Possibilities of using Pulsed Electric Field in leaf bio-refinery:

Influence on protein extraction and Pulsed Electric Field efficiency

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Possibilities of using Pulsed Electric Field in leaf bio-refinery: influence on alkaline protein extraction and leaf protein quality

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Summary

Pulsed Electric treatment is an electroporation technique. Pulsed electric field treatment can potentially increase extraction values. Fresh grass and freeze dried grass were both investigated. Extractions were one with water, phosphate buffer and NaOH solutions. PEF treatment was done in demi-water. Field strength, capacitor strength and resistance were viewed as parameters and varied. Strong electric fields and high ion concentration in the sample have high impact on PEF effectiveness. RC time gives feedback on pulse effectiveness quickly. PEF increase protein extraction values from fresh grass. Dried, low water or low ion concentration samples will not react well to PEF. RC time shows that these samples have less effective pulses.

Chapter 1: Introduction

For a long time, plants have been grown for their compounds. However, using plants for the production of proteins is relatively new. In the 1960s plants have first been viewed as a source for the production of protein. Grass is an abundant resource that is naturally occurring on almost every area of the World. Its abundance and ease of growth makes it an attractive crop for bio-refinery. Bio-refinery of grasses will mainly yield sugars, mainly as fibres, and leaf proteins[2]. Leaf protein has high nutritional value and is commercially attractive for food and feed industries. With the growth of the human population, novel sources of protein are needed. The advantage that leaf protein extract has over consuming the plant itself is the ease of digestion. As mentioned before, bio-refinery will separate protein and fibres and hereby increase the digestibility, and therefore protein can be digested more efficiently without fibres. This protein can then be used in food and in animal feed. Whole plant bio-refinery and leaf bio-refinery are both possible for grass. Leaf bio-refinery is the preferred method, since the leaf is the part of the plant that is the easiest to harvest. Furthermore, the meristem, the part where new cells are formed, is located at the bottom of the leaf. Regeneration after harvest of grass is more efficient this way.

The focus of this research is on the extraction of protein as could be used in a grass bio-refinery. The weight of the protein yield as percentage of the total weight of the treated grass is the most important factor in this case. Digestion and yield of cellulose, pectin and extractives would be important factors for a set-up of a whole bio-refinery. However, as protein yield is the main factor in this paper all other substances will be treated as either an impurity or a hindrance.

Historically protein extraction from grass can be done by pressing out its aqueous fraction. Proteins, sugars, oils and cell components are present in the aqueous fraction. Evaporating the liquid yields a lot of proteins and other dissolved components[1]. The solid fraction contains of mainly lignocellulose, most of which can be processed further[3]. A different way to extract protein from grass is by treatment with alkaline solution. Alkaline treatment is most effective on ground or chopped grass. Alkaline extraction of leaf protein is shown to be an effective way of extraction high concentrations of protein[4]. Its drawbacks are that the process either takes a long time or uses large amounts of alkali material. Furthermore,

alkaline extraction yields a colour formation through various reactions. Racemization can also occur at higher alkaline concentrations.

Proteins are partially soluble in water. An increase in pH, up to a pH of 8, increases protein solubility. Alkaline material increases the pH, which increases diffusion of protein from inside to outside the cell. Alkaline solutions also solubilise lignin, which makes extraction more rapid. If diffusion of the wanted material increases, the effectiveness of the extraction also increases. Increase in diffusion can be done by Pulsed Electric Field (PEF), a technique that uses an electric field for a wide array of applications. Usage on cells allows for disruption of cell membrane, creating pores[5]. PEF can also be used to lyse cells, due to its disruptive nature. PEF might coagulate proteins with sulfuryl sidechains[6]. Depending on the method of extracting proteins, PEF could also help increasing yield[7]. The pore formation of PEF allows more interaction between the extraction fluid and the intercellular components. The turgor pressure in fresh plant cells in combination with PEF increase the flux from inside to outside the cell. Both of these effects could increase extraction.

The main aim of this research is to investigate if PEF affects protein extraction yield when used on grass. Furthermore, optimal conditions for extraction after PEF treatment are a major concern. The following points will be investigated:

1. What parameters in PEF treatment influence extraction?
2. Does the material, which is to be PEF treated, affect the extraction?

The initial experiments will be used to investigate previous work as well as becoming familiar with the techniques used. The initial experiments will also give a baseline for different solvents. PEF will be introduced as a pre-treatment after the different solvents have been investigated. After these steps, PEF will be used as a pre-treatment, while extraction will be done with different solvents. Lastly, the influence of the sample material is investigated.

PEF could possibly assist in the extraction of the compounds that amount to the colouring of proteins. For the extraction part PEF could also be viewed as an extraction technique. If the pores formed by PEF can be kept at specific diameters the cell walls and membranes could act as a filter. The PEF formed pores' size could be increased after the extraction of the small compounds to increase diffusion of protein to the solvent. Increasing the pore size might

also lead to a reduced need for strong alkaline conditions. Less alkaline for the extraction of proteins will also lead to less chirality changes and makes the harvested protein more digestible in general.

Chapter 2: Theory

This thesis utilizes a set of different techniques with a focus on pulsed electric field technology. This technique has a wide application range and different effects on materials. The technique dates back to the 19th century and has been used in different processes with varying results. The application of PEF is widely reported on for its use in bacterial transformation. Its use on multicellular organisms is not well documented.

2.1 Pulsed Electric Field

The first time a paper has been published on the impact of electric currents on cells was in the 1890s. The paper reported on the effect of electric fields on solutions with bacterial cells. Observed was that the bacterial cells were killed by discharging an electric field. Over the years different applications were created that use PEF. Strong electric fields have the ability to kill (micro-) organisms in the solution. These fields could kill up to 95% of the organisms in the solution[8]. On the other hand PEF is used to create pores which allow foreign DNA uptake by micro-organisms.

2.1.1 History

In the late 1930s milk pasteurized by electric treatment was being sold. Early work on plant cells was done by Flaumenbaum who published his findings on electro plasmolysis in 1949[9]. Plasmolysis means the separation of cell wall and cell membrane. Plasmolysis occurs after a plant cell has lost water and the cell shrinks. In electro plasmolysis this process is done by an electric field. After 1960 most research began on the effects of electric fields on micro-organisms in solution. The resulting technique was called electrohydraulic treatment. Electrohydraulic treatment uses two electrodes to send high voltage pulses through a liquid medium, which disrupts the cells in it. The electrohydraulic treatment had however multiple secondary effects, mainly arcing of electric pulses. The arcs make electrohydraulic treatment unreliable, as samples can be mistreated as a result. This technique was never used in large industrial applications because of these effects.

The problems that had arisen with the electro plasmolysis were solved a few years later by using homogeneous pulses without arcing. This new technique was dubbed pulsed electric field. It doesn't have the drawbacks that electro plasmolysis has and is thus overall more reliable. The permeabilization that occurred when using PEF was first reported by

Doevenspeck in 1975[10]. Differences between soft, below 2 KV/cm, and hard pulses, which resulted in cell death, were first distinguished at that time as well. Soft pulses are used to make membranes permeable for short amount of time, while hard pulses are used to disrupt cells completely. This discovery gave way for different applications of the same technique. Either permeability or sterilisation could now be achieved. Doevenspeck patented the PEF technique that he created in 1960. This machine is essentially a high voltage pulse generator that stores energy in a capacitor. The capacitor can be discharged over a treatment chamber evening out the charge over the cell membrane.

2.1.2 Pore formation

Application of PEF results in the formation of pores. Higher field strengths will result in higher charges over cell membranes. High potential differences over the membrane will result in a more disruptive form of pore formation. Higher potential differences will result in larger pores. A longer pulse length will cause the potential difference over the membrane to discharge over a longer time. A longer time to discharge the capacitor will result in a less acute force that disrupts the membrane. This will result in smaller pores. Combining low field strength with a high pulse length will result in small pores. A strong field with an acute discharge will create larger pores and will have more disruptive force[5].

Nowadays, electroporation is used to make cells temporarily permeable. It is mainly used on bacterial cells to introduce DNA to that cell. Although a lot of cells are destroyed, the technique has the desired effect. The gene transfer aspect and the sterilisation aspect of PEF will not be reviewed in this paper. The research on the effects of PEF on plant cells is limited. It is however still unclear how this technique specifically influences plant cells. It is however known that PEF will form pores in cells and that can be used.

2.1.3 The cellular disintegration index

The primary method to measure the amount of damage done to the cell wall is by the Z_p value. The Z_p is a measurement on basis of the conductivity or your sample. The Z_p was defined by Rogov and Gorbatov in 1974[11] and is as follows:

$$Z_p(t) = \frac{\sigma(t) - \sigma_i}{\sigma_f - \sigma_i}$$

$$Z_p(t) = \frac{\sigma(t)-\sigma_i}{\sigma_f-\sigma_i} \quad (1)$$

$$0 \leq Z_p(t) \leq 1 \quad (2)$$

Where σ(t) is the measured electrical conductivity at low frequency (~1 kHz), σ_i is the initial conductivity, the conductivity of the non-treated sample, and σ_f is the final conductivity, the conductivity of the totally destructed sample. σ(t), σ_i, σ_f all in $\Omega^{-1}m^{-1}$. The Z_p value can give information on the state of the PEF treated sample[12]. If the measured Z_p is close to 1 almost all cell walls will be permeable. A Z_p closer to 0 the sample is almost completely intact. A strong electric field will have a higher Z_p associated with it than a weak electric field[13]. The Z_p is a measurement over the total process.

2.1.4 Resistor-Capacitor time constant

The RC time constant has a multitude of uses, mainly in the field of electronics. The RC time constant is defined as the time it takes to charge or discharge a capacitor of an RC circuit. In such a circuit the RC time constant is found by multiplying the resistance and the capacitor strength.

$$\tau = R * C \quad (3)$$

In which τ , the RC time constant in seconds. R, the resistance, in Ω. C, the capacitor strength in μFD. The RC time gives information about the effectiveness of one pulse in PEF, instead of the overall effectiveness which is information given by the Z_p. The RC time constant depends on capacitor strength and resistance[12].

2.2 Composition of grass

The most important substances for bio-refinery will be reviewed in the following paragraphs. Most important in this case means substances that could either hinder extraction or digestion in the bio-refinery or substances that are important products of grass bio-refinery.

All of these paragraphs will deal with one substance or one group of substances. After a short introduction the different aspects of each substance or group of substances will be viewed.

2.2.1 The cell wall

The first obstacle in every extraction from plants is the cell wall. This physical barrier specifics vary per species, but almost all cell walls consist of cellulose, hemicellulose and lignin[14, 15]. Pectin secures the different cells to each other. This combination gives plants their strength and in some plants also flexibility. The importance of the cell wall cannot be overlooked when working with plants. Cell walls are sturdy barriers that defend plant cells from outside threats. Cell walls might have to be digested or destroyed before extraction to be effective.

2.2.1.1 Cellulose

The organic substance cellulose is a glucose polysaccharide which consists of hundreds linked D-glucose units in a linear chain. Cellulose is produced mostly by plants and algae as a means of support for their internal structure and, e.g. in trees, their external structure. Cellulose is, as opposed to other glucose based polymers, hard to digest and near insolvable in conventional solvents. Ruminants, as well as some beetles, termites and fungi, have the necessary enzymes to digest cellulose. Nearly all of these ruminants and insects use micro-organisms to fully digest cellulose.

Currently, cellulose is used mainly in the production of paper and cardboard and the production of different polymers. Cellulose is the most abundant organic polymer on the earth. Its availability makes it an interesting substance for bio-fuel production. However the low digestibility of cellulose is the main challenge in this field.

2.2.1.2 Hemicellulose

In contrast to cellulose, hemicellulose is a group of heteropolymers. These polysaccharides consist of xylose, mannose, galactose, rhamnose, and arabinose in addition to glucose. While cellulose is stable and strong, hemicellulose is amorphous and easily susceptible to hydrolysis. Unlike cellulose, which is linear, hemicellulose has polysaccharide side groups.

Hemicellulose supports the cellulose cell wall base by bonds in between the cellulose matrix-like structure which is present in most cell walls. Hemicellulose is also the first target of wood rot as it is easier to digest and disrupting the hemicellulose will partly disrupt cellulose as well.

2.2.1.4 Lignin

This heteropolymer consists of aromatic alcohols and is almost as an abundant source of organic polymer as cellulose is. Lignin is an integral part of wood strength and functions as a support along with hemicellulose. Lignin is hydrophobic because of its aromatic structure and acts as a hurdle for water absorbance by the hydrophilic polysaccharides. This characteristic makes lignin an important part of the water upkeep of plants. In lumber the lignin content tells something about the durability of the specific type of wood. Lignin is used in a large number of negligible processes.

2.2.1.5 Pectin

Pectin is a heteropolysaccharide that is a binding agent in plant cell walls. The combination of cellulose, hemicellulose, lignin and pectin can be found in most plants that grow on land. The combination of these four substances gives strength and durability to the middle lamella of plants. Pectin has been used for a long time as a gelling agent to make jams and jellies. It also has uses in medicines, candy and juices and drinks.

2.2.2 Protein

Most processes in organisms are regulated and carried out by proteins, enzymes. Proteins form the main part of the metabolic systems in cells by specific binding affinities to certain molecules. Proteins can also serve as storage, e.g. in seeds. Proteins are made of amino acids. Each amino acid is bound to two other amino acids by a peptide bond, the only exception to this rule are the amino acid at the start and the one at the end of the polymer.

Proteins are essential as a dietary element in mammalian life, because mammals cannot synthesize all amino acids. Some amino acids, the essential amino acids, can only be gathered from protein sources such as meat, fruits and vegetables. Most plants have the ability to synthesize all amino acids, this could make them an excellent source for protein.

2.2.3 Extractives

This group of substances is substantial and does include all substances that can be used for various applications. Extractives include but are not limited to colorants, fragrances, flavours and other additives to different processes. These substances vary greatly in sell price and usability. The production of extractives in bio-refinery is beyond the scope of this paper.

Chapter 3: Materials and Method

3.1 Materials

In this thesis, freeze dried grass and fresh (untreated) grass were used. Freeze dried grass was obtained from the biobased chemistry and technology (BCT-) group (Wageningen, the Netherlands). Freeze dried grass was harvested locally as fresh grass and freeze-dried by the BCT-group. The freeze dryer used was Christ Alpha 2-4 LD plus. Fresh grass was harvested locally. Grasses were not analysed for maturity, protein content or growth conditions. All chemicals used for the extraction were of analytic quality and supplied by Sigma-Aldrich. PEF pre-treatment done by BIO RAD Gene Pulser Apparatus. The BIO RAD machine used in this thesis has different variables. Field strength, capacitor charge and resistance can all be set to different values. All experiments are done in triplicate, mean values are given. Previous work shows that 20% of the dry weight of grass is protein and that 15% of fresh grass is dry weight. Percentages were obtained orally from Chen Zhang, work not published. These values were used in the quantification of all experiments in this thesis.

3.2 Aqueous extractions

3.2.1 Extraction solvent test

Extractions were done in an Eppendorf thermomixer comfort (40 ^{o}C, 600 rpm) for 1h. 50 mg was used per sample. To repeat previous work, extraction efficiencies of 2 ml 0.1 M NaOH, 0.05 M NaOH, or 0.067 M phosphate buffer (pH 7.5) on 50mg freeze dried grass were tested. Protein contents were analysed by Lowry.

3.2.2 Influence of sample size on alkaline protein extraction

All extractions were done in an Eppendorf thermomixer comfort (40 ^{o}C, 600 rpm) for 1h. Extractions efficiencies of 2 ml 0.1 M NaOH (pH 13, solution) on a range from 10 mg to 50 mg freeze dried grass were tested. Protein content analysed by Lowry.

3.3 Pulsed electric field pre-treatment

Extractions on 10 cm fresh grass, roughly 8.5 mg, in either water or 0.1 M NaOH, pH 13. PEF treatment used a field strength of 2.5 kV/cm, a resistance of 1000 Ω and a capacitor strength of 25 µFD. Samples were compared with water extraction without PEF. Analysis done with Bradford.

3.4 Influence of parameters on pulsed electric field effectivity

To investigate the different parameters of the PEF machine (BIO RAD Gene Pulser Apparatus) the field strength (0.5 kV/cm and 1.5 kV/cm), resistance (800 Ω and 600 Ω) and capacitor strength (3 µFD and 1 µFD) were varied. Pre-treatment was done on 10 cm fresh grass, roughly 8.5 mg. The parameters were compared with the standard PEF treatment, described in 3.3, while varying one parameter at a time. RC time measured by the BIO RAD Gene Pulser Apparatus.

Field strength (2.5 kV – 0.5 kV) effect on extraction was investigated further. Samples were pre-treated with PEF and subsequently protein was extracted with water, 0.067 M phosphate buffer or 0.1 M NaOH. The samples were compared with a blank, untreated extraction. Analysis done with Dumas.

Pulse frequency influence was investigated discharging the electric field multiple times. PEF treatment used a field strength of 2.5 kV/cm, a resistance of 1000 Ω and a capacitor strength of 25 µFD. Pre-treatment was done on 10 cm fresh grass, roughly 8.5 mg. RC time measured by the BIO RAD Gene Pulser Apparatus.

3.5 Influence of freeze drying on pulsed electric field effectivity

The difference between freeze dried grass and fresh grass was investigated by discharging the electric field multiple times. PEF treatment used a field strength of 2.5 kV/cm, a resistance of 1000 Ω and a capacitor strength of 25 µFD. Pre-treatment was done on 10 cm fresh grass, roughly 8.5 mg. RC time measured by the BIO RAD Gene Pulser Apparatus.

3.6 Analytic methods

The Lowry, Bradford and Dumas method were used to analyse protein content. The Lowry and the Bradford method used are based on BSA calibration curves. The methods itself as well as the calibration curves used will be shown below.

3.6.1 Lowry protein assay

Lowry assay kits were obtained from the Sigma-Aldrich. For more information see the kit's manual. The Lowry method determines the amount of protein in a solution. The assay is based on a change in colour due to reaction of copper with protein[16]. The colour

formation can be measured by spectrophotometer, Beckman-Coulter DU720, with wavelengths between 500 nm and 800 nm. Table 1 shows the standard curve used.

In this thesis three wavelengths were used, all within the previously stated range. As shown above, some wavelengths have a steeper curve than others. The steeper the curve the more unreliable the measurement at high protein concentrations. A wavelength of 750 nm would be the most accurate measurement of the protein concentration. 750 nm is outside of the visible spectrum and will thus have less interference of pigment and polyphenol coloration. 550 nm and 650 nm, yellow and red respectively, could have more interference of these substances.

3.6.2 Bradford protein assay

Bradford assay kits were obtained from Sigma-Aldrich. For more information see the kit's manual. The Bradford method determines the amount of protein in a solution as well. The assay is based on the change in colour due to a reaction with Coomassie brilliant blue, a dye, and protein[17]. This colour formation can be measured using standard colorimetric techniques, Beckman-Coulter DU720, as well. Table 2 shows the standard curve used.

The Bradford method uses Coomassie brilliant blue to form a complex with protein[17]. This complex turns the solution, which is initially brown, to blue. The formation of this blue colour can be measured by absorption.

3.6.3 Dumas protein assay

Dumas is a protein assay based on the combustion of chemicals to analyse their nitrogen content.[18] Dumas measures all nitrogen, dumas results could thus be higher than the actual values.

Chapter 4: Results and discussion

4.1 Aqueous extractions

4.1.1 Extraction solvent test

Initial protein extractions were done to repeat literature data and to investigate the influence of pH on protein extraction. As shown in Table 1 the 0.1 M NaOH extraction extracts 5.66% of total protein in 1 h. The high pH and the large amount of alkaline material that the 0.1 M NaOH, pH 13, solution has, aided protein extraction significantly[4]. A NaOH concentration of 0.05 M, pH 12.7, yields 5.25% of total protein extracted in 1 h. The small difference in pH already results in a decrease in extraction value. The results, Table 1, are in compliance with previous work [4, 19, 20].

Basic phosphate buffer extraction yields 1.08% of total protein after 1 h. When compared to water, the phosphate buffer extraction increases yield. Phosphate buffer extraction was used to circumvent the destructive character of high concentrations of NaOH[14, 21]. The pH of the phosphate buffer is favored as this increases protein solubility without damaging proteins.

Extraction solution	Percentage of total protein extracted
Water	0.6 ± 0.1
NaOH 0.05 M	5.2 ± 0.6
NaOH 0.1 M	5.7 ± 1.0
Buffer	1.1 ± 0.4

Table 1: Protein extraction values of freeze dried grass with different solvents

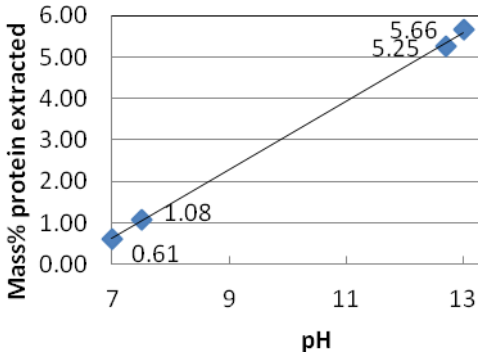


Figure 1: Mass% protein extracted at increasing pH.

Figure 1 shows the extraction values as a function of the pH of the extraction solution. The percentage protein extracted here shows a linear correlation with the pH[22]. However, this linearity observed here does not imply that protein solubility has a linear correlation with pH. Rather it means that extraction from plant material could be linear. Higher pH increases the solubility of protein in water. Higher pH will thus increase protein extraction yield.

4.1.2 Influence of sample size on alkaline protein extraction

Protein was extracted from different amounts of grass to test for a suitable sample weight. As shown in Figure 2, no significant change in extraction was found when experimenting with different weights. Increase in extracted amount has an almost linear correlation with increase in sample weight. Protein extraction values can be higher than real protein extraction values. The Lowry method can be easily distorted by polyphenols and other organic molecules in the sample. Assuming extraction took place at the same rates and similar initial values, this data can still indicate suitable amounts of sample weight.

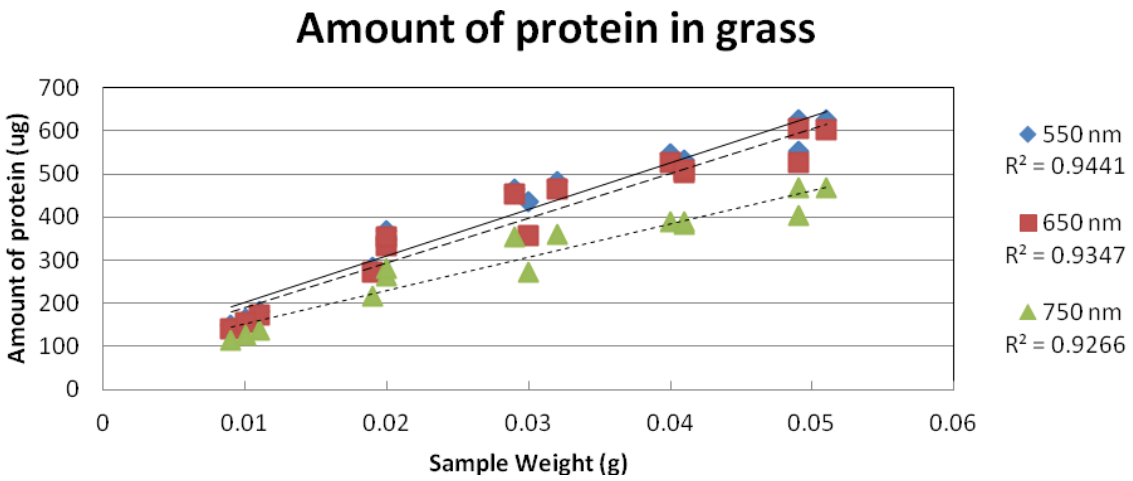


Figure 2: Amount of protein extracted from different amount of grass. Solid line; 550 nm trend line R^2=0.9441, medium dashes; 650 nm trend line R^2=0.347, short dashes; 750 nm trend line R^2=0.9266

4.1.3 Lowry assay versus Bradford assay

The first results, which were analysed with the Lowry assay, were inaccurate. Lowry didn't have the expected results and the range used to make the standard curve wasn't in accordance with the protein range of the experiments. This was most likely due to interference of other compounds, such as salts and organic matter. Lowry is known to have interference due to these compounds[16]. Later Bradford was introduced as an alternative means to analyse protein. Bradford does not have the disadvantages that Lowry has. Bradford has other disadvantages, such as interference from detergent and only short range linearity in its standard curve. However, these disadvantages did not play a role in the quantifications. All experiments were thus analysed with Bradford, with the exception of some of the early experiments.

4.2 Exploratory pulsed electric field application

To further increase extraction yield PEF was introduced. The pore formation[5] increases diffusion which may result in a higher yield at milder conditions. Table 2 shows the percentage protein extracted and that PEF pre-treatment increases extraction in water.

PEF increases yield if used before a water extraction[13, 23]. However, the impact of PEF is weaker on the 0.1 M NaOH extractions. PEF affects the cell wall[5] as does NaOH[14]. Both substances affecting the cell membrane could lower effectiveness of either one. This will result in lower effectiveness of either technique.

Extraction solution	Percentage of total protein extracted
Water	0.94 ± 0.5
PEF water	4.65 ± 2.7
NaOH 0.1 M	10.60 ± 2.4
PEF NaOH 0.1 M	13.74 ± 0.3

Table 2: Protein extraction values of fresh grass; water extraction (blank), PEF pre-treated water extraction, 0.1 M NaOH extraction, PEF pre-treated 0.1 M NaOH extraction. Quantification done with Bradford.

4.3 Influence of parameters on pulsed electric field effectivity

4.3.1 Pulse controller parameters

The RC time was measured to investigate the influence of different parameters on the effectiveness. Table 3 shows the influence of field strength, capacitor strength and resistance on the measured RC time. A larger difference between the RC time constant and the measured RC time indicates a more effective pulse. As shown in Table 3 a stronger electric field has a larger drop in RC time associated with it. This means that a stronger electric field is more disruptive than a weaker electric field. Changes in capacitor strength have a similar RC time drop associated with them. In both cases the RC drop is roughly 10 %. The change in resistance yields a larger relative drop in RC time at lower resistances. Meaning that smaller resistances will yield more effective PEF.

Field strength (kV)	Capacitor strength (µFD)	Resistance (Ω)	Calculated RC time (s)	Measured RC time (s)
2.5	25	1000	25	21.0
1.5	25	1000	25	21.6
0.5	25	1000	25	23.8
2.5	3	1000	3	2.7
2.5	1	1000	1	0.9
2.5	25	800	20	17.4
2.5	25	600	15	12.5

Table 3: Resistor-Capacitor time constants at different parameters compared to the measured RC time

To accurately investigate the effect of field strength on increase in extraction yield Dumas was introduced as an analytic technique. However, samples sizes were too small to properly analyse with the Dumas-method. Manual peak selection was done by Chen Zhang in an effort still find useable data (appendix 3). The Dumas data doesn't hold up with data from literature and data from previous experiments. Water is the most efficient extraction solvent and PEF treatment doesn't improve extraction according to this data. Dumas should be an accurate protein quantification technique, however extraction values might not have been in the range of the Dumas protocol. If samples sizes were too small, Dumas quantification could be inaccurate.

4.3.2 Pulse frequency

The pulse effectiveness was measured to investigate the influence of pulse frequency on effectiveness. More pulses will result in changes to the measured RC time. As shown in Table , the increase in pulses will increase the permeability of the cell membrane while decreasing the measured RC time drop. Using more pulses on one sample increases the treatment time. Treatment time is the cumulative RC time. Both the increase in the amount of pulses and the treatment time increase the inactivation of micro-organisms. According to literature the increase in treatment time is a more important factor than the increase in pulses[24].

n_{pulse}	Measured RC time (s)
1	21.2
2	17.5
3	15.9

Table 4: Change in measured RC time from increase in pulses

4.4 Usage of pulsed electric field treatment

4.4.1 Freeze dried grass versus fresh grass

Untreated, fresh, grass and freeze dried grass react differently to PEF treatment. Fresh grass shows a larger drop in RC time than freeze dried grass, Table . A larger drop in RC time denotes more ions outside the grass sample, meaning more cells are disrupted. It was assumed that fresh and freeze dried grass have the same dry matter composition and that the only difference between freeze dried and fresh grass is the amount of water.

n_{pulse}	Measured RC time	
	Freeze dried grass	Fresh grass
1	21.0	21.2
2	20.1	17.5
3	19.3	15.9

Table 5: Difference in measured RC time between freeze dried and fresh grass

Fresh grass can only have a larger drop in RC time than freeze dried grass if the PEF treatment is more effective. In freeze dried grass the drop in RC time is less than the drop in RC time in the fresh grass. Freeze dried grass should start with more cells ruptured than fresh grass due to its pre-treatment. Freeze drying damages cells, the initial drop in RC time should be lower for freeze dried grass.

In PEF, ions are the reason for pore formation. In freeze dried grass the lack of water makes ions immobile. Figure 3 shows the structure inside a grass leaf. The picture shows canals that transport water when the plant is alive. These water canals are the veins of plants, they transport nutrients, etc. A similar picture of freeze dried grass, which was impossible to produce, is expected to have smaller canals and thinner leaves. X-ray tomography pictures of freeze dried grass are had to make. The freeze dried samples couldn't be properly fixated in the machine. The pictures were thus unclear.

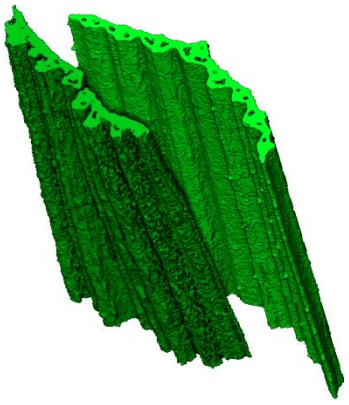


Figure 3: X-ray tomography picture of fresh grass. Picture taken by Remco Hamoen.

Chapter 6: Conclusion

In this thesis, PEF was successfully used to increase protein extraction yield on fresh grass. Solvents with specific affinity, e.g. protein affinity, can increase diffusion of extractives even further. Alkaline solutions can be used for even faster extraction after PEF. Using both PEF and alkaline material increases the overall effectivity.

Most leaf material can be treated with PEF to form pores. Freeze dried leaves do not respond well to PEF treatment. The dried leaves lack water which enables the electric field to become more homogeneous. A more homogeneous electric field will result in a less pore formation.

Pore formation results in a drop of measured RC time. Stronger electric fields over the treatment chamber result in more effective pulses and a larger RC time drop. The capacitor strength and the resistance, however, do not significantly influence the roughly 10% in RC time.

Pulse effectiveness decreases with every subsequent pulse. The ion diffusion after pore formation will increase the homogeneity of the electric field. Freeze dried grass has a larger initial drop in conductivity, while fresh grass has a larger subsequent measured RC time drop.

Chapter 7: Recommendations

During this thesis, mainly during the experimental part, there have been some issues or challenges. Some experiments did not work, as a result of the low capacity of the PEF machine. A few new challenges came up during the experiments. Most of these new challenges are ideas that could prove helpful in the future. These recommendations for future work will be discussed in this chapter.

7.1 Protein solubility as a function of pH

In this thesis the link between protein solubility and pH has been made. PEF treatment increases diffusion speed. Higher protein affinity of solutions will result in higher extraction values in a shorter time as well. As shown before higher pH will correlate with higher extraction values, probably on basis of solubility. In some cases increase of buffer concentration increases reactions, e.g. general acid/base catalysis.

Increasing buffer concentration will increase the reactivity of the buffer, meaning it will be less influenced by addition of other acids and bases. Protein might solve more readily in higher concentrated buffers, because of the increased reactivity. Protein could also solve less readily in higher concentrated buffers, because of the already high concentration of minerals. Both scenarios are worth looking into as they can potentially increase the process's efficiency.

Ideally, this can be done by extractions with a few different types of buffer solutions, all with different pH, different ionic strength and different concentrations. A 3-dimensional plot of the pH and buffer concentration versus the amount of protein extracted will show this well. The plot can give an optimum for both values, the pH and the buffer concentration, or single out one value as the critical one. A nitrogen based protein measurement, e.g. Kjeldahl or Dumas, should be the most accurate protein analysis assay.

7.2 Pore formation

The pore formation associated with PEF treatment can be studied in 2 different ways. The first way this can be viewed is through the pores itself. A protocol for studying these pores

has already been made[5]. Rapid freezing and electron microscopy are used in this case. The other way this can be done is through diffusion. The increase in diffusion as a result of the pore formation comes associated with a flux. Changes in flux can be associated with changes in both the number of pores and the size of pores.

For experimental purposes the diffusion will do, however more sophisticated results can be found with rapid freezing and electron microscopy. The diffusion can be studied in 2 main ways, either by checking what comes out of the sample or what goes into the sample. Colorants like polyphenols and pigments can be extracted with either pure EtOH or a diluted solution. Measuring the content of these compounds over time gives an indication in pore size and amount. More and/or larger pores will result in a larger open area. The larger area will increase the flux.

On that same note, solving PEF treated grass in a solution of substances of different sizes can give an indication of the pore size as well. Substances of different sizes that can easily be identified using a spectrophotometer, IR-spectroscopy or in some cases NMR or GCMS can help check this. Using these techniques to check the solvent compounds before and after letting the grass rest in the solution for some time. The difference between the two measurements gives information about the pore size. Small molecules that disappeared between the 2 measurements will have diffused into the leaves.

7.3 Treatment chamber size

The first issue that was encountered during PEF treatment of grass was the size of the treatment chamber. The treatment chamber used in this thesis has a workable volume of approximately 0.8 mL (not measured). In this volume sample sizes up to 10 cm can be treated without problems, e.g. arcing or short circuits. 10 cm, roughly 8.5 mg, of grass holds approximately 0.28 mg of protein, 15% of fresh grass weight is dry matter and 22% of dry matter is protein. Extractions were done in 2 mL solvent, meaning the maximum concentration of protein is 0.14 mg/mL solvent. This concentration is correct if 100% of the protein is extracted. 30% protein extracted after 1h at 40 ^{o}C is an accurate estimation[4]. 0.04 mg protein per mL solvent is the protein concentration, however this should be lower due to lower alkaline content ideally used after PEF.

The problem with these values is the end concentration of protein. Higher protein concentrations can be achieved with either running more samples in the same solvent or increasing the amount of grass per extraction. Both of these methods are time intensive, based on empirical data 10 cm of grass can be ran in roughly 2,5 minutes. If this technique is to be made less time consuming the volume of the treatment chamber has to increase. A new machine with a larger treatment chamber could be the answer.

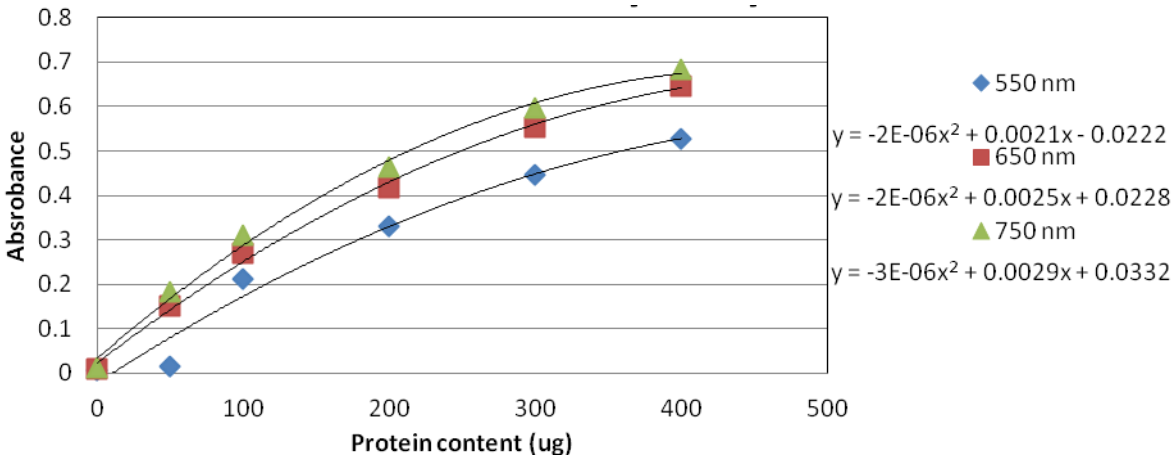
Starting from the analytic part of the process to the initial pre-treatment. Bradford can measure proteins effectively up to about 1 mg/mL. Higher concentration can be used in Bradford analysis, but those have to be diluted before use. If 1 mg/mL is the desired concentration, taking into account the amount that can be extracted over the course of 1 h in 2 mL solvent with the grass weight to dry matter and the protein content to dry matter percentages. The sample size will be approximately 200 mg fresh grass. Under ideal circumstances a PEF machine that can hold 200 mg fresh grass would work very well. If 8.5 mg fresh grass is suited for 0.8 mL than a PEF machine with a volume of approximately 20 mL would be well suited for these experiments.

7.4 Cellular disintegration index

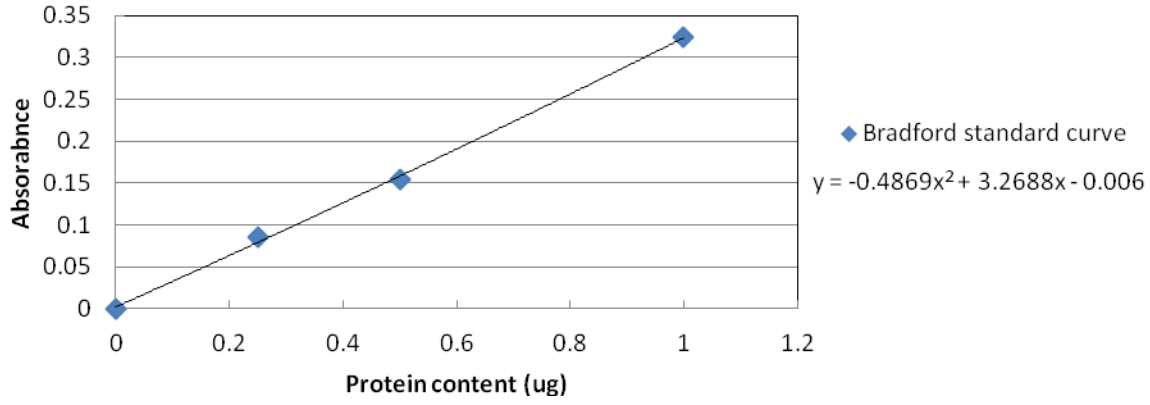
The efficiency of the PEF treatment, the amount of cell disrupted, mentioned in this thesis is the Z_p. The effects of different parameters on the Z_p have already been studied. A Z_p of around 1 will have a larger flux than a sample with a lower Z_p. It is important that this is checked otherwise protein could be lost.

Further investigation into Z_p could lead to more efficient treatment by PEF. The Z_p is a general measurement of how disrupted the samples is. The route to get to a specific Z_p can be different though. Some leaf material could have many, short pulses as preferred treatment. While other leaves could have a preference for a few, long pulses. The Z_p can then be used as a more accurate measurement for pulse effectiveness than the RC time constant.

Chapter 8: Appendix



Appendix 1: Standard curve Lowry analysis. Standard curves made at different wavelengths; yellow light (550 nm), red light (650 nm) and infrared light (750 nm). Formulas for protein content as a function of absorbance given.



Appendix 2: Standard curve Bradford analysis. Formula for protein content as a function of absorbance given.

Extraction solvent	Treatment		
	Non treated	PEF 2.5 kV	PEF 0.5 kV
Water	2.5	1.4	1.8
Buffer	1.4	3.2	1.2
0.1 NaOH	2.0	1.0	1.3

Appendix 3: Dumas results, percentage protein extracted from fresh grass after PEF treatment

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