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# Prolongation of shelf life of minimally processed spinach by combining vacuum impregnation and electric field treatments


Presented by

Sophia Leffler



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Examiner Lund University: Prof. Marie Wahlgren

Examiner TUM: Prof. Dr. Wilfried Schwab

Supervisor: Prof. Federico Gómez Galindo

## Abstract

A continuous growth of the world population raises the importance of preserving food resources while maintaining their nutrient value. Leafy vegetables such as spinach are a food category which decay quickly, and a large amount ends up as food waste. Therefore, shelf life extension while keeping a good product quality is of great interest in food processing.

In this study, the effect of combining vacuum impregnation (VI) and electric pulses on eco-spinach leaves was investigated aiming to prolong their shelf life. Shelf life extension was to be achieved by provoking different metabolic processes and reactions as stress response. Various pulsed electric field (PEF) and moderate electric field (MEF) electroporation parameters were studied as stressors. In the first part of the study, protocols achieving reversible electroporation to ensure cell survival after treatment were identified. Vitamin $B_1$, $B_3$, $B_5$ or $B_9$ was introduced into the plant tissue by VI to analyse their ability to delay leaf senescence. For the shelf life evaluation treated spinach leaves were packed in perforated plastic bags and stored at 5 °C until spoilage. As retail shelf life, a decay of >10% spinach leaves per bag was defined. The atmosphere in the bags was controlled by measuring $O_2$ and $CO_2$ concentrations with a gas analyser, L*, a* and b* values were measured with a spectrophotometer for detection of colour changes and pictures were taken under controlled conditions to record the deterioration process.

VI led to a weight gain of 58-65% due to volumetric expansion since air spaces in the spinach tissue were filled up with the VI solution. VI with vitamin $B_3$ showed the longest shelf life among the different vitamins and was used for the combined treatment. Results show that the composition of $O_2$ and $CO_2$ did not significantly change during storage time. PEF and MEF treated samples preserved their colour. VI led to a decrease of L* value and leaves appeared darker. The different treatments resulted in a significant faster deterioration of the leaves probably due to increased metabolic activity. The combined treatment of VI with vitamin $B_3$ and PEF or MEF showed the longest retail shelf life with 12 days. Cells might be protected better through a synergistic effect of different metabolic processes. However, the shelf life was still significant lower compared to untreated leaves with 14 days.


*Keywords*: Spinach, pulsed electric field, moderate electric field, reversible electroporation, vacuum impregnation, shelf life, metabolic activity, respiration

## Statutory Declaration

Hereby I declare that I have written this thesis by my own. Furthermore, I confirm that no other sources have been used than those specified in the thesis itself.

This thesis, in same or similar form, has not been available to any audit authority yet.

Lund, 25.04.2022

(Sophia Leffler)

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## Abbreviations

| Abbreviation | Description |
|---|---|
| AC | Alternating Current |
| ANOVA | Analysis of Variance |
| ATP | Adenosine Triphosphate |
| $Ca^{2+}$ | Calcium Ions |
| Chol | Choline |
| $CO_2$ | Carbon dioxide |
| CoA | Coenzyme A |
| DC | Direct Current |
| DI water | Deionized water |
| DNA | Deoxyribonucleic Acid |
| DRP | Deformation-Relaxation Phenomena |
| FDA | Fluorescein Diacetate |
| Fol | Folate |
| $H_2O_2$ | Hydrogen Peroxide |
| $H_2S$ | Hydrogen Sulphide |
| HDM | Hydrodynamic Mechanisms |
| LED | Light Emitting Diode |
| MEF | Moderate Electric Field |
| NaCl | Sodium Chloride |
| NO | Nitric Oxide |
| $O_2$ | Oxygen |
| OTR | Oxygen Transfer Rate |

| | |
|---|---|
| $p_{atm}$ | Atmospheric pressure (1000 mbar) |
| PBS | Phosphate Buffered Saline |
| $p_e$ | External pressure |
| PEF | Pulsed Electric Field |
| PI | Propidium Iodide |
| $p_i$ | Internal Pressure |
| RDA | Recommended Daily Allowance |
| ROS | Reactive Oxygen Species |
| SAR | Structure-Activity Relationship |
| VI | Vacuum Impregnation |
| wb | Wet basis |

| Symbol | Description |
|---|---|
| °C | Degree Celsius |
| µg | Microgram |
| µM | Micromolar |
| µm | Micrometre |
| µs | Microsecond |
| µS | Micro Siemens |
| cc | Cubic Centimetre |
| cm | Centimetre |
| d | Day |
| g | Gram |
| h | Hour |
| Hz | Hertz |
| L | Litre |
| M | Molar [mol/L] |
| mbar | Millibar |
| mS | Milli Siemens |
| mg | Milligram |
| min | Minute |
| mM | Millimolar |
| Mm | Millimetre |
| s | Second |
| T | Temperature |
| V | Volt |

# 1. Introduction

According to the United Nations population projections the global population, especially from developing countries, is growing continuously (Roser, 2013). In this regard, the demand of saving food resources, avoiding nutrient losses, and reducing the economic impact of food waste increases. Therefore, the development of sustainable food systems is essential to face challenges as malnutrition or food-related environmental issues by reducing food loss and waste (Boz & Koelsch Sand, 2020).

Consumers in households contribute for more than 50% of the total food waste in Europe (Janssen et al., 2017). Especially leafy vegetables belong to a food category which spoils quickly (Boz & Koelsch Sand, 2020). In the United Kingdom, around 40% of bagged salad, which accounts for approximately 37.000 tons, is thrown away every year (The Guardian, 2017). This can be countered with improved food preservation by extending the shelf life and can therefore be one important way to contribute to the reduction of food waste (Janssen et al., 2017).

Novel technologies such as the application of electric pulses or vacuum impregnation (VI) have already gained a lot of interest in the food processing area to improve functional and nutritional values of foods and food products. Since no heat supply is needed, heat sensitive food components can be preserved and food properties enhanced. Moreover, it is possible to delay tissue decay of fruits and vegetables (Koutchma, 2014). The prolongation of the shelf life has been already successfully applied in the flower industry by combining pulsed electric field (PEF) and VI. In cooperation with the company “OptiCept Technologies” an increase of the shelf life and enhancement of the quality of cut flowers was achieved (OptiCept Technologies, 2021).

The main aim of the present study was to analyse the effect of the combined application of VI and electrical treatment on minimally processed spinach to delay senescence and therefore prolong the shelf life. The electrical treatments PEF and moderate electric field (MEF) were applied. For VI different vitamins of the B-complex were used as impregnation solutions.

## 2. Aim of Study and Hypothesis

The overall aim of the thesis is assessing the impact of combining vacuum impregnation (VI) and electric induced pulses, i. e. Pulsed Electric Field (PEF) and Moderate Electric Field (MEF), on minimally processed eco-spinach leaves aiming to prolong their shelf life. Colour, visual appearance as well as oxygen ($O_2$) and carbon dioxide ($CO_2$) measurements are used as an indicator for the impact on the shelf life.

The specific aims of this thesis are:

- To evaluate a PEF and MEF protocols leading to reversible electroporation, ensuring leaf viability after the treatment
- To analyse different B-vitamins, i. e. vitamin $B_1$, $B_3$, $B_5$ or $B_9$, as VI solution in their ability to extend the shelf life of spinach leaves
- To assess if the combination of VI and PEF or MEF shows additional improvements in the delay of leaf senescence

# 3. Theoretical Foundations

## 3.1 Spinach

Spinach (*Spinacia oleracea*) is one of the five most produced leafy vegetables worldwide. It can be consumed raw, boiled, or baked. Spinach belongs to the family of Chenopodiaceae and it can be cultivated in regions with temperate climate all year long. Temperatures between 16-22 °C are considered as optimum air conditions (Aramrueang et al., 2019; Pandey & Kalloo, 1993). Minimally processed vegetables such as spinach became increasingly popular since the 1980s because of the higher demand for convenient and high quality foods and food products (Conte et al., 2008).

### 3.1.1 Nutrient Composition and Health Benefits

In general, fresh vegetables mainly consist of water with a ratio of 75% to 95% referring to fresh weight (Aramrueang et al., 2019). Spinach has an approximate water content of 91.4% and is low in protein (~2.9%), carbohydrate (~3.6%) and fat (~0.4%). Moreover, spinach has a diverse mineral and vitamin composition compared to other leafy green vegetables (**Table 1**). The vegetable has especially high amounts of magnesium, potassium, and iron. However, only around 10% of the iron in leafy vegetables can be absorbed by the human body because of the presence of several dietary inhibitors like fibre. These inhibitors decrease the bioavailability of iron (Coad et al., 2016). By consuming 100 g spinach it is possible to get enough of various vitamins according to their respective recommended dietary allowance (RDA). It is even exceeded for vitamin K with 604% and vitamin A with 188% (Roberts & Moreau, 2016). Due to their composition, leafy green vegetables such as spinach contribute to a balanced diet since they are a good source for various substances required for essential body functions and the human health (Hedges & Lister, 2007; Roberts & Moreau, 2016).

**Table 1.** Vitamins (Value Per 100g wb, wet basis) of different Leafy Vegetables (Aramrueang et al., 2019)

| | C (mg) | B6 (mg) | Fol (µg) | Chol (mg) | A (µg) | E (mg) | K (µg) |
|---|---|---|---|---|---|---|---|
| Spinach | 28.10 | 0.20 | 194.00 | 19.30 | 469.0 | 2.03 | 482.90 |
| Cabbage | 36.60 | 0.12 | 43.00 | 10.70 | 5.00 | 0.15 | 76.00 |
| Cauliflower | 48.20 | 0.18 | 57.00 | 44.30 | - | 0.08 | 15.50 |
| Broccoli | 89.20 | 0.18 | 63.00 | 18.70 | 31.00 | 0.78 | 101.60 |
| Iceberg Lettuce | 2.80 | 0.04 | 29.00 | 6.70 | 25.00 | 0.18 | 24.10 |

Description of vitamin abbreviations: C, vitamin C (total ascorbic acid); B6, vitamin B-6; Fol, folate; Chol, choline; A, vitamin A (retinol activity equivalents); E, vitamin E (alpha-tocopherol); K, vitamin K (phylloquinone).

### 3.1.2 Structure

A spinach leaf is around 0.4 mm thick and as the cross section shows in **Figure 1**, it can generally be divided into four layers. These layers consist of different tissues. The first layer, called upper epidermis, is built from star-shaped cells arranged in a singular layer. The epidermis is covered with a cuticular wax layer and contains stomata which are pores with a pair of guard cells (Dymek et al., 2015; Prabhakar, 2004). Stomata have two main functions – regulating the flow of water through perspiration and enabling intake of carbon dioxide ($CO_2$) and release of oxygen ($O_2$). By adapting to different environmental factors, stomata provide optimal photosynthesis conditions in plants (Sack, 1987). Thus, the cuticle protects the leaf against water loss and regulates gas exchange. Furthermore, it provides protection, for example against mechanical injuries from the environment or against microorganisms (Domínguez et al., 2011). The next layer is the palisade mesophyll consisting of two layers of elongated cells. This section is followed by a spongy mesophyll which is a multi-cell layer. It contains round cells and most of the intercellular air. Of the total leaf volume, the air fraction makes up approximately 30%. Due to the high air content in the spongy mesophyll, this tissue structure is the most random and irregular compared to the other leaf layers. The final layer is the lower epidermis which is similar in its structure to the upper epidermis (Dymek et al., 2015).

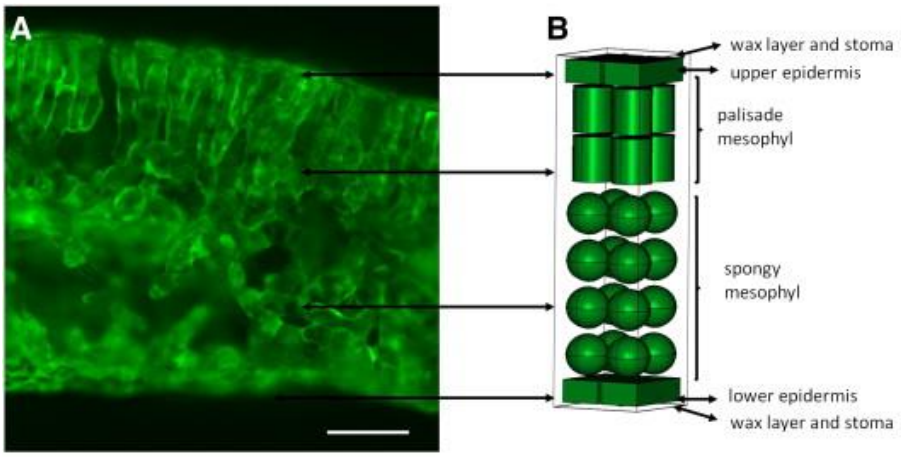


**Figure 1**. Representation of the cross section of spinach in a microscopic picture (**A**) compared to a simplified model (**B**). The scale bar in A measures 100 µm adapted from (Dymek et al., 2015).

### 3.1.3 Quality Aspects and Food Waste

The quality characteristics of spinach comprise an optically appealing appearance as well as nutritional and safety aspects (Conte et al., 2008). One of the biggest challenges for the food industry is to extend the shelf life of minimally processed leaves such as spinach while preserving adequate product quality. Operations applied on spinach are washing, cutting, and packing. Due to the processing of the leaves the rate of deterioration rises because of physiological defects and degradation (Glowacz et al., 2013).

In Britain, around 40% of bagged salad equivalent to approximately 37.000 tons per year end up as food waste since leafy vegetables spoil quickly (The Guardian, 2017). According to Janssen et al. (2017), over 50 % of the total European food waste comes from private households. It is therefore necessary to develop a sustainable food system and prevent food waste as the global population is continuously growing. By avoiding huge amounts of food waste, problems as a negative environmental impact which is connected to the carbon dioxide footprint and the need to feed the world population can be faced (Janssen et al., 2017; Roser, 2013).

A possible way to lower food waste is to extend the shelf life of the respective food (Janssen et al., 2017). A technology which gained more interest over the last 50 decades is the non-thermal treatment such as pulsed electric field (PEF) (Donsì et al., 2010).

## 3.2 Electric Field Induced Electroporation

### 3.2.1 Pulsed Electric Field

As an alternative to traditional thermal processes, PEF can be applied for the preservation of food. With this approach, it is possible to prevent quality degradation as a result of heat treatment, such as decomposition of vitamins and aromatic substances (Lebovka et al., 2005). PEF is a method which is based on electroporation and results in an increased permeabilization of the cell membrane (Donsì et al., 2010).

#### 3.2.1.1 Mechanism and Influencing Factors

Mostly, short electric pulses with high field strengths are applied to the product which leads to electroporation. This subsequent opening of pores improves the mass transfer in the cell membrane and allows the transition of various molecules which otherwise cannot pass through to leave or enter the cells (Gómez Galindo, 2016).

PEF in an electric field leads either to reversible or irreversible electroporation. The process is termed reversible electroporation if the cell can recover and survives. Pores created in the membrane are then resealed after some time and homeostatic semi-permeability is restored. In contrast, irreversible electroporation occurs if the disturbance of homeostasis by molecular transport is too high, so cells cannot recover which then leads to cell death (Miklavcic & Davalos, 2015).

Gómez Galindo (2016) pointed out that only by accurately controlling the parameters of electric pulses, electroporation can be reversible. By using specific parameters adapted to the product, cell death can be avoided and the functionality of the cells can be restored. Parameters which show a crucial impact on the reversibility of the electroporation are the pulse polarity (bipolar or monopolar pulses), the pulse duration and the pulse frequency (Gómez Galindo, 2016). Because of the non-conductive character of air, the current flow is strongly influenced by the spongy mesophyll layer (Dymek et al., 2015). Furthermore, Dymek et al. (2014) has shown that in rucola leaves different cell structures are electroporated by choosing the pulse polarity type. By applying bipolar pulses, electroporation was mainly efficient for puzzle-shaped epidermal cells whereas monopolar pulses have electroporated these as well as the round mesophyll cells. For the pulse duration it is important to find a balance between an overly short period of pulse application whereby the desired disruption of the membrane does not occur and an overly long period which leads to irreversible electroporation. At last, it can be said that with a higher frequency of the electric pulses,

the level of electroporation declines. However, it is generally the interaction of the various parameters which is essential and must be adjusted individually depending on the product (Gómez Galindo, 2016).

#### 3.2.1.2 Advantages and Applications of Irreversible PEF

PEF is a suitable alternative technology to the traditional ways of improving food quality and changing food properties. Through PEF-treatment, the properties of the plant-tissue are modified which impacts the functionality and the possibility to process the product (Gómez Galindo, 2016). Due to its non-thermal approach, this technology is a useful option especially for heat-sensitive foods. PEF opens possibilities to alter the competitiveness on the food market by introducing new products with improved quality. Another promising objective of PEF is the reduction of energy costs since PEF is characterised by a short processing time and a low amount of energy is required (Donsì et al., 2010; Thamkaew, 2022).

The type of PEF, which leads to irreversible electroporation, has already gained great interest in research for over 50 years, initially with the aim of inactivating microorganisms (Donsì et al., 2010). In the past few years, PEF also gained more interest in food processing. For example, PEF has been applied in the treatment of liquid foods such as pasteurisation (Fincan & Dejmek, 2002). Furthermore, the efficiency of methods such as drying or extraction of valuable compounds can be enhanced due to the improved mass transfer of water and other molecules (Donsì et al., 2010; Fincan & Dejmek, 2002).

### 3.2.2 Moderate Electric Field

A method which can also lead to cell permeabilization is Moderate Electric Field (MEF). It is another promising technology of electrical treatment to change tissue properties and improve the quality of food and food products (Thamkaew & Gómez Galindo, 2020).

#### 3.2.2.1 Mechanism and Food Applications for Irreversible Electroporation

Compared to PEF, the applied electric current is simpler. For example, pulse-forming networks or capacitors are not used for MEF. Instead of a direct current (DC) as for PEF, an alternating current (AC) with much lower field intensity is applied. It is crucial for the MEF treatment to set specific parameters based on the present product. Depending on the frequency, field strength and the duration of the treatment, reversible or irreversible electroporation occurs (Thamkaew & Gómez Galindo, 2020).

With MEF, it was already possible to improve drying processes because of an increased permeability of the tissue (Thamkaew & Gómez Galindo, 2020). Kulshrestha and Sastry (2010) also mentioned a positive effect on juice extraction processes. Furthermore, the extraction yield of fresh mint leaves was higher when using the MEF treatment compared to conventional heating. Therefore, MEF could be a good alternative to traditional heating methods for extraction of cell metabolites such as macromolecules, sugars or pigments from cellular systems (Sensory & Sastry, 2004).

### 3.2.3 Reversible Electroporation for Electrical Treatments

Reversible electroporation of plant cells is not fully understood yet and is lacking research, however it is an area which opens potentials for new innovations and possibilities of food treatments. Cells of plant tissues are different in their shape, size, structure of the cell walls as well as the air spaces in between. Therefore, one of the biggest challenges when electroporating plant tissues is their heterogeneity. Depending on the to be electroporated target tissue, different protocols with adapted parameters are necessary to achieve the desired degree of electroporation (Gómez Galindo, 2016).

#### 3.2.3.1 Metabolic Responses and the Influence of Reactive Oxygen Species on Senescence

Applying PEF leading to reversible electroporation results in changes of tissue properties due to several metabolic reactions (Demir et al., 2018). After the pores have been opened by an electric pulse, thus allowing molecules to either leave or enter the cell, the resealing process begins. One of the first responses to biotic and abiotic stress is a higher production of reactive oxygen species (ROS) with hydrogen peroxide ($H_2O_2$) as a major constituent (Sabri et al., 1996). Among other ROS, $H_2O_2$ is considered the most stable with the least reactivity and it can pass membranes easily. All these properties make $H_2O_2$ a good signalling molecule. Therefore, $H_2O_2$ is crucial for the regulation of various physiological reactions under stress conditions (Jajic et al., 2015). Depending on the amount of ROS production, especially $H_2O_2$, it can either lead to cell damage, or it can facilitate the adaption to abiotic stress (Huang et al., 2019). Liao et al. (2012) pointed out that with an $H_2O_2$ concentration of 600 µM the vase life of Oriental x Trumpet hybrid lily *“Manissa”* was increased, whereas a concentration of 800 µM already showed negative effects. As a conclusion, cell death can occur with high concentrations of $H_2O_2$ by damaging the cells because of oxidative modifications

of crucial cellular elements. Whereas lower ROS production leads to a better stress tolerance which may be reflected in an acclimation to drought or photooxidative stress or a delay of senescence. ROS in a specific amount can also lead to a better resistance against pathogen penetration or has an essential function during early reactions to injuries (Jajic et al., 2015). Furthermore, the concentration of free $Ca^{2+}$ in the cytosol increases during the recovery process. The ROS production and an influx of $Ca^{2+}$ may induce pathways which lead to a higher protein expression and the production of secondary metabolites. The complex metabolic processes also include energy release because of the movement of ions and the hydrolysis of adenosine triphosphate (ATP). ATPase activity is necessary for the recovery process since it is needed to take up the ionic species against the concentration gradient (Gómez Galindo, 2016).

The various stress provoked responses may cause modifications in the proteome and metabolome. Moreover, the reciprocal influence of the different signalling pathways may result in co-expression of the different stress responses. Therefore, cells may be more protected against further abiotic stress through cross tolerance (Demir et al., 2018). In total, all these stress induced responses can be possibly used to specifically influence the characteristics of a product (Gómez Galindo, 2016).

#### 3.2.3.2 Use of Reversible Permeabilization in Food Processing

Reversible electroporation has already been researched in many different fields. In biotechnology and medicine for example, various molecules can be introduced into cells like genetic material into living cells (Fincan & Dejmek, 2002). Although little is known about the specific impact of reversible electroporation, it has also gained more interest in the field of food processing. Applying electric pulses is a potential pre-treatment method to enhance properties of food material e.g. after drying or freezing processes (Thamkaew et al., 2021). Kwao et al. (2016) studied the effect of treating basil leaves with electric pulses before drying them at 50 °C. It was possible to shorten the subsequent drying process by applying PEF before, whereas the cells recovered with time. Within this study an even better effect was observed when the guard cells were electroporated and stomata stayed open during the drying process. Forced opening of the stomata during the process may ease the release of water and thus, increase the degree of dehydration. Hereby, not only the drying time was clearly reduced, also aromatic compounds and colour were preserved better (Kwao et al., 2016). Thamkaew et al. (2021) also observed an improvement of the drying process

of basil leaves at 40 °C after reversible PEF was applied. For PEF treatment with a following resting period of 24 h before drying, it is assumed that the electrical treatment facilitates a phase of hardening which has a protective function on the cells (Thamkaew et al., 2021). Furthermore, it was demonstrated that PEF changes the texture of potatoes by strengthening their cell walls (Gómez Galindo, 2016).

## 3.3 Vacuum Impregnation

Vacuum Impregnation (VI) is a method which enables mass transfer in porous materials such as plant tissues. It has already been widely researched to change physicochemical, nutritive, and sensory properties of food products. Since VI can lead to a new product quality it has gained a lot of interest in the food industry sector (Radziejewska-Kubzdela et al., 2014; Yusof et al., 2017). In general, VI is a technology which is based on mechanically caused pressure gradients. Within this process, air is removed from the tissue to introduce a solution to the pores of the matrix with a defined composition. Among other things, the used solutions can contain firming, antimicrobial or antioxidant compounds (Panarese et al., 2013).

### 3.3.1 Mechanism and Influencing Factors

During the VI process, fruits or vegetables are placed in a solution with a specific composition. The material is then exposed to a two-stage pressure variation (Yusof et al., 2017). The impregnation that takes place is a result of two mechanisms – hydrodynamic mechanisms (HDM) and deformation-relaxation phenomena (DRP) (Radziejewska-Kubzdela et al., 2014).

When the material is initially immersed in solution, the internal ($p_i$) and external ($p_e$) pressure of the capillary are in equilibrium and correspond to atmospheric pressure ($p_{atm}$). On this stage, the volume of the capillary is loaded with gas. Gas is released when the pressure is reduced below $p_{atm}$. Due to the lower pressure from outside, the first part of DRP takes place. The capillary deforms and expands and therefore, the volume in the capillary rises. Once a balance between internal and external pressure of the capillary ($p_i = p_e$) occurs, the fluid partially enters the capillary. This step is based on the action of HDM. At last, the pressure is returned to atmospheric pressure where the DRP transits to the relaxation phase. In this step, the impregnation of the tissue takes place. The capillary shrinks to a lower final size compared to the beginning of the treatment. Due to an intense flow of liquid from outside into the capillary the level

of liquid of intracellular capillaries increases whereas the gas volume declines (Radziejewska-Kubzdela et al., 2014).

For a successful outcome and to achieve the desired effects it is important to know that the VI procedure is influenced by several variables. The process is based on various extrinsic factors such as the vacuum pressure and the duration of the impregnation steps (Gómez Galindo & Yusof, 2015). When carrying out VI, it is crucial to not remove the vacuum too fast. If the transition from vacuum to atmospheric pressure occurs too quickly, the capillary vessels may contract and therefore inhibit HDM (Radziejewska-Kubzdela et al., 2014). Other extrinsic determinants are the temperature, the viscosity, and the osmotic pressure of the surrounding solution. VI is also affected by many intrinsic factors such as the pore size and distribution which affect the capillary pressure. Further parameters are the size and shape of the product as well as the wetting angle between liquid and pore walls and the surface tension of the solution (Gómez Galindo & Yusof, 2015). Key factors of VI are the ratio of porosity and pore cross-linking which influence the characteristics of the intracellular air spaces. Due to the heterogeneity of the tissue and the dynamism of VI, the impregnation may not be homogenous (Panarese et al., 2016). Gómez Galindo and Yusof (2015) pointed out that cells with a smaller size and less cross-linking of intercellular spaces might result in a lower impregnation effect. For the tissue of strawberries as well as for the tissue of spinach, it was observed, that the impregnation of the outer part was more difficult since the tissue there is more compact and the cells are smaller (Gómez Galindo & Yusof, 2015; Velickova et al., 2013).

### 3.3.2 Advantages and Food Applications

VI is a promising technology regarding the modification of physicochemical and sensory properties as well as the enhancement of the quality of food (Yusof et al., 2017). Depending on which components are introduced in the material, this approach may be used to improve texture, colour, and taste, lower the pH and water activity, or modify thermal properties. Furthermore, enriching the diet by introducing bioactive substances, health benefits of the product can be enhanced (Radziejewska-Kubzdela et al., 2014).

Substances which have been typically introduced in food matrices are e.g. calcium lactate, ascorbic acid, citric acid, trehalose or sucrose (Radziejewska-Kubzdela et al., 2014; Yusof et al., 2017). An improvement in texture of minimally

processed lettuce, apples and carrots was achieved by performing VI with calcium lactate at concentrations between 0.5 to 2.5 g/L. VI with ascorbic acid or citric acid in a concentration range of 10 mg/L to 20 g/L was applied to lower bacterial growth due to the decreased pH and to prevent enzymatic browning of the product during storage. A VI solution with sucrose is commonly used to decrease the water activity prolonging the shelf life (Yusof et al., 2017). Moreover, vitamins can be added to the material to improve their nutritive value and to influence the metabolism of the product (Radziejewska-Kubzdela et al., 2014).

### 3.3.3 Vitamins for Vacuum Impregnation

Generally, vitamins are organic nutrients which are crucial for the human health since they are necessary for several vital body functions. Since most vitamins are either not synthesized in sufficient concentrations or not present in the human body, they must be taken up through the diet (Chand & Savitri, 2016).

Vitamins have already been successfully introduced through VI into cell matrices whereby certain advantages can be obtained. On top, Radziejewska-Kubzdela et al. (2014) pointed out that the bioavailability of Vitamin E was clearly increased by consuming vitamin enriched food compared to the intake as a supplement in capsules.

In contrast to fat-soluble vitamins like vitamin E which are stored for a certain period in the liver, water-soluble vitamins are excreted through the urine. Therefore, water-soluble vitamins need to be supplied daily through the diet. These include among others the vitamin B-complex group (Chand & Savitri, 2016).

#### 3.3.3.1 Properties and Health Benefits of Several B-Vitamins

Vitamin $B_1$, also known as thiamine, is involved in energy metabolism as well as cerebral metabolism. Since it can be stored in the body for only a very short time and it is partially lost by cooking, it is beneficial to consume the vitamin in foods that are not processed as much (Fattal-Valevski, 2011). Vitamin $B_9$ (Folate) is among other things involved in DNA metabolism and thus involved in the decrease of the risk of neural tube deficiencies (Fenech, 2012; Sobczyńska-Malefora & Harrington, 2018). Vitamin $B_5$ (pantothenic acid) belongs to the nutritional supplement class of medications and is used to treat dietary deficiencies. It has already been introduced in foods as an additive (Sanvictores & Chauhan, 2022). Vitamin $B_3$, also known as niacin or nicotinic acid, is regarded as most important B vitamin for a healthy life. However, in nature it can only be found in small amounts. It is classified GRAS (Generally

Recognized as Safe) and has already been used as a food additive. Among other things, vitamin $B_3$ is required for the metabolism of carbohydrates, lipids and proteins or cell respiration (Chand & Savitri, 2016).

#### 3.3.3.2 Influence on Senescence

Over many years of research has been made on introducing substances into food products to delay senescence and therefore, extend their post-harvest life. For example, nitric oxide (NO) or hydrogen sulfide ($H_2S$) showed beneficial effects to the inhibition of both ripening and senescence of several postharvest products like for example strawberries (Hu et al., 2012). Nevertheless, by adding these kinds of compounds into foods the consumer acceptance is questionable because of their reputation of being toxic (Sohail et al., 2021). Therefore, introducing vitamins would be a good alternative. However, a plant that is packed with a lot of vitamin has no advantage if it cannot develop properly or is impaired in its resistance to environmental influences (Smith et al., 2007).

Vitamins are synthesized by plants because of their crucial functions in plant metabolism. Hereby, in the biosynthesis of for example vitamin $B_1$, $B_3$, $B_5$ and $B_9$, the amino acid metabolism is involved (Miret & Munné-Bosch, 2014). Several vitamins which play a role in the central metabolism are involved in metabolic reactions if proteins are not present. As mentioned above, different vitamins carry out specific tasks but overall, especially water-soluble vitamins mostly act as co-factors for enzymes (Smith et al., 2007). Of these, the vitamins of the B-complex possibly have the greatest biological similarity because of their importance in enzyme reactions which makes them a key factor in the protein, fat, and carbohydrate metabolism (Marks, 1993). Furthermore, the presence of the vitamin B-complex shows improved water and nutrient uptake and enhances stress tolerance of plants – especially oxidative stress (Farouk et al., 2021). Plants that are exposed to abiotic stress accumulate vitamin $B_1$ and increase the production of important biosynthetic enzymes. This way an enrichment of thiamine concentration improves the stress resistance of plants. Vitamin $B_1$ also shows a potential in protecting plants against pathogens since it is known to trigger a systemic acquired resistance (SAR) against fungal, bacterial, and viral infections (Miret & Munné-Bosch, 2014). Higher concentrations of Coenzyme A (CoA) – main active form of vitamin $B_5$ – leads to a higher salt and osmotic resistance. Furthermore, vitamin $B_3$ is a precursor of pyridine alkaloids which are accumulated as

osmoprotectants in response to stress. All in all, the biosynthesis and influence of the individual vitamins is very complex and depends on many internal and external effects. Nevertheless, all these protective mechanisms of the different vitamins might delay spoilage of food or food products (Rubio et al., 2008).

#### 3.3.3.3 Food Applications

In a study of Farouk et al. (2021) it was possible to improve the yield and the quality of superior grapevines by spraying them with a vitamin B-complex containing vitamin $B_1$, $B_2$, $B_6$ and $B_{12}$ (Farouk et al., 2021). In another study of Xu et al. (2021) the quality as well as the nutrient content of broccoli has been successfully maintained and senescence has been delayed by immersing the vegetable in a 5 mg/L solution of folic acid. The positive effect of folic acid on broccoli is correlated to several aspects such as a reduced respiration rate, a suppression of ethylene formation, an increase in the activity of antioxidant enzymes, and an inhibition of the gene expression of chlorophyll-degrading enzymes (Xu et al., 2021). Foliar sprays containing thiamine were applied on maize (*Zea mays* L.) in a study of Sahu et al. (1993). Hereby, senescence was delayed by enhancing the photosynthetic efficiency and photosynthesis (Sahu et al., 1993).

## 3.4 Combined Vacuum Impregnation and Electrical Treatment

As described above, VI leads to a replacement of the air by a solution containing a specific composition of substances. By the subsequent application of electrical treatments such as PEF or MEF, a homogeneous electroporation of the cells of the different tissues is desirable. In the end, through the combination of these processes the aim is that the solution is not only homogeneously distributed extracellularly but also intracellularly in the tissue (Dymek et al., 2016).

### 3.4.1 Metabolic Responses and Research in Food Processing

Yusof et al. (2017) showed that after applying VI the metabolism of the cells can be increased depending on the introduced substances. So, for example, shortly after the impregnation with sucrose or calcium lactate a higher metabolic heat production was examined. On the other hand, for VI with ascorbic acid or citric acid no changes were observed. It is assumed that substances such as sucrose are taken up by the cells and then metabolized by only applying VI. So, Yusof et al. (2017) suggested that different molecules might be taken up and metabolized differently by cells which leads to different effects (Yusof et al., 2017).

A study of Dymek et al. (2016) pointed out that the metabolism in spinach leaves is already increased after the VI treatment with cryoprotectant trehalose but is even further increased by a following PEF application. Hereby, the electroporation of the cell membrane and the subsequent resealing process not only leads to structural modifications of the lipid matrix but also includes several complex metabolic responses. These can involve for example energy release caused by the flow of ions or ATP hydrolysis. As already mentioned, one of the goals is to additionally have a higher intracellular concentration of the substances introduced into the tissue. However, in this study no raise of trehalose in the cytosol was detected (Dymek et al., 2016). Demir et al. (2018) have shown that an enhancement of the freezing tolerance of spinach through the combined pre-treatment of VI using a solution containing cryoprotectants and PEF is possible. Applying VI results in the reduction of freezable water and therefore, lowers the tissue damages caused by freezing. The effect of substances like sugars cannot enhance the freezing tolerance of the cells by itself. Further physiological adaptions are necessary to enable cell viability after freezing and thawing processes. Nevertheless, the cryoprotectants are introduced in the apoplast after the VI treatment, but not intracellularly as naturally. A subsequent PEF treatment may facilitate the introduction and distribution of the molecules inside the cells. Intracellularly the cryoprotectants can then develop their protective properties better (Demir et al., 2018). Also, Yurttas et al. (2014) has already explored the combined use of VI and electrical treatments as a pre-treatment to improve food properties and to prolong the shelf life of food. The extension of the shelf life of mushrooms was researched using an anti-browning solution for VI followed by an electrical treatment. In this study, it was shown that the shelf life of sliced mushrooms could be extended by using a VI solution containing 2 g/100 g ascorbic acid and 1 g/100 g calcium lactate. The combined use of the technologies also scored best in sensory tests in which the product appearance counted as one of the most crucial characteristics (Yurttas et al., 2014).

# 4. Materials and Methods

## 4.1 Raw Material

The spinach was provided by the company Vidinge Grönt AB (Sweden). It was Eco-spinach that was supplied from Italy to Sweden within 4-5 days after harvesting. The temperature during delivery, storage, and the following processes at the storage location Vidinge was always kept at 4 °C. As the product arrived at the company, the quality was controlled and afterwards the spinach was washed. These two processes were carried out within a maximum period of 4 days. Before packing, the spinach was stored for a maximum of 2 days. At last, the product was packed in plastic bags with a shelf life of 9 days starting from the packaging date. In summary, the spinach remained in the company for a maximum of 6 days. However, an internal average value of the company from receiving to packing the spinach leaves is claimed to be about 2-3 days.

For the experiments, the spinach was delivered after the washing process, e.g. within maximum 11 days after the spinach was harvested. For the transportation of the spinach, the cooling chain was interrupted for around 1 h. The spinach was stored for approximately 12 h at 5 °C before it was treated further. For the experiments, spinach leaves with no visible damages were used.

The equipment and chemicals used for the experimental part of this thesis are listed in the appendix (**Table 10**, **Table 11**).

## 4.2 Treatments

### 4.2.1 Electrical Treatments

For the electrical treatment, two different types of electro-technologies were applied on the spinach leaves. Leaves were electroporated with PEF using a DC power source and applying rectangular pulses. Whereas, during MEF treatment, AC with lower field strength was used and sinusoidal waves were applied.

#### 4.2.1.1 Pulsed Electric Field

A sodium chloride (NaCl) solution with a conductivity of 130 µS/cm was prepared by solving NaCl in distilled water (DI water). An electroporation chamber was filled with 50 ml of the NaCl solution, and two spinach leaves were placed in face down. The treatment chamber consisted of two parallel stainless-steel electrodes with a gap of 0.5 cm between them. Both electrodes and the samples were covered fully with the solution. For the treatment it was also crucial to avoid overlapping of the leaves and to

prevent bubbles when closing the lid. By connecting the chamber to a pulse generator CythorLab™ (ADITUS Medical AB, Lund, Sweden) the electric pulses were delivered. One train of 500 bipolar, rectangular pulses with an amplitude of 200 V (400 V/cm) was applied on the samples. The pulse width had a value of 200 µs and the pulse space was 1600 µs.

After the treatment, the leaves were taken out of the chamber and washed with DI water to remove the remaining NaCl solution. Then, the water at the surface of the leaves was carefully removed with absorbent paper before they were packed.

#### 4.2.1.2 Moderate Electric Field

The MEF treatment was carried out in the same way as the PEF treatment with few modifications. Instead of the pulse generator CythorLab™, the treatment chamber was connected to a high-power programmable AC power source (B&K Precision Corp., Yorba Linda, California). Then, the electric pulses were delivered to the chamber. Bipolar sinusoidal waves with 90 V (180 V/cm) and a frequency of 50 Hz were applied for 5 s per treatment. Subsequently, the leaves were washed and dried before packing.

### 4.2.2 Vacuum Impregnation

For the VI treatment, four different vitamins of the B-complex – vitamin $B_1$, $B_3$, $B_5$ and $B_9$ – were used. More detailed characteristics and used concentrations are shown in **Table 2**. The different vitamin solutions were prepared by solving the vitamin powder in DI water.

**Table 2.** Used vitamins with their properties and applied concentrations for VI experiments.

| Vitamin | Chemical name | Molecular Weight [g/mol] | Used Concentrations [mM] |
|---|---|---|---|
| Vitamin $B_1$ | Thiamine Hydrochloride | 337.28 | 1.48 |
| Vitamin $B_3$ | Nicotinamide | 123.11 | 8.12 |
| Vitamin $B_3$ | Nicotinamide | 123.11 | 40.61 |
| Vitamin $B_5$ | Pantothenic Acid | 219.23 | 1.51 |
| Vitamin $B_9$ | Folic Acid Dihydrate | 441.40 | 0.40 |

Around 10-13 g of spinach leaves were weighed in a beaker. The exact weight of the spinach leaves per beaker was noted before and after the treatment to determine the weight change of the leaves after the VI impregnation with the following formula:

$$Weight\ Change\ [\%] = \frac{Weight\ before\ treatment\ [g]}{Weight\ after\ treatment\ [g]}\ x\ 100\% \qquad (1)$$

The spinach leaves were then covered with the prepared vitamin solution. A thin plastic net was placed on the surface of the leaves to prevent them from not being completely immersed in the solution. Seven beakers were then placed in the VI chamber which was covered in aluminium foil to ensure that dark conditions prevail during the treatment. In this way, light sensitive vitamins were protected. Using a Teflon tube, the chamber was connected to a Vacuum Controller IT2010-05 (S.I.A. di Panarese Angelo, Italy) to regulate the pressure within the VI chamber. A vacuum pump was used to decrease the pressure. The protocol consisted of one cycle that lasted approximately 32 min. This cycle was repeated once for a complete VI treatment and therefore, the whole treatment took around 64 min. The vacuum impregnation was carried out at room temperature.

**Figure 2** shows the detailed protocol which was set by using a Cimplicity HMI software (PLC, Series 90-30, General Electric, Charlottesville, VA, USA) which was connected to the vacuum controller. The initial pressure of one cycle was 1000 mbar (atmospheric pressure) which then was decreased gradually to 150 mbar within 11 min. After the pressure of 150 mbar was kept for 1 min, the pressure was stepwise increased back to 1000 mbar within 7 min. After atmospheric pressure was reached, it was kept for further 13 min.

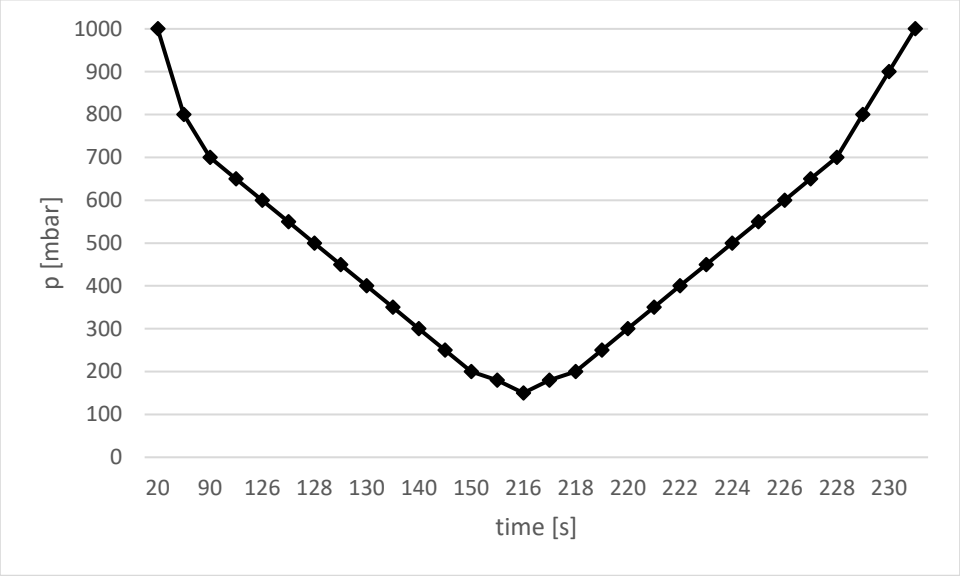


**Figure 2**. Detailed protocol set with Cimplicity HMI software. One cycle is shown.

After vacuum impregnation was completed, the beakers were removed from the chamber. Spinach leaves were then taken out from the beaker and carefully dried with absorbent paper to remove the remaining vitamin solution on the surface. Afterwards, the spinach leaves were packed for shelf life evaluation and further analyses.

### 4.2.3 Combination of Vacuum Impregnation and Electrical Treatment

First, VI treatment with a vitamin $B_3$ solution with a concentration of 8.12 mM was performed as described above. Secondly, the leaves were treated with either PEF or MEF in a 130 µS/cm NaCl solution. After the electrical treatment, the leaves were washed and dried with absorbent paper before packing.

### 4.2.4 Packing

The material for the plastic bags was obtained from OptiCept AB (Lund, Sweden). The plastic is made of polypropylene with a thickness of 30 µm. The material has approximately 104 holes/$m^2$ with a diameter of 80 µm. The Oxygen Transmission Rate (OTR) is around 1300 cc/$m^2$/24hr/atm. Bags with dimensions of 20 cm x 16 cm were

prepared. Approximately 17 g of sample were weighed in the bags and sealed with a Heat Sealer with Cutter (Fisherbrand, Sweden). While sealing, atmospheric air was pumped into the bag with an electrical air pump (Likey, NY, USA) to provide the leaves with as much air as possible during storage (**Figure 3**). The aim was to come as close as possible to industrial conditions. Treated and non-treated leaves were packed. Non-treated leaves were washed with DI water and then gently dried with a soft tissue before packing to have the same conditions as for treated leaves for further analyses and comparisons.


**Figure 3**. Process of sealing packages while pumping air in the bag with an electrical air pump.

#### 4.2.5 Storage

The bags containing the spinach leaves were stored at 5 °C. To get close to retail conditions the lights were left on.

### 4.3 Analysis

All analyses after packing the spinach leaves, including measurement of atmospheric composition, visual observations, and colour measurements, were done for both treated and non-treated spinach leaves. For each analysis, three bags containing the spinach leaves were used and discarded afterwards.

### 4.3.1 Microscopic Observations

#### 4.3.1.1 Effect of Electrical Treatment

The effect of the electrical treatments on electroporation was analysed with fluorescence microscopy using the method described by Dymek et al. (2014).

First, a propidium iodide (PI) solution with a conductivity of 130 µS/cm was prepared. 0.17 g of PI were weighed in a 1000 mL screw neck bottle and 800 mL DI water were added. After the PI was completely dissolved, 4.3 mL of 1x Phosphate Buffered Saline (PBS) solution with a conductivity of 15 mS/cm were added. Then, the bottle was filled up with DI water to a total volume of 1 L. With a conductivity meter (Orion Research Inc., Jacksonville, FL, USA) the conductivity of the prepared solution was double checked. Afterwards, the treatment chamber for the electrical treatment was filled with the PI solution and two spinach leaves were placed in face down. After closing the chamber, pulses with specific parameters were applied on the leaves as described for the PEF treatment with NaCl solution. PI binds to the DNA of the cells and increases it's fluorescence by around 20-30 times. Therefore, it is possible to analyse the level of membrane electroporation (Dymek et al., 2014). Immediately after the electrical treatment, the leaves were taken out and rinsed with DI water to remove remaining PI solution on the surface. Then, the spinach leaves were observed under a fluorescence microscope (Elipse Ti-U, Nikon, Japan) with a magnification of x 5 and x 10. Micrographs were taken in different areas of the leave. This was done in duplicates.

Different protocols were tested until a good protocol for reversible permeabilization was found. For PEF treatment one train of rectangular pulses with different combinations of amplitudes (100-300 V) and number of pulses (250-500 pulses) were observed. Hereby, the pulse width always had a value of 200 µs and the space between the pulses was 1600 µs. For MEF treatment bipolar sinusoidal waves with different combinations of frequency (50-300 Hz), amplitude (20-200 V) and time of treatment (2 s, 5 s) were examined.

Micrographs were modified by using the software ImageJ (Wayne Rasband, MD, USA). First, the pictures were converted into 8-bit images and second, the colour balance and brightness of the 8-bit images were adjusted to visualize the results better. To not falsify the results for comparisons of the different treatments, identical changes were made for micrographs with x 5 and x 10 magnification respectively.

#### 4.3.1.2 Cell Viability

The cell viability of the spinach leaves after the electrical treatment was observed after 24 h with fluorescein diacetate (FDA) following the method of Dymek et al. (2014).

First, the leaves were electroporated in a 130 µS/cm NaCl solution. Damp paper covered with a plastic net was placed in a transparent box for the storage of the leaves. Secondly, the leaves were removed from the chamber, rinsed with DI water, and then placed in the box. The box was closed with a lid and kept in a 5 °C refrigerator 24 h.

To test the cell survival of the spinach leaves, a stock solution of 12 mM FDA was prepared in acetone. This solution was stored at 4 °C in a screw neck bottle covered with aluminium foil. Shortly before the experiment, the stock solution was diluted with DI water to reach a concentration of $12 \times 10^{-4}$ µM. Samples treated with different parameters were tested of cell viability. 6 leaves were examined per treatment. First, the leaves were incubated in the diluted FDA solution in darkness at room temperature for 30 min. Then, the samples were taken out of the solution, rinsed with DI water, and immediately observed under the microscope. FDA micrographs were also modified by using the software ImageJ. The micrographs were manipulated in the same way as it was done for the images of spinach leaves stained with PI. Thus, the micrographs were converted into 8-bit images and the colour balance and brightness were manipulated for an improved visualization. The same changes were made for micrographs with x 5 and x 10 magnification respectively.

### 4.3.2 Measuring Atmospheric Composition

The $O_2$ and $CO_2$ concentration inside the bags was measured in triplicates using the CheckMate 3 (Dansensor A/S, Denmark). The bags, stored at 5 °C, were punched with a needle whereby the device absorbed the gas and measured the concentrations.

During storage, measurements were done every second or, for the combined treatment, every third day until the spinach lost its freshness and was considered as not consumable anymore. The spinach in the bags was used for the following visual observations and colour measurements and then discarded.

### 4.3.3 Visual Observations

After the measurement of the atmosphere, the packages were opened, and the total amount of decayed leaves were counted to record the state of decay of the spinach bags. A spinach leaf was considered as decayed when at least half of the leaf was hanging. Representative examples can be seen in **Figure 4**.


**Figure 4**. Representative pictures of the state of decay of spinach leaves. (**a**) Fresh, not decayed leaf. (**b**) Head hanging leaf. (**c**) Completely decayed leaf.

Furthermore, on measuring days pictures of the leaves were taken under controlled conditions with a digital camera (Digital sight DS-Qi1Mc, Nikon Co., Japan). Identical conditions for pictures taken on different days were ensured by having a cabinet with uniform lighting. LED lights were placed on all four sides in the cabinet which was closed for taking the picture. Hereby, influences from, for example, daylight were excluded. Also, the same camera settings as flash, zoom etc. were used for all images.

### 4.3.4 Colour Measurements

The colour measurements were performed for treated and non-treated spinach leaves by using a Portable Spectrophotometer CM-700d with a target mask with 8 mm diameter (Konica Minolta Sensing, Inc., Japan). From each bag, 6 intact leaves were taken, and the colour was measured on the surfaces. The parameters L*, a* and b* could be taken from the device. Hereby, L* specifies the lightness and a* and b* indicate the chromatically coordinates, i.e. the difference in red and green or in yellow and blue respectively.

### 4.3.5 Statistical Analysis

Statistical analysis between treatments and within methods was performed by means of one-way-ANOVA on a significance level of $p<0.05$ using MS Excel (Microsoft Office, Redmond, WA, USA). Post hoc tests were carried out using Tukey´s confidence intervals ($p<0.05$).

# 5. Results

The analytical results are expressed as means ± standard error with minimum of two measurements.

## 5.1 Microscopic Observations of Leaf Electroporation and Cell Viability

The effect of electroporation is not only characterized by the occurrence of electroporation but also by the maintenance of cell viability. Therefore, these two aspects were observed under the microscope by staining the leaves with PI and FDA for different PEF and MEF protocols.

### 5.1.1 Pulsed Electric Field

It was possible to achieve homogenous electroporation while maintaining leaf viability for the PEF protocols 2, 3, 6 and 7 (**Table 3**). The protocol applying 500 bipolar pulses with a pulse amplitude of 200 V (protocol 3) was chosen for further analyses. With these treatment parameters not only uniform electroporation (**Figure 5**) was achieved, but the leaves also showed the highest percentage of viable cells (**Figure 6**). Furthermore, for all protocols replicates of 6 leaves were prepared for the FDA test. By using 500 pulses and 200 V, all leaves visually showed an equally good quality after a storage period of 24 h.

**Table 3.** Electroporation occurrence and preservation of viability of spinach leaves after 24 h for different PEF protocols. One train of rectangular bipolar pulses with a pulse width of 200 µs and a pulse space of 1600 µs was applied for all protocols. Pulse amplitude and the number of pulses were changed individually as indicated.

| Protocol number | Pulse amplitude [V] | Number of pulses | Homogenous Electroporation | Viability after 24 h |
|---|---|---|---|---|
| 1 | 300 | 500 | Yes | No |
| 2 | 250 | 500 | Yes | Yes |
| 3* | 200 | 500 | Yes | Yes |
| 4 | 100 | 500 | No | - |
| 5 | 300 | 400 | Yes | No |
| 6 | 300 | 300 | Yes | Yes |
| 7 | 300 | 250 | Yes | Yes |

*Protocol used for further analyses and combined treatment

Representative micrographs for the effect of reversible electroporation for protocol 3 are shown in **Figure 5**. The DNA of nuclei of permeabilized cells which have taken up the PI appear as red dots in the micrographs. Therefore, a uniform distribution of red dots indicates homogenous electroporation. It is assumed that the electroporated cells which were able to absorb the PI were at least temporarily permeable.

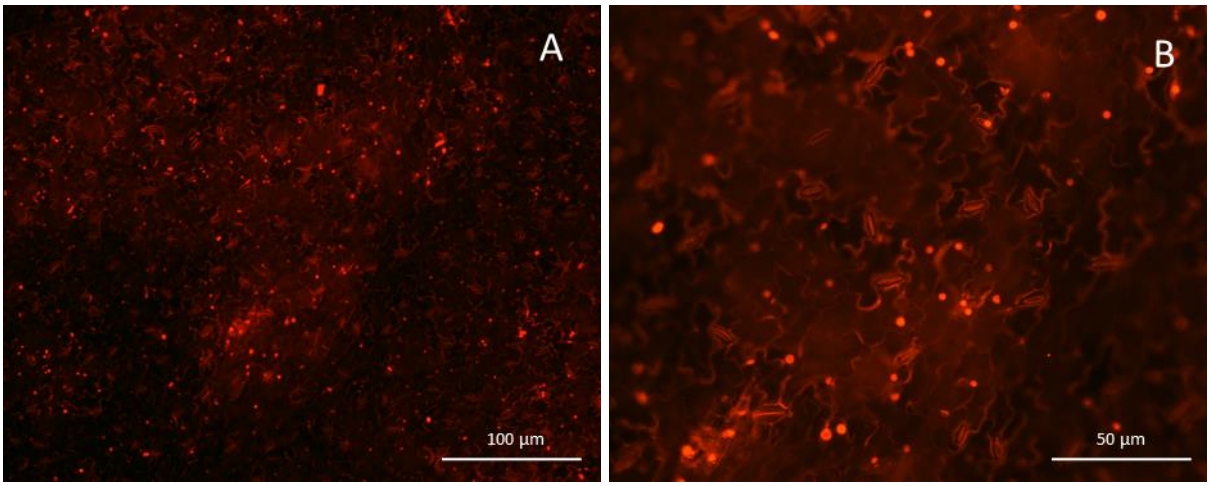


**Figure 5**. Representative micrographs of the surface of electroporated spinach leaves stained with PI. Samples were treated with one train of 500 rectangular bipolar pulses with an amplitude of 200 V, whereas the pulse width was 200 µs and the pulse space 1600 µs. The penetration of PI into permeabilized cells can be seen as red dots. (**A**) Magnification x 5. (**B**) Magnification x 10.

**Figure 6** shows the results of the FDA test for leaves treated with protocol 3 compared to an untreated control sample and a dead leaf. Viable cells stained with FDA are visible by bright green fluorescence, whereas non-viable cells lack fluorescence and appear black. The leaves treated with 200 V and 500 pulses kept their viability after 24 h.

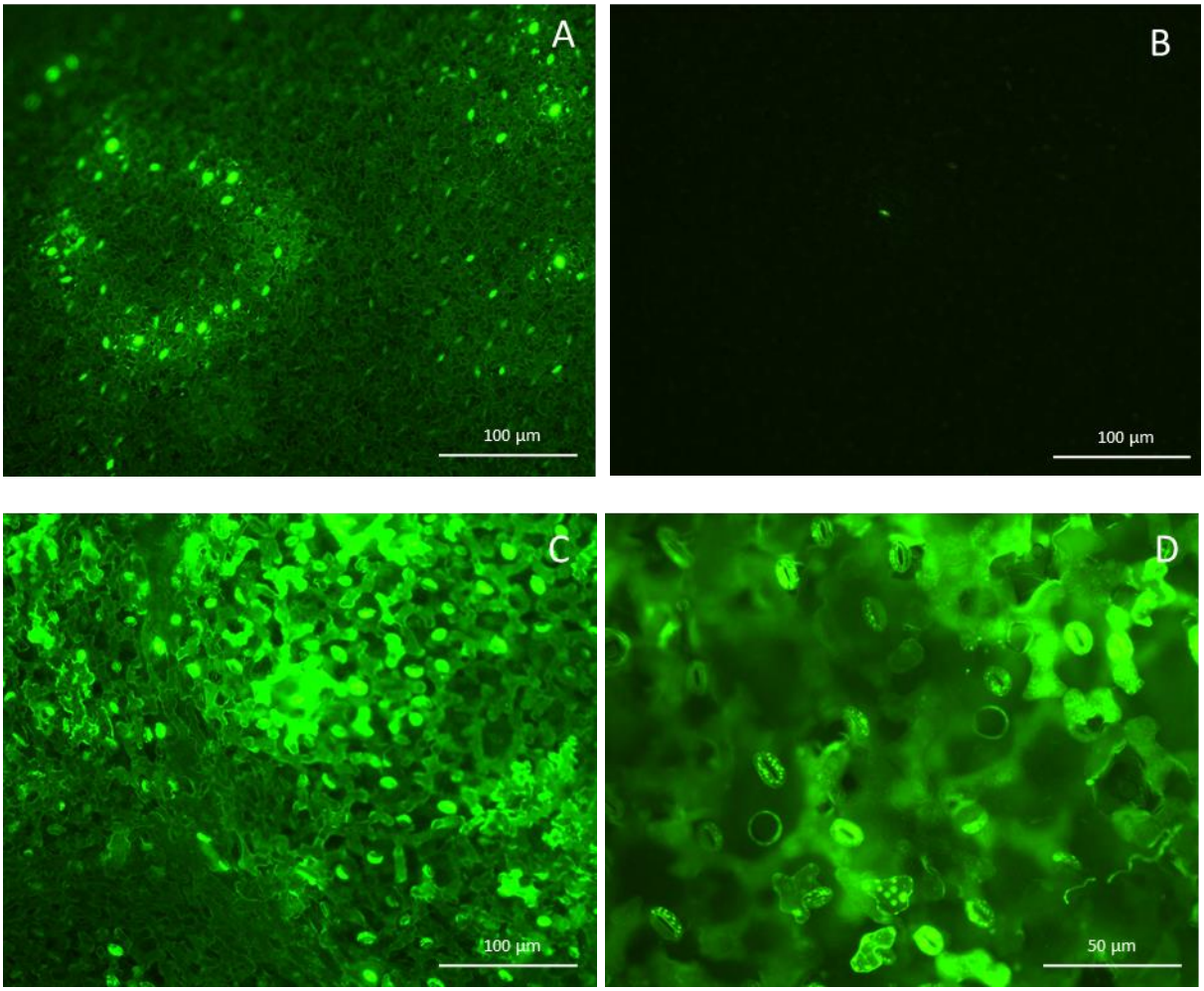


**Figure 6.** Representative micrographs of the surface of untreated and treated spinach leaves stained with FDA. (**A**) Untreated fresh leaf. (**B**) Dead leaf treated with one train of 500 bipolar pulses with an amplitude of 300 V, a pulse width of 200 µs and a pulse space of 1600 µs. (**C**) and (**D**) treated with one train of 500 bipolar pulses with an amplitude of 200 V, a pulse width of 200 µs and a pulse space of 1600 µs. (**C**) Magnification x 5. (**D**) Magnification x 10.

### 5.1.2 Moderate Electric Field

For MEF treatment, protocol 3, 4 and 6 (**Table 4**) showed homogenous electroporation (**Figure 7**) where cell viability was kept for at least 24 h after the treatment (**Figure 8**).

**Table 4.** Electroporation occurrence and preservation of viability of spinach leaves after 24 h for different MEF protocols. For all protocols, bipolar sinusoidal waves were applied. Pulse amplitude, frequency and time of application were changed individually as described.

| Protocol number | Pulse amplitude [V] | Seconds [s] | Frequency [Hz] | Homogenous Electroporation | Viability after 24 h |
|---|---|---|---|---|---|
| 1 | 20 | 2 | 50/100/300 | No | - |
| 2 | 50 | 2 | 50 | Yes | - |
| 3 | 90/100 | 2 | 50 | Yes | Yes |
| 4 | 110/120 | 2 | 50 | Yes | Yes |
| 5 | 200 | 2 | 50 | Leaves dead after treatment | |
| | | | | - | - |
| 6* | 90 | 5 | 50 | Yes | Yes |

*Protocol used for further analyses and combined treatment

The protocol applying 90 V and 50 Hz for 5 s was selected for further analyses. This protocol was chosen by comparing the micrographs for the leaves. For those stained with FDA the entire leaves were fluoresced green indicating the highest cell viability.

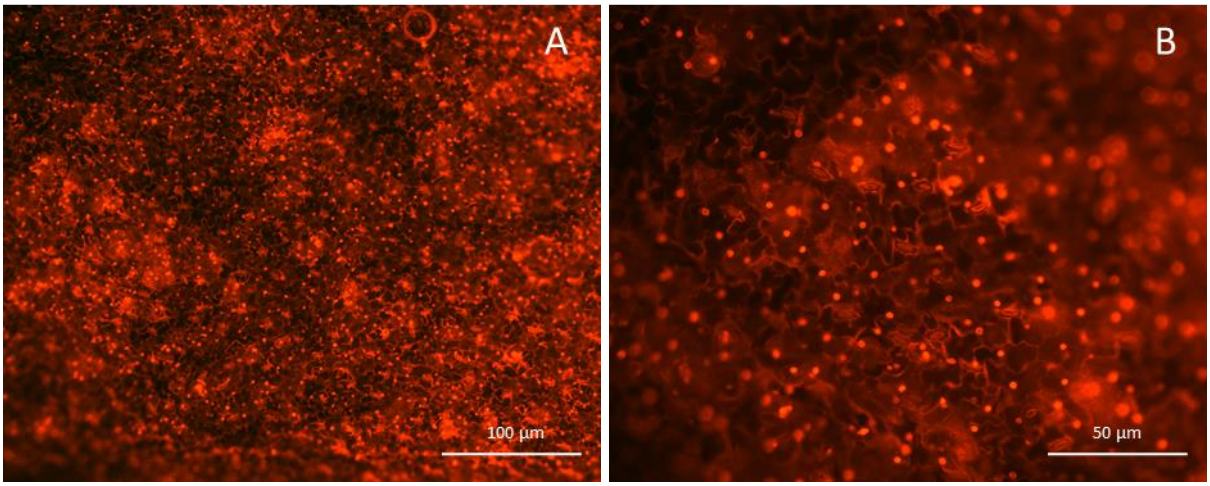


**Figure 7.** Representative micrographs of the surface of spinach leaves treated with bipolar sinusoidal waves with 90 V and 50 Hz for 5 s and stained with PI. The penetration of PI into permeabilized cells can be seen as red dots. (**A**) Magnification x 5. (**B**) Magnification x 10.

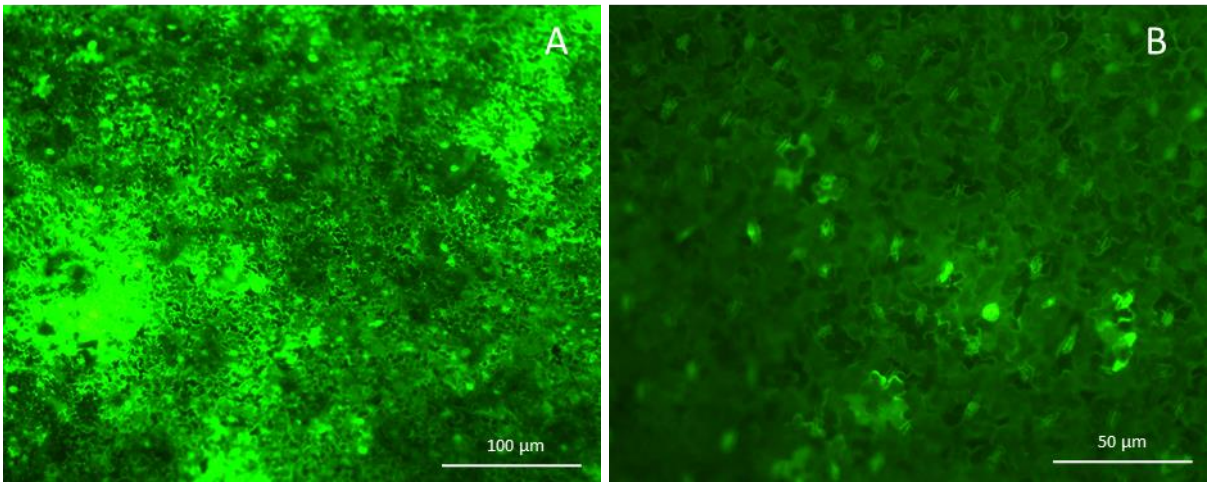


**Figure 8**. Representative micrographs of the surface of spinach leaves treated with bipolar sinusoidal waves with 90 V and 50 Hz for 5 s and stained with FDA. Viable cells appear in form of a bright green fluorescence, non-viable cells are black. (**A**) Magnification x 5. (**B**) Magnification x 10.

## 5.2 Weight Change after Vacuum Impregnation

Impregnation of spinach leaves with different B-vitamin solutions increased the weight of the leaves. **Table 5** shows the average weight change per vitamin after VI treatment.

**Table 5**. Weight change [%] of spinach leaves after impregnation with different B-vitamin solutions.

| Impregnated Vitamin | Concentration [mM] | Weight Gain [%] |
|---|---|---|
| Thiamine Hydrochloride ($B_1$) | 1.48 | 63.64 ± 0.29 a |
| Nicotinamide ($B_3$) | 8.12 | 58.39 ± 0.21 b |
| Nicotinamide ($B_3$) | 40.61 | 61.49 ± 0.34 c |
| Pantothenic Acid ($B_5$) | 1.51 | 64.59 ± 0.29 ad |
| Folic Acid Dihydrate ($B_9$) | 0.40 | 63.23 ± 0.39 ae |

The analytical results are expressed as means ± standard error; Different letters next to the weight gain demonstrate statistical differences ($p<0.05$) between different vitamin solutions ($n=21$).

The impregnated leaves all gained weight at a level of 58-65%. According to one-way ANOVA (Analysis of Variance), impregnating spinach leaves with each B-vitamin increased their weight significantly ($p<0.05$). Spinach leaves impregnated with pantothenic acid ($B_5$), thiamine hydrochloride ($B_1$), or folic acid dihydrates ($B_9$) gained the most weight. In contrast, the lowest increase was observed for leaves impregnated

with nicotinamide ($B_3$). Here, spinach leaves which were exposed to the higher vitamin $B_3$ concentration gained significantly more weight.

## 5.3 Atmospheric Composition

The material used for the bags to pack the spinach leaves was perforated. Therefore, the $CO_2$ and $O_2$ level should always be around a stable value corresponding to the composition of the air. **Figure 9** shows an exemplary diagram of the proportions of $O_2$ and $CO_2$ inside the bags for with PEF and MEF treated samples compared to control samples.

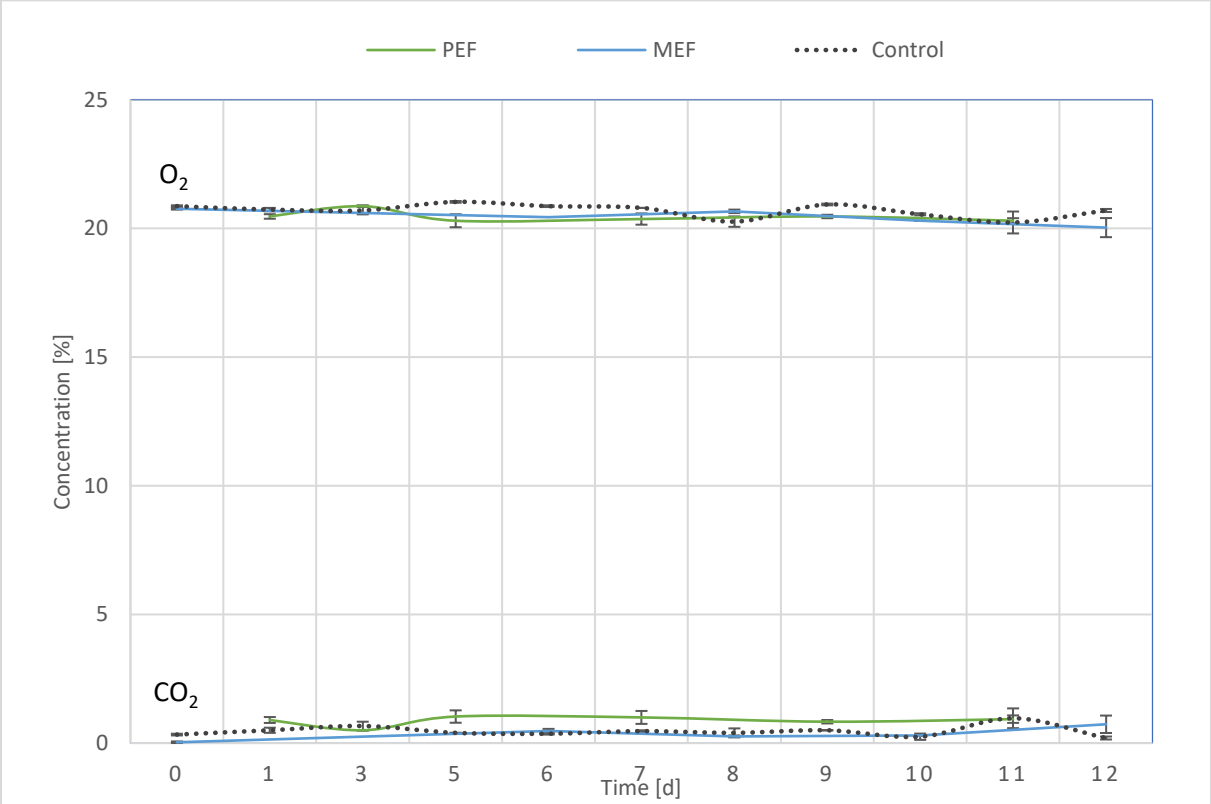


**Figure 9**. Composition of oxygen and carbon dioxide inside bags containing untreated and treated spinach leaves. Per observation day, a mean value of three measurements is shown. Upper lines indicate $O_2$, the lower ones $CO_2$ percentage. Error bars represent standard error.

Generally, there were no significant differences in the atmospheric composition between the two different treatments nor to the control samples. In conclusion, the atmosphere in all bags approximated the surrounding composition of the air during their time of storage. Similar observations were made for leaves impregnated with different vitamins of the B-complex and for the combined treatment. The following table (**Table 6**) shows an overview for all different treatment methods representing the lowest and highest $O_2$ and $CO_2$ concentration.

**Table 6.** Overview of lowest and highest $O_2$ and $CO_2$ concentrations in bags for treated and untreated samples. Per observation day, a mean value of three measurements was calculated.

| Method | Concentration Range of $O_2$ [%] | Concentration Range of $CO_2$ [%] |
|---|---|---|
| *Electrical Treatment* | | |
| PEF | 20.30 – 20.87 | 0.50 – 1.03 |
| MEF | 20.03 – 20.77 | 0.03 – 0.73 |
| *Vacuum Impregnation* | | |
| Thiamine Hydrochloride ($B_1$) | 19.63 – 21.07 | 0.23 – 0.97 |
| Nicotinamide ($B_3$) | 19.15 – 20.43 | 0.10 – 1.05 |
| Pantothenic Acid ($B_5$) | 20.20 – 20.80 | 0.47 – 0.93 |
| Folic Acid Dihydrate ($B_9$) | 19.53 – 20.90 | 0.40 – 1.10 |
| *Combined Vacuum Impregnation and Electrical Treatment* | | |
| VI+PEF | 18.97 – 20.67 | 0.07 – 1.70 |
| VI+MEF | 18.20 – 20.60 | 0.10 – 2.30 |
| *Untreated* | | |
| Control | 20.10 – 21.0 | 0.20 – 0.97 |

## 5.4 Colour Measurements

L*, a* and b* values were measured to visualize possible colour changes of the treated and untreated leaves with progressing storage time until spoilage. Furthermore, the colour after the respective treatment was compared to untreated samples to examine whether the treatment affected the spinach colour.

### 5.4.1 Electrical Treatments

Regarding the PEF treatment, no significant differences with progressing storage time could be detected for L*, a*, and b* values. Moreover, no significant colour difference compared to the control sample was observed (**Figure 10**, **Table 7**). After MEF treatment, the L* value of the sample at day 4 was significantly higher compared to day 0. Values of a* and b* did not change over the storage time. Right after the treatment (day 0), MEF treated leaves did not show a significant difference in colour compared to the untreated leaves (**Figure 10**, **Table 7**).

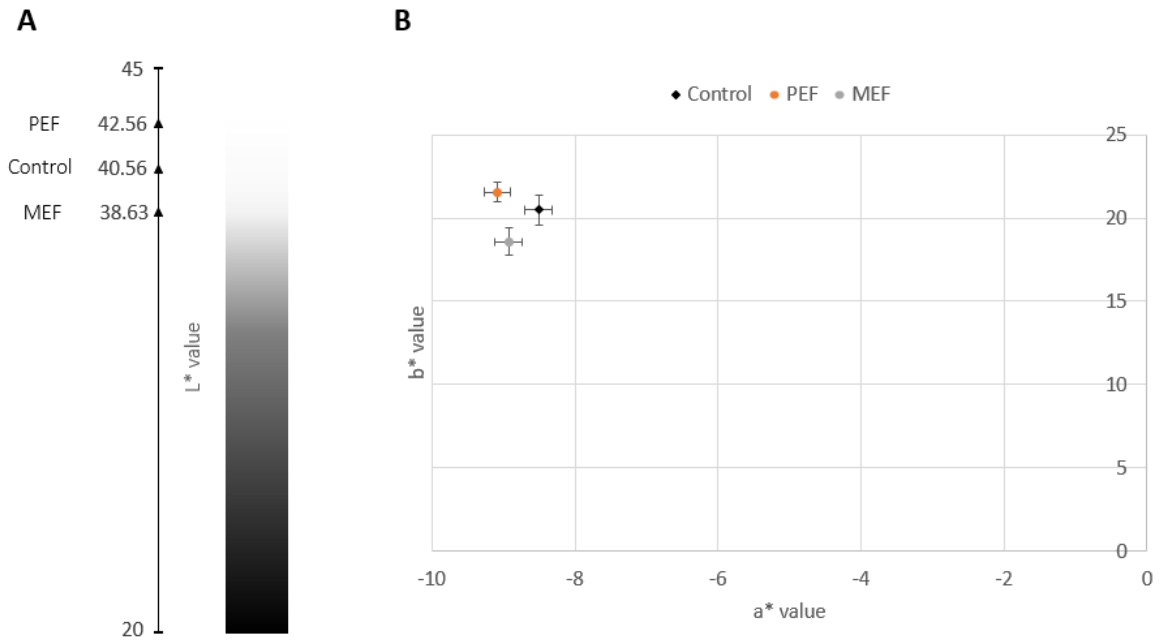


**Figure 10**. Colour measurements ~8 h after electrical treatments compared to untreated spinach leaves (n=18). (**A**) Lightness scale for L* value (**B**) Colour coordinates a* and b*. Error bars represent standard error.

**Table 7**. Statistical analysis for L*, a* and b* values for untreated as well as PEF and MEF treated spinach leaves.

| Method | L* value | a* value | b* value |
|---|---|---|---|
| Control | 40.56 ± 0.55 a | -8.51 ± 0.19 a | 20.47 ± 0.90 a |
| PEF | 42.56 ± 0.45 a | -9.08 ± 0.19 a | 21.56 ± 0.62 a |
| MEF | 38.63 ± 0.76 a | -8.92 ± 0.19 a | 18.56 ± 0.82 a |

The analytical results are expressed as means ± standard error; Different letters next to the standard error demonstrate statistical differences ($p<0.05$) between PEF or MEF treated and untreated spinach leaves (n=18).

### 5.4.2 Vacuum Impregnation

Only for leaves treated with vitamin $B_3$ and $B_9$, significant differences in L*, a* and b* values over storage time were observed. For vitamin $B_3$, the L* value of day 6 was significantly higher compared to day 0. For vitamin $B_9$ samples, a* value of day 6 was significantly lower and b* value significantly higher compared to day 0. Otherwise, no variations and therefore no trend was detected.

Compared to the untreated leaves, L* and b* values were always lower and a* values higher for the impregnated leaves. (**Figure 11**). **Table 8** shows which of the values differed significantly from the control.

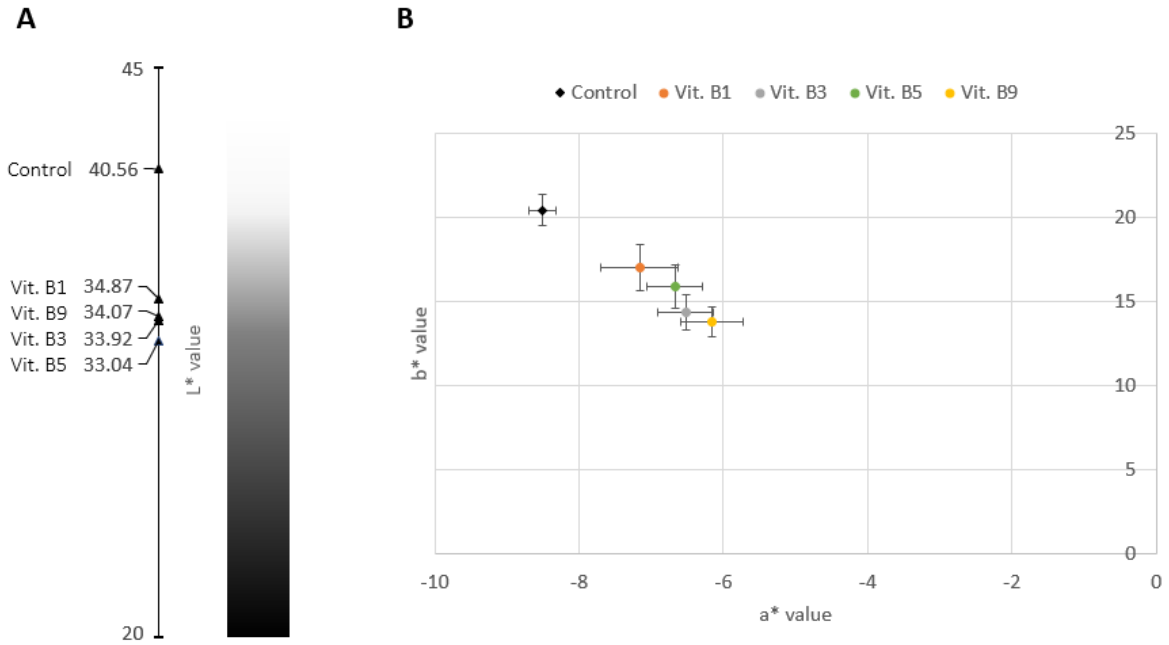


**Figure 11**. Colour measurements ~8 h after impregnation with different vitamins compared to untreated spinach leaves (n=18). (**A**) Lightness scale for L* value (**B**) Colour coordinates a* and b*. Error bars represent standard error.

**Table 8**. Statistical analysis for L*, a* and b* values for untreated as well as with different B-vitamins impregnated spinach leaves

| Method | L* value | a* value | b* value |
|---|---|---|---|
| Control | 40.56 ± 0.55 a | -8.51 ± 0.19 a | 20.47 ± 0.90 a |
| Vit. B1 | 34.87 ± 1.19 b | -7.17 ± 0.53 a | 17.03 ± 1.38 a |
| Vit. B3 | 33.92 ± 0.50 b | -6.52 ± 0.39 b | 14.41 ± 1.03 b |
| Vit. B5 | 33.04 ± 0.73 b | -6.67 ± 0.39 b | 15.94 ± 1.29 b |
| Vit. B9 | 34.07 ± 0.99 b | -6.16 ± 0.43 b | 13.79 ± 0.89 b |

The analytical results are expressed as means ± standard error; Different letters next to the standard error demonstrate statistical differences ($p<0.05$) between with different B-vitamins impregnated and untreated spinach leaves (n=18).

### 5.4.3 Combined Vacuum Impregnation and Electrical Treatment

For the combined application of VI and electric pulses, differences for a* and b* value were only observed with a subsequent MEF treatment over storage time. For the a* value, day 6, 9 and 12 showed significantly lower values than day 0, whereas for b* value day 6 and 9 were significantly higher.

As for the treatment with VI L* and b* values were always significantly lower and a* values significantly higher compared to untreated leaves (**Figure 12**, **Table 9**).

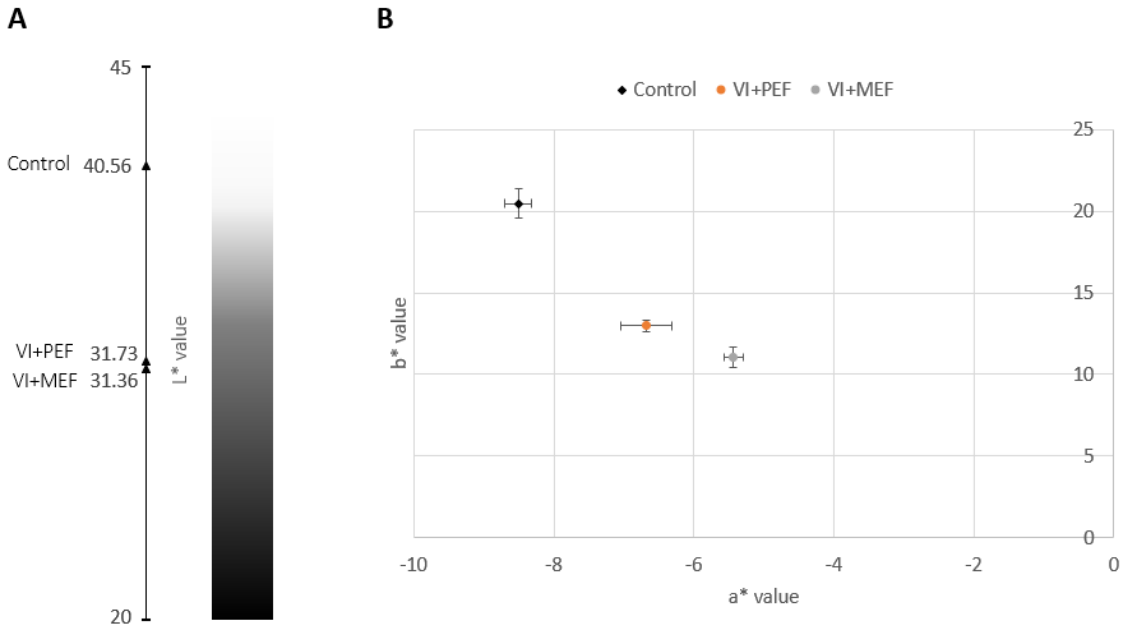


**Figure 12**. Colour measurements ~8 h after combined treatment compared to untreated spinach leaves (n=18). (**A**) Lightness scale for L* value (**B**) Colour coordinates a* and b*. Error bars represent standard error.

**Table 9**. Statistical analysis for L*, a* and b* values for untreated as well as with vitamin $B_3$ impregnated and PEF or MEF treated spinach leaves

| Method | L* value | a* value | b* value |
|---|---|---|---|
| Control | 40.56 ± 0.55 a | -8.51 ± 0.19 a | 20.47 ± 0.90 a |
| VI+PEF | 31.73 ± 0.35 b | -6.68 ± 0.36 b | 12.97 ± 0.38 b |
| VI+MEF | 31.36 ± 0.29 b | -5.43 ± 0.14 b | 11.05 ± 0.60 b |

The analytical results are expressed as means ± standard error; Different letters next to the standard error demonstrate statistical differences ($p<0.05$) between combined treatment of VI with vitamin $B_3$ and PEF or MEF and untreated spinach leaves (n=18).

### 5.4.4 Comparison of Different Methods

**Figure 13** shows representative pictures of spinach leaves taken around 1 h after the respective treatment documenting changes visible to the naked eye.

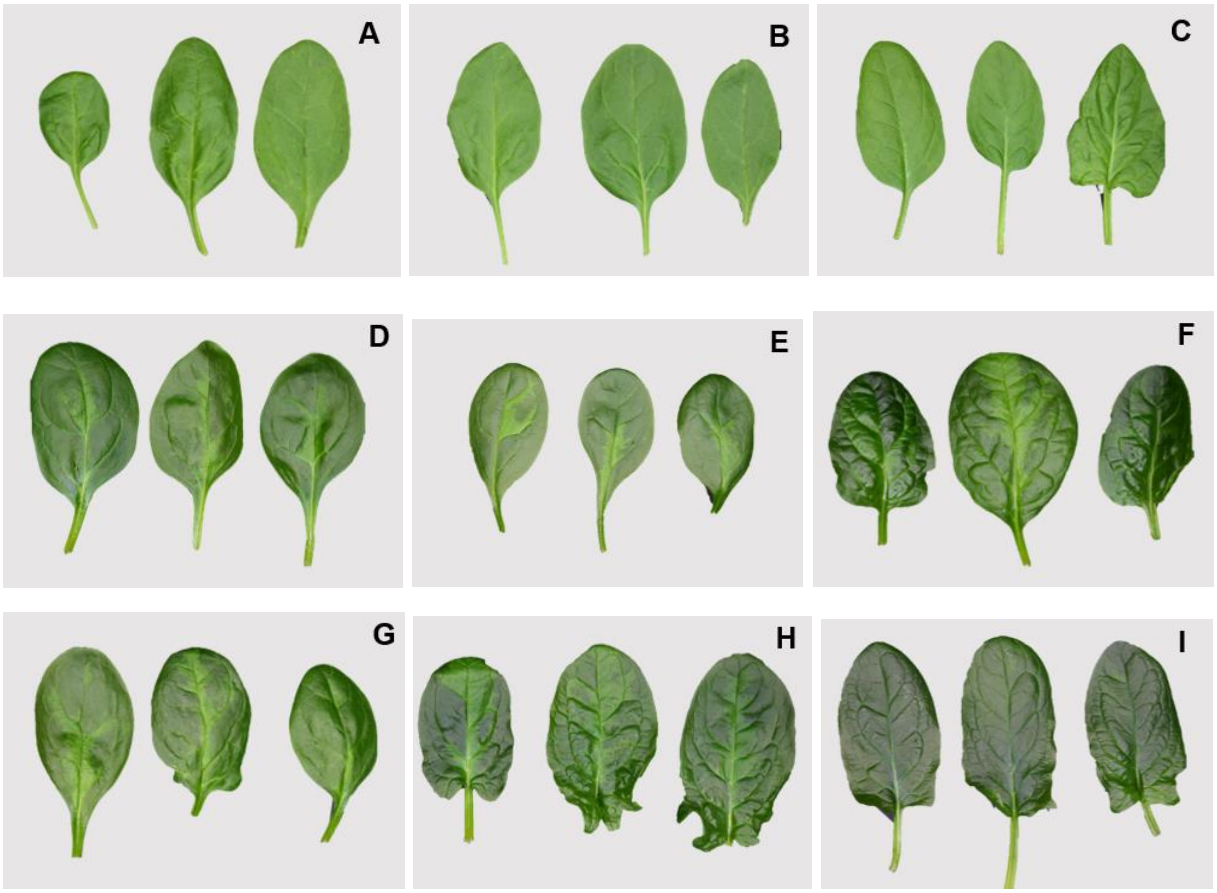


**Figure 13**. Representative pictures of spinach leaves taken around 1 h after the respective treatment. (**A**) Untreated fresh leaves. (**B**) PEF treatment (500 pulses, 200 V, 50 Hz, pulse width 200 µs, pulse space 1600 µs). (**C**) MEF treatment (50 Hz, 90 V, 5 s). (**D**) VI with vitamin $B_1$. (**E**) VI with vitamin $B_3$. (**F**) VI with vitamin $B_5$. (**G**) VI with vitamin $B_9$. (**H**) VI with vitamin $B_3$ and subsequent PEF treatment. (**I**) VI with vitamin $B_3$ and subsequent MEF treatment.

Leaves treated with PEF and MEF had no visible differences compared to the untreated ones. In contrast, the green colour appeared darker when VI was used on its own or in combination with electric pulses. The samples treated by combining VI with PEF or MEF seemed to be even darker compared to the exclusive application of VI. For the use of different vitamins only the leaves treated with vitamin $B_5$ showed a slightly darker green colour.

The visual observations regarding the lightness of the green colour coincide with the measured L* values (**Figure 10**, **Figure 11**, **Figure 12**). The L* values of untreated leaves as well as with PEF and MEF treated leaves were the highest corresponding to

a lighter green colour. This is followed by the samples impregnated with the different B-vitamins whereas the treatment with vitamin $B_5$ showed the lowest L* values. The combined use of the two technologies displayed the lowest L* value reflected in the green colour appearing the darkest.

## 5.5 Visual Observations of Quality Deterioration during Storage

The following graphs (**Figure 14**) show an overview of the deterioration progress of spinach leaves treated with the different methods.

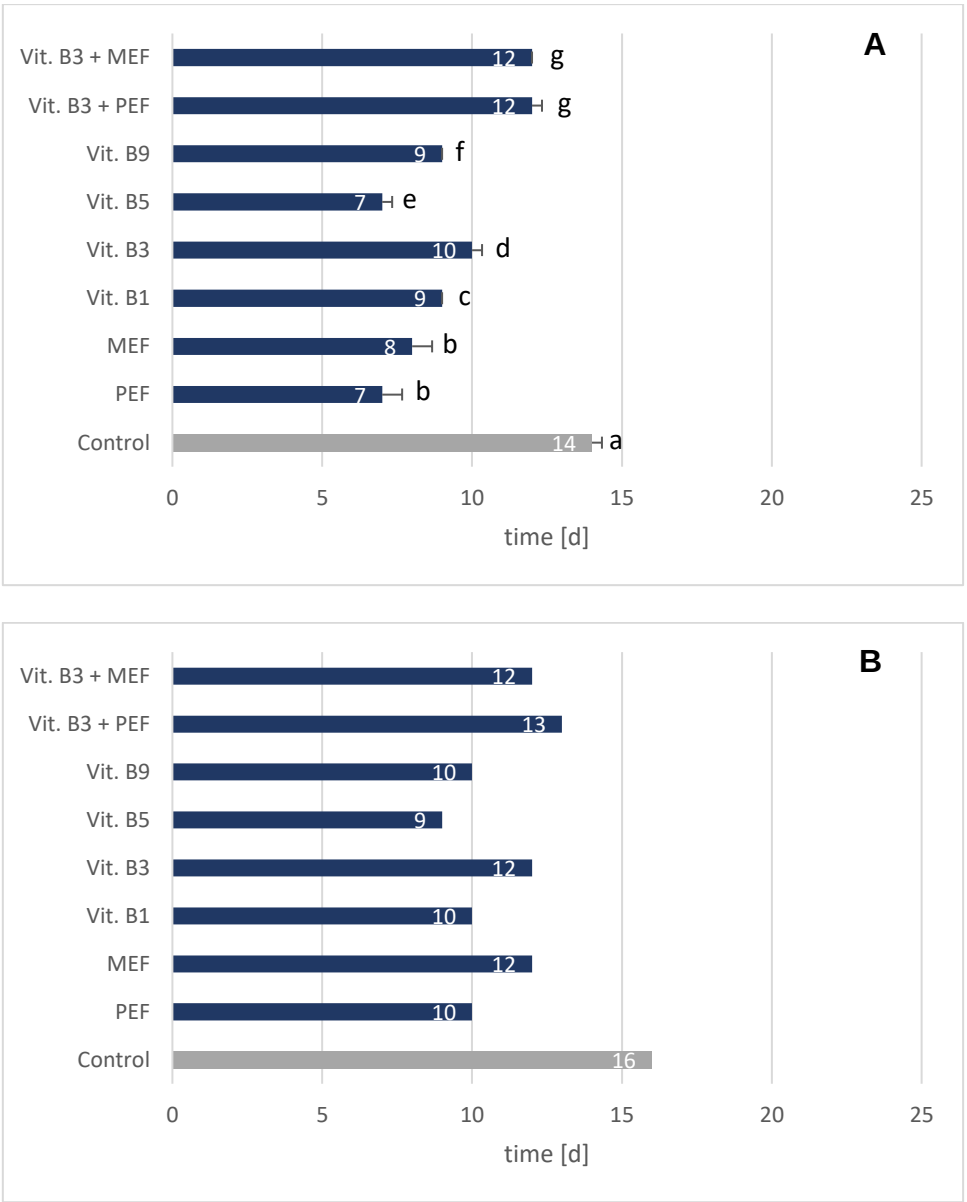


**Figure 14**. Deterioration progress of spinach leaves for different treatment methods over time. Average values of three measurements per measuring day are reported (n=3). (**A**) Time at which >10% were decayed (retailer shelf life). Error bars represent average standard errors. Different letters next to bars demonstrate statistical differences ($p<0.05$). (**B**) Time at which package was considered completely spoiled

(100%). No error bars are seen in **B** since leaves in completely spoiled bags were not counted and the replicate measurements concurred.

**Figure 14 A** shows the day where more than 10% of the leaves were decayed. 10% were chosen since this is an internal acceptable value of the company Vidinge for deviations and defects (retail limit for shelf life) (C. Prieto-Jiménez, personal communication, 2022). If a product contains a higher percentage of defects, it will be discarded. **Figure 14 B** shows at what stage the whole package was completely spoiled and no longer consumable. For treated spinach leaves, the bags counted as spoiled as soon as yellow liquid was seen in the bags, for untreated ones when leaves were greasy and oily.

As shown in **Figure 14**, spinach leaves have a different survival time depending on the treatment method. According to the results of retailer shelf life (exceeding 10% of decayed leaves), the control samples had a significant higher shelf life. By comparing the different treatments the combination of VI with vitamin $B_3$ and electric pulses showed the best results. The shortest survival time was displayed by the samples impregnated with vitamin $B_5$.

The day the treated samples were considered completely spoiled, yellow liquid was detected in the packages (**Figure 15**). The smell of spinach became more intense two days before the packages went bad. No yellow liquid was visible in the bags containing untreated samples. Instead, the leaves became greasy and oily making them unsuitable for consumption.

**Figure 15**. Representative picture of spoiled spinach with yellow liquid visible in package. Spinach in the picture was treated with PEF (500 pulses, 200 V, 50 Hz, 200 µs pulse width and 1600 µs pulse space).

Further observations were that on one hand some leaves had a different back side after the electrical treatment compared to the control (**Figure 16 A**). They were limper and oilier. Generally, those leaves also spoiled more quickly than leaves which had the same appearance as untreated ones. On the other hand, all spinach leaves impregnated with various vitamins showed an uneven colour distribution (**Figure 16 B**). Lighter and darker areas could be detected which was especially apparent on the back side of the leaves. At last, more water accumulated inside the bags with leaves impregnated with the B-vitamins.


**Figure 16**. (**A**) Spinach leaves with different back side after electrical treatment compared to untreated leaf. (**a**) Untreated leaf. (**b**) and (**c**) leaves treated with PEF (500 pulses, 200 V, 50 Hz, 200 µs pulse width and 1600 µs pulse space). (**B**) Representative picture of back side of spinach leaves treated with VI with vitamin $B_5$.

# 6. Discussion

## 6.1 Microscopic Observations of Leaf Electroporation and Cell Viability for Electrical Treatments

Regarding the electrical treatments, microscopic observations were made (**Figure 5**, **Figure 7**) before choosing a suitable protocol to visualise the effect of electroporation. Since plant tissues have a heterogenous structure and differ in their cell size, morphology, amount of air in extracellular spaces etc. it is challenging to find an appropriate protocol for homogenous electroporation (Dymek et al., 2014). Within this thesis, the pulse amplitude and number of pulses were changed for the PEF treatment (**Table 3**). Both parameters had an impact on the electroporation effect. The research of Dymek et al. (2014) on the electroporation effect of rucola leaves also shows that various PEF parameters including number of pulses have a strong impact on the electroporation and survivability of the cells. In this thesis, no homogenous electroporation occurred with a low pulse amplitude while keeping the number of pulses high (e.g. 100 V and 500 pulses). A possible explanation for this observation could be that the current flow of the electric pulses was not strong enough to electroporate the cells. By exceeding a voltage value of 300 V while keeping 500 pulses uniform electroporation was possible but the cells lost their viability within 24 h. Hereby, the disturbance of homeostasis by molecular transport might have been too high. Thus, cells were not able to recover from the treatment leading to cell death (Miklavcic & Davalos, 2015). In conclusion, homogenous electroporation while keeping cell viability can be achieved when voltage and number of pulses are properly adjusted to each other.

The same procedure was used to find a protocol for the MEF treatment (**Table 4**). In this case, it was demonstrated that different parameters had a different influence on the electroporation effect. Generally, the pulse amplitude and the time of pulse application had a greater impact than the frequency. For a pulse duration of 2 s and a frequency of 50 Hz, homogenous electroporation occurred from a pulse amplitude of 50 V. The higher the voltage, the more cells were electroporated. However, exceeding 200 V led to cell death since cells were not able to recover from the treatment. By applying the electric pulses for a longer time (5 s), a uniform electroporation of a large number of cells was possible even with a lower pulse amplitude (90 V).

## 6.2 Weight Gain after Vacuum Impregnation

Spinach leaves impregnated with different B-vitamin solutions showed a significant increase in their weight (**Table 5**). Panarese et al. (2014) pointed out that by introducing substances into plant tissues, the leaf volume changes. The volume expands during generation of vacuum, whereas when atmospheric pressure is restored, the volume decreases. Due to the deformation of the leaf tissue during the VI process, the weight of the spinach leaves might increase to a certain extent (Panarese et al., 2014). Moreover, most of the weight gain is due to volumetric expansion. By introducing the different B-vitamin solutions the spaces in the spongy mesophyll are filled up with the liquid leading to an increased weight (Radziejewska-Kubzdela et al., 2014). The significant difference in the weight gain depending on the vitamin used can be caused by various factors. Not only the heterogeneity of the spinach tissue but also the properties of the vitamins might have an influence. The vitamins vary in their composition and have a different molecular weight which can cause differences in the uptake of substances. Furthermore, the different B-vitamins were applied in different concentrations during the VI treatment possibly leading to differences in the weight change.

## 6.3 Atmospheric Composition

Respiration is the most important metabolic process that affects the shelf life of fruits and vegetables. Thereby, among other things, the respiration rate depends on the amount of $O_2$ and $CO_2$ the products are exposed to. For a high respiration rate, spinach needs high levels of oxygen and low levels of carbon dioxide (Inam-ur-Raheem et al., 2015). Since during storage ca. 21% $O_2$ and <2% $CO_2$ were measured in the bags (**Table 6**), the spinach leaves kept a high respiration level which leads to senescence. Nevertheless, since the atmospheric conditions in the bags did not significantly differ either in comparison to untreated leaves or between the various treatment methods, influences from the air composition during storage regarding variations in the life span could be excluded.

## 6.4 Colour Measurements

For electrical treatments, only for MEF a significant higher $L^*$ value on day 4 during storing was observed. In general, all leaves differed in their characteristics, including colour, even within the same batch which could have led to significant variations. Since no trend in colour change was discernible, it is assumed that the difference on day 4

was due to the heterogeneity of individual spinach leaves. For irreversible electroporation, Kwao et al. (2016) detected colour changes as a consequence of the oxidation of chlorophyll. With the set parameters for PEF and MEF treatment which led to reversible electroporation the extent of cell membrane damage was probably not high enough to obtain changes in the colour (Kwao et al., 2016).

For the impregnation with all B-vitamins, an uneven colour distribution was observed after the treatment (**Figure 13**). Panarese et al. (2016) found that by impregnating trehalose to spinach leaves, it was easier to introduce the VI solution into the free volume of the spongy mesophyll compared to the palisade mesophyll. This observation was associated with the fact that most of the intracellular air is located in the spongy mesophyll. Presumably this is also the case for the impregnation with other substances such as B-vitamins. Furthermore, the different plant layers vary in their porous structure. This might result in leave parts being impregnated more or less effectively which might be reflected in the appearance of the spinach leaves having lighter and darker parts (Panarese et al., 2016).

## 6.5 Shelf Life Evaluation and Visual Observations of Quality Deterioration during Storage

It could be shown that it was not possible to prolong the shelf life of minimal processed spinach by applying PEF and MEF with the stablished parameters, VI with different vitamin solutions or by combining these methods (**Figure 14**).

For PEF and MEF, the microscopic observations showed that it was possible to achieve reversible electroporation (**Figure 5**, **Figure 7**) which probably led to a transient enhancement of the mass transfer and an increase in metabolic activity. Hereby, the ability to restore biochemical balance after a temporary increased permeabilization is crucial to prevent cell death (Dymek et al., 2014). As described by Huang et al. (2019), the presence of ROS affects the product differently depending on the produced amount of ROS. For the electrical treatments in this thesis, the increased production of ROS as a stress response might have been too high which caused the spinach to decay more quickly. The higher ROS concentration might have led to irreversible DNA damage which caused faster cell death (Huang et al., 2019). Furthermore, plant tissues have a protective outer layer with a hydrophobic surface. By applying external stress, the surface might be damaged leading to a loss of the natural barrier against microorganisms resulting in a faster quality deterioration and

senescence of the treated spinach leaves (Ragaert et al., 2007). Dymek et al. (2014) also pointed out that if the cell membrane is damaged permanently metabolic responses which would take place after resealing might not occur. Leaves showing a different backside after the treatment (**Figure 16**) spoiled quicker than leaves which did not show any visual changes. It is likely that these leaves might have been more sensitive to the electrical treatment due to structural differences and the applied protocol may have killed some parts of the leaves. This resulted in a stronger damage of the plant tissue and thus, a faster deterioration.

During VI, when exchanging extracellular air with a VI solution, respiration of the target tissue should be reduced by restricting respiration to cell compartments filled with gas (Panarese et al., 2014). Nevertheless, within this thesis the shelf life of the impregnated samples was decreased. Yusof et al. (2017) pointed out that depending on the substances which are introduced into the plant tissue, the metabolism is effected differently. This was also confirmed by studies of Panarese et al. (2014) who showed that the metabolic activity was increased by introducing metabolizable molecules such as sucrose. In contrast, no change in gross metabolic activity was detected after the impregnation of spinach leaves with e.g. citric or ascorbic acid (Yusof et al., 2017). The introduction of the various B-vitamins probably stimulated and therefore, raised metabolic processes. Furthermore, with the VI protocol used in this thesis it might not be possible to completely replace the air with the respective vitamin solution and thus, achieve complete impregnation. This allows leaves to continue the metabolism and respire. Panarese et al. (2016) found that the metabolic activity was even increased if leaves used mitochondrial oxygen consuming pathways. Moreover, the intracellular amount of calcium increases due to the mechanical deformation of the plant tissue. This might have led to an increased metabolism since calcium is a signalling molecule which among other things triggers a faster oxygen consumption (Panarese et al., 2014). In addition, it was observed that the different B-vitamins have a different impact on the shelf life of the spinach leaves (**Figure 14**). The metabolism might be affected differently, depending on the characteristics of the introduced substances. The specific vitamins play different roles in plant tissues and have different functions in various metabolic processes. For example vitamin $B_5$ is involved in intermediary metabolism processes whereas vitamin $B_3$ is required for metabolising carbohydrates, lipids and proteins (Chand & Savitri, 2016; Sanvictores & Chauhan, 2022). These factors might result in a different life span depending on the B-vitamin used. In order to make more

specific conclusions, it would be necessary to further investigate the influence on metabolism.

With two days difference, a decay of >10% spinach leaves occurred earlier for the combined treatment compared to the untreated samples. However, for the combination of the treatments the leaves showed the longest survival time compared to using the methods individually. Vitamin $B_3$ was used for the VI solution since it showed the longest life span of spinach leaves in the single treatment procedures. Due to the combined treatment it might be that the two applications trigger different metabolic responses which interplay in a way that leads to longer survival times (Demir et al., 2018). A higher ROS production caused by external stress can lead to oxidation of vitamins which are crucial for several metabolic processes. By introducing the vitamins externally to the plant tissue the tolerance to oxidative stress may be enhanced (Sharma et al., 2012). It is speculated that there is a possibility that the vitamins can also be introduced intracellularly due to the following electroporation of the plant tissue. This might provoke metabolic processes differently and thus, delay senescence (Demir et al., 2018). Individual application of VI with the used VI protocol probably resulted in incomplete tissue impregnation. This may have been improved by a subsequent electrical treatment leading to a longer leaf viability. More research is necessary to prove if the combination of VI and electroporation is significantly advantageous regarding the cell survival compared to single application of the treatments. Moreover, the metabolic consequences induced by the treatments are extremely complex and require more detailed investigations to understand which metabolic and other consequences occur in the plant tissue.

# 7. Conclusions and further Considerations

The results presented in this thesis show that it is not possible to extend the shelf life of eco-spinach leaves using VI, PEF and MEF individually or by combining them. But it was demonstrated that by combining VI and PEF or MEF, the survival time of spinach leaves until a decay of >10% is significantly higher compared to applying the treatments individually.

With PEF and MEF reversible electroporation can be achieved by controlling different parameters. In general, more cells were electroporated by increasing pulse amplitude, number of pulses or time of pulse application. However, the interaction of the individual parameters, which vary in their strength of influence, is decisive for achieving reversible electroporation while maintaining leaf viability. To obtain homogenous electroporation, at least 500 pulses with 200 V (for a frequency of 50 Hz, a pulse width of 200 µs and a pulse space of 1600 µs) are necessary for PEF and at least 50 V, 50 Hz for 2 s for MEF. However, to ensure cell viability after the electrical treatment, 400 pulses with 300 V for PEF and 200 V, 50 Hz for 2 s for MEF should not be exceeded.

The metabolism and thus the respiration rate in the plant tissue is probably affected by every treatment. A faster deterioration of the spinach leaves may be the result of an increased metabolic activity. For electrical treatments it was suggested that a high ROS production led to irreversible DNA damage causing earlier cell death. Furthermore, since the electrical treatment resulted in a stronger damage of some leaves and thus, a faster spoilage of these, the protocols might need some modifications to achieve improved shelf life. For VI, the metabolism might be affected differently depending on the introduced substances. In this case, the B-vitamins probably stimulated and therefore, increased metabolic processes. Moreover, an uneven colour distribution for all leaves treated with VI was observed. Presumably this is a consequence of variations of the porous structure in the different plant layers and may indicate incomplete impregnation. By improving the VI protocol ensuring homogeneous impregnation of the leaf tissue the shelf life might be enhanced. For the combined treatment, which had the longest retailer shelf life of all treatments, cells might be protected better through a possible synergistic effect of different metabolic processes. Also, the oxidation of vitamins might counteract consequences of oxidative stress. Through further research and a deeper understanding of reactions as stress response, an extension of shelf life through the combined treatment might be possible.

# 8. Future Work

Further studies are needed to have a more detailed look at metabolic processes taking place as a stress response during and after electric treatments and VI. E. g. the amount of produced ROS. This might provide information on the influence on the respiration rate and metabolic activity. Moreover, a deeper knowledge is needed on protective mechanisms developed in cells after electrical treatments. Furthermore, more investigations on microbial infections and enzymatic processes might be helpful to understand the deterioration process of the leaves better.

Within this thesis, pulse amplitude and number of pulses for PEF were changed to find a protocol achieving homogenous electroporation. Pulse polarity, pulse space and pulse width are further parameters which can be adjusted and may lead to an optimised protocol affecting the metabolism differently.

For VI different vitamins of the B-complex were used as impregnation solutions. Introducing other vitamins might cause different stress responses and therefore, affect the metabolism in another way. To achieve homogeneous impregnation of the spinach leaves adjustments of the VI protocol might be necessary. In addition, introducing a solution by combining substances could lead to beneficial cross-reactions. Lukhava (2020) for example showed an enhancement in freezing tolerance of Arugula leaves by using a VI solution containing vitamins $B_1$ and $B_9$ as secondary metabolites in a glycerol solution.

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# 10. Appendix

**Table 10**. List of used equipment and materials

| Equipment and materials | Provider |
|---|---|
| Advanced Conductivity Meter | Orion Research Inc., Jacksonville, FL, USA |
| Analytic Scale | Mettler Toledo AB, Switzerland |
| Beakers 50 mL, 100 mL, 250 mL, 500 mL | SIMAX Chez Republic; Th. Geyer GmbH & Co KG |
| CheckMate 3 | Dansensor A/S, Denmark |
| Digital Camera | Digital sight DS-Qi1Mc, Nikon Co., Japan |
| Electric Air Pump | Likey, NY, USA |
| Electroporation chamber | - |
| Florescence Microscope | Elipse Ti-U, Nikon, Japan |
| Heat Sealer with Cutter | Fisherbrand, Sweden |
| High Power Programmable AC Power Source | B&K Precision Corp., Yorba Linda, California |
| ImageJ Software | Wayne Rasband, MD, USA |
| NIS-Elements Advanced Research | Nikon, Japan |
| Portable Spectrophotometer CM-700d | Konica Minolta Sensing, Inc., Japan |

| | |
|---|---|
| Pulse Generator CythorLab$^{TM}$ AM 033-033 | ADITUS Medical AB, Lund, Sweden |
| Spectrophotometer target mask with plate, 8 mm diameter (MAV) | Konica Minolta Sensing, Inc., Japan |
| Transparent Lunch Box | Xeonic Co., LTD, Korea |
| Vacuum Controller IT2010-05 | S.I.A., Bologna, Italy |
| Vacuum Pump | D.V.P. vacuum technology s.p.a, Italy |

**Table 11**. List of used chemicals

| Chemicals | Provider |
|---|---|
| Deionized Water | - |
| Fluorescein diacetate | Sigma-Aldrich, USA, $\lambda_{ex}$ = 492 nm, $\lambda_{em}$ = 517 nm |
| Propium Iodide | Sigma-Aldrich, USA, $\lambda_{ex}$ = 535 nm, $\lambda_{em}$ = 617 nm |