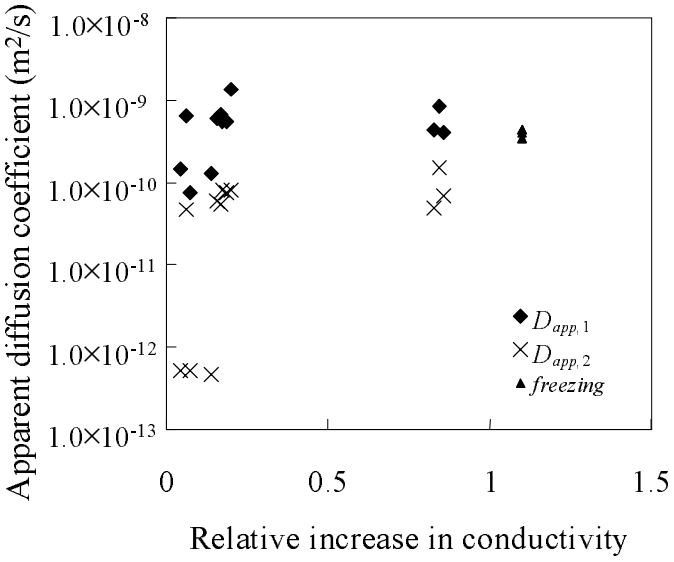

Fig. 4. *The distribution of the apparent diffusion coefficients of ionic species over the range of PEF treatment in terms of relative increase in conductivity.*

It can be seen from these figures that the increase in the duration of electrical treatment does not affect the rate of mass transfer, as the values of the diffusion coefficient are more or less the same. This was observed in both the diffusion of red pigment and ionic species. It should be borne in mind that, as shown in the previous paper, the total amount of extractable matter, i.e. the process yield, increased with the duration of treatment.

The results of the simulation gave us the fractional amounts of the species extracted by the fast and slow processes of diffusion, which are shown in Figs. 5 and 6. These results showed the contribution of the two kinds of diffusion to the total observed concentration in the external solution. At short and medium treatment (PN=27 and PN=54), most of the components were extracted by the contribution of the slow process while the fast process played the major role in the extraction following long-duration treatment (PN=270 and when the tissue was frozen and then thawed).

There is a good correlation between the fractional amounts of red pigment and conductive matters extracted by the faster and slower process, confirming our expectation that red pigment and ionic species are extracted from the same part of the tissue.

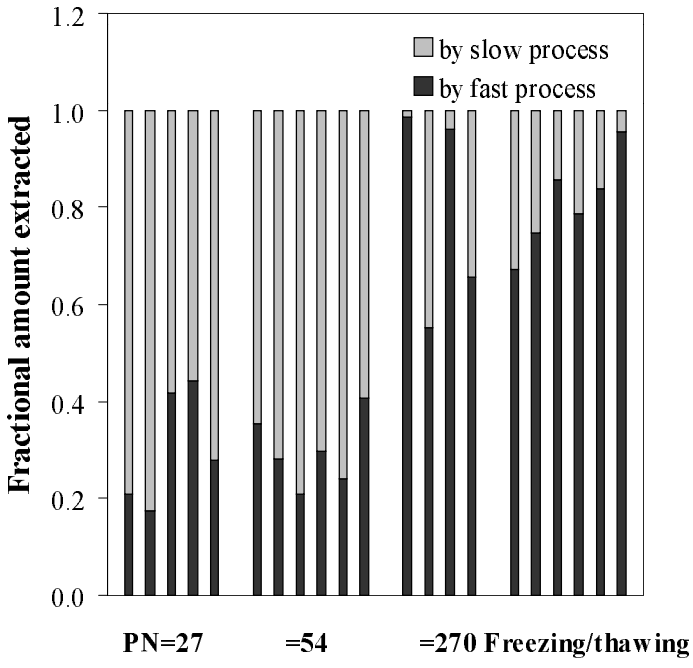

Fig. 5. *The extractability of the red pigment by the two different diffusion processes, fast and slow.*

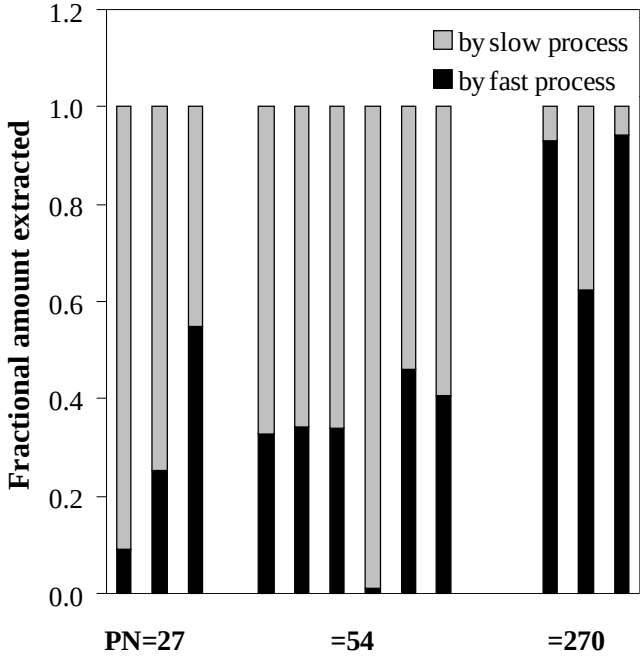

Fig. 6. *The extractability of the ionic species by the two different diffusion processes, fast and slow.*

3.2 Analysis of calculated diffusion coefficients

The major red pigment component (75-95%) in red beetroot is betanin (Simon, 1993) which has a molecular weight of 564 g/mol. Therefore, the diffusion coefficients of betanin can represent those of the red pigment. The diffusion coefficient for sucrose (MW=342 g/mol) in dilute aqueous solution is 0.52×10^{-9} m^2/s (Lewis, 1990). To a first approximation, diffusion coefficients scale with the inverse radius of the diffusing species, or the inverse cubic root of molecular weight, so we would expect the diffusion coefficient for betanin in dilute aqueous solution to be 0.45×10^{-9} m^2/s. Following the longest treatment (PN=270), the values of the diffusion coefficients were in the range $1.2 \times 10^{-10} – 1.9 \times 10^{-10}$ m^2/s, i.e. 3-4 times lower. Apparent diffusion coefficients even in fully permeabilized plant tissue are expected to be hindered because of the decrease in the effectively accessible solvent volume and because of path tortuosity, which is particularly affected by the relatively dense cell walls and small pores. In blanched potato tissue, Anderson, Gekas & Öste (1994) found hindrance factors of the order of 2. In other published data, the tissue anatomically closest to red beetroot would be sugar beetroot. The diffusion coefficient of sucrose (MW=342 g/mol) in sugar beet

plasmolyzed by mechanical shear stress and strong pressure (Vukov, 1977), was found to be in the range 0.7×10^{-10} to 1.23×10^{-10} m^2/s i.e. a hindrance factor of 4, which is in reasonable agreement with our data. However diffusion coefficients might be different following different methods of plasmolysis (Vukov, 1977).

The diffusion coefficient in water at infinite dilution of potassium ions, which are the most common ions in red beet and considered as the representative ions in the beet, was calculated and found to be 1.75×10^{-9} m^2/s. This calculation was based on the values for chloride ions, reported by Anderson, Oliveira, Gekas & Öste, (1994) and for potassium chloride salt by Geankoplis (1993), using the formula reported by Cussler (1984). Following harsh treatment, we found the diffusion coefficients of ionic species to be 4.0×10^{-10} m^2/s to 8.5×10^{-10} m^2/s. They were then compared to those of potassium ions calculated and a hindrance factor of 2-4 was found, more or less the same as for betanin.

Our results suggest that the level of PEF treatment determined the fraction of tissue that was permeabilized, and thus determined the yield. When the kinetics of extraction was described by bimodal diffusion, the fastest transport mode, corresponding to fully permeabilized tissue, was often, but not always, observed following low treatment levels corresponding to a 5-15% increase in relative conductivity. The fraction of the total yield resulting from the fast transport increased with treatment duration. At maximum treatment duration and in frozen and thawed samples, only this fast mode was observed and the extraction was satisfactorily described by a single diffusion coefficient, with a hindrance factor of 2-5.

4. Conclusions

The mass transfer in red beet root treated with pulsed electric fields during solid-liquid extraction can be described satisfactorily by bimodal Fickian diffusion. At high degrees of permeabilization achieved by pulsed electric fields or freezing/thawing, a single mode is sufficient. Two processes of diffusion resulting from partial permeabilization allowed us to determined diffusion coefficients by fitting the model to experimental data. The diffusion

coefficients were approximately constant following electrical treatment of varying duration, but the yield from the slower one decreased.

Acknowledgement

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