STUDY OF THE EFFECT OF VACUUM IMPREGNATION WITH DIFFERENT SUBSTANCES ON RESPIRATION AND COLOR OF PACKED SPINACH

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Study of the effect of vacuum impregnation with different substances on respiration and color of packed baby spinach leaves.

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Abstract

In this study, vacuum impregnation (VI) was used to investigate the effect of different solutions: sucrose 21 % (w/v), calcium lactate 1 % (w/v), ascorbic acid 0.1 % (w/v), and GABA 0.075 % (w/v) on atmospheric composition, color, and shelf life of packed baby spinach leaves. VI was performed at two different temperatures: 21 °C and 7.5 °C. The packed, impregnated leaves were stored for 8 days at 21 °C and 7.5 °C. The efficiency of VI was evaluated by measuring the weight gained after impregnation, color changes of the leaves during the storage period, and the modification of the atmospheric composition in the packages. The influence of GABA impregnated into baby spinach leaves was studied further for 22 days at a storage temperature of 7.5 °C, in order to study its effect on the shelf life of the spinach leaves. The metabolic activity of GABA impregnated leaves was measured for a short term (14 h) with isothermal calorimetry at 7.5 °C, in order to evaluate the changes on the heat production created inside the packed leaves. Results show that respiration (O_2 consumption and CO_2 production) of baby spinach leaves increased after VI and this increase depends on the substance used for impregnation as well as the temperature. Sucrose played a major role at both the studied temperatures, by displaying the highest alteration of the atmosphere created inside the packed spinach during storage. Impregnation with ascorbic acid leads to faster deterioration of packed spinach leaves in comparison to the other substances used. Impregnation with GABA extended the shelf life of the packed baby spinach leaves at 7.5 °C, till 22 days, while the control samples were discarded after 10 days. Measurement of the metabolic activity by isothermal calorimetry showed less heat production from the treated spinach with GABA comparing to the untreated control at 7.5 °C. The calorimetry studies showed higher heat production of the unpacked spinach comparing to the packed leaves at the same storage temperature. The measurement of metabolic activity was in correspondence with the respiration measurements (O_2 consumption and CO_2 production) for short time scales, where the lower respiration in the GABA impregnated samples was followed by less heat production, regardless if the sample was packed or not.

Keywords: Spinach, modified atmosphere, minimal processing, metabolic activity, quality, GABA.

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Introduction

Spinach is one of the dark green leafy vegetables, which is an excellent source for many types of food nutrients such as folate, minerals (especially calcium and Iron) and vitamins (such as vitamins C or K). It is considered a good source of carotenoids and antioxidants, which helps our body to get rid of radicals before they become harmful. Many investigations have reported that consuming carotenoids from dark green vegetables such as spinach can protect the human body, and stop different types of cancer e.g. breast, skin, stomach and lung cancer (Yan, 2016).

Like all vegetables, spinach can be consumed fresh, with salad or cooked. Studies showed that teens and adults bodies need about ½ to 2 cups of dark leafy vegetables weekly. Children between 2 to 3 years old require at least ½ cup of dark leafy vegetables per week (Adames, 2013).

Spinach and vegetables quality is a general term, it could be described in different ways. From the consumer view of point, quality of spinach means that spinach must have good appearance and shape, and offer high nutritional value, with good flavor, taste and structure and without any physical damage (Diezma et al., 2013). However, fresh spinach leaves have a very short shelf life, especially fresh cut spinach, unless properly packed and refrigerated, may lose their nutritional value very quickly (Cocetta et al., 2014).

Spinach and all other vegetables are biologically active organisms. Their metabolic activity continues after harvesting. Respiration of plants after harvesting might be defined as a process in which the plant cells absorb oxygen and produce carbon dioxide.

Depending on the respiration rate, horticultural products can be classified into 4 groups:

- Low respiration rate. Nuts, dates, apples, grape, garlic, onion and sweet potato.
- Moderate respiration rate: Such as banana, cherry, and few types of vegetables e.g. cabbage, lettuce.
- High respiration rate: such as cauliflower, avocado, blueberry, and green onion.
- Extremely high respiration rate: spinach, broccoli, and sweet corn (Robertson, 2013).

In order to preserve vegetables and prolong their shelf life, it is necessary to reduce both physical damage and respiration rate. Packaging of spinach is one solution which is used widely to protect spinach leaves and preserve their quality. Packaging methods might be influenced by

many different factors such as packaging materials, humidity, microbial growth, and heat accumulation (Robertson, 2013).

Many methods have been developed in vegetables processing to extend shelf life. Most of them are focused on reducing the respiration rate by lowering temperature, modified atmosphere packaging (MAP), browning, dipping, and edible coatings (Lu, 2007).

The key point for prolonging the shelf life is to reduce the respiration rate. The main factors which might affect respiration rate are the temperature and the permeability properties of the packaging material for O_2, CO_2 and water vapor. The MAP technology seems to be the most efficient method to preserve fresh cut vegetables and prolong their shelf life. However, it needs the adequate composition of gases inside the packages to get the optimal conditions for aerobic respiration. Otherwise, an anaerobic respiration might take place, leading to fermentation, production of undesirable metabolites and physiological disruption (Castelló et al., 2006).

The demand to develop new and cheap methods which can prolong product shelf life and preserve its freshness has increased recently. These methods must ensure preserving vegetables quality and safety, and consumer´s money simultaneously. One of these methods, which have been raised up in the last decades, is to apply vacuum impregnation (VI). This method can be used to introduce many types of micronutrients to the vegetables/fruits, or incorporate preservatives or texture enhancers into the products. Moreover, many organoleptic characteristics might be modified by applying this technique, and increase the product stability comparing to the non-treated products (Castelló et al., 2006). VI is used widely nowadays as preserving processes, for getting the desirable characteristics of food products and enhance the products appearance and sensory properties.

2. Aims

The main aims of this thesis are:

- To study the influence of impregnating different substances on respiration and color changes of packed spinach leaves.
- To study the effect of impregnating γ aminobutyric acid (GABA) on packed spinach shelf life at 7.5 °C.
- To test isothermal calorimetry to measure the metabolic activity of the leaves inside the package.

3. Background

3.1 Spinach

The scientific name of spinach is *Spinacia oleracea* and belongs to the *Chenopodiaceous* family. Spinach grows in a dry soil which contains rich organic matter and pH 6.5 - 7. It grows quickly and can be harvested as soon as they become big enough for eating, In general, after 37-45 days. (Carberry, 2017).

3.1.1 Structure

Plant tissues consist of highly interconnected intercellular air spaces forming a complicated network, contributing to their anisotropy and heterogeneity (Mendoza et al., 2007). The structure of this complex network depends on factors such as the species, cultivar, tissue functionality and maturity (Vincent, 1989). In leaves, the orientation and structural geometry of the intercellular spaces play a fundamental role in efficient light capturing and facilitating liquid and gas transport in the plant tissue (Schotsmans et al., 2004; Yusof, 2017).

A microscopy image of spinach leaves shows that it consists mainly of epidermis, palisade mesophyll, spongy mesophyll, vein and air spaces (Figure 1).

The epidermis is the outermost layer of the leaf. It consists of flat, closely packed cells and plays a protective role in the spinach leaf. Epidermis produces a waxy layer known as cuticle which covers the leaf surface and prohibit water lose by regulating gas exchange and protecting the internal cells (Domínguez et al., 2011). Moreover, epidermis contains stomata which are pores which facilitate gases exchange into and out of the leaf. They are provided with a pair of guard cells which can open and close the pore under different conditions.

The ground layers of the plant tissue consist of two different parts: the palisade mesophyll and the spongy mesophyll. The palisade mesophyll is placed near to the leaf surface and consist of rod-shaped cells contain a huge number of chloroplasts which is used during the photosynthesis. Palisade cells are organized and packed tightly in order to increase the area for light absorption (Vogelmann and Evans, 2002).

Spongy mesophyll cells are occupying the lower part of the leaf tissue. They are small cells and there is a massive air space between them which enable gases exchange between the leaf and

the surrounding area by means of stomata during photosynthesis and respiration (Winter et al., 1994).

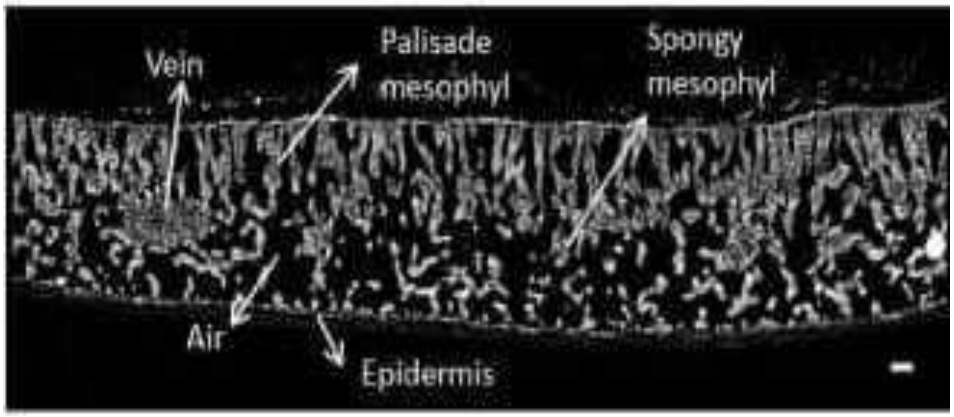


Figure 1. Image of a cross-section of as spinach leaf (Yusof, 2017).

3.2 Vacuum impregnation (VI)

In the last decades, VI started to be used widely in the field of food processing, thanks to a deeper understanding of the food microstructure and recognition of the roles of physical-chemical phenomena occurring at microscopic scales (Derossi et al., 2012).

VI is a non-destructive method which is based on the fact that the intracellular space of the plant tissue contains liquids and gases. The main goal of VI process is to replace the intracellular air with an external solution which has a desired composition. Properties of the plant tissues include the porosity fraction and pore connectivity which are the main structural factors playing an important role in this technology (Radziejewska-Kubzdela et al., 2014a).

During the VI process vacuum is applied to raw materials immersed in the desired solution. Vacuum will provoke gases in the extracellular space to escape from the tissue to the surrounded solution. Hereafter, when the atmospheric pressure will be restored again the solution will penetrate the matrix of the tissue replacing the air (Radziejewska-Kubzdela et al., 2014a).

3.2.1 VI process principle

Two phenomena dominate the impregnation mechanism resulting with the infusion of a solution into the tissues:

- Hydrodynamic mechanism (HDM).
- Deformation – relaxation phenomena (DRP) (Radziejewska-Kubzdela et al., 2014a).

After the product is placed in a closed chamber and immersed under the desired solution (time = t_0), the pressure inside the chamber or inside the capillaries of the leaf tissue (p_i) will be equal to the pressure outside the chamber (p_e), which, at the same time, equals to the atmospheric pressure (p_{at}) 1000 Mbar ($p_i = p_e = p_{at}$). At this particular time (t_0) the volume of the capillaries consisting of gas and solution is Vg_0 (Figure 2).

When the pressure is reduced ($p_1 < p_e$), the initial gas and solution inside the capillaries will start to flow out from the tissue to the surrounded solution. The movement of the gas and solution will cause deformation and expansion of the capillaries of the cell which is known as the first step of the deformation–relaxation phenomena (DRP). This action will lead to increase the free capillaries volume as it is interpreted by the following equation:

$$(Vg_{1A} = Vg_0 + X_{c1}) \qquad (1)$$

where $X_{c1:}$ is the volume of the capillaries which increases after the initial gas and solution flow out the cell.

This part of the process will finish when the previous equilibrium is restored again (when $p_i = p_e$) (Figure 2 step A1). The pressure inside the capillary increases slightly, while the free volume inside decreases to:

$$(Vg_{1B} = Vg_0 + X_{c1} - X_v) \qquad (2)$$

where X_v: is the volume of the solution which penetrates the capillaries.

In the second phase of vacuum impregnation, the atmospheric pressure gets restored again and this leads to the relaxation phase. Hence the capillary volume will expand and become even bigger than before (Figure 2, Step 1B).

Upon exerting pressure and then decompressing, the external solution tends to flow into the capillaries with much more ease. This in turn reduces the final volume of gas inside the capillaries. (Figure 2, Step 2).

$$(Vg_2 = Vg_0 - X_c - X_v) \qquad (3)$$

Removal of the vacuum during the process should occur slowly, since a fast and extreme pressure change might close capillary vessels and hinder of the HDM phenomena (Derossi et al., 2012; Radziejewska-Kubzdela et al., 2014a).

The speed of the impregnation is not the only factor which might affect the VI process. There are many other factors such as:

- The plant tissue microstructure: porosity (including size, shape, dimensions and distribution of the pores).
- Sample resistance to the gas and liquid flow.
- Sample shape: sample size, sample weight, and volume.

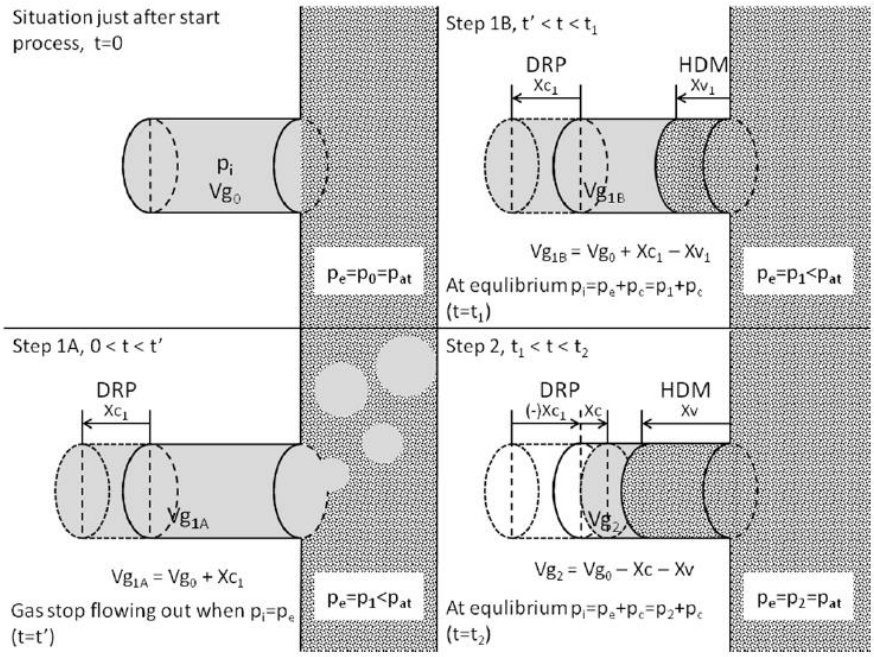


Figure 2. The hydrodynamic mechanism (HDM) and deformation–relaxation phenomena (DRP) which leads to filling of the capillaries during the vacuum impregnation process, from (Radziejewska-Kubzdela et al., 2014).

3.2.2 Applications of vacuum impregnation

The impregnated substance in the intracellular space and capillaries of the plant tissue might provide various advantages, such as increase the food nutritional value (e.g., enrichment with polyphenols, probiotics or micronutrients), and extension of the product shelf life (e.g. reduce pH value) (Radziejewska-Kubzdela et al., 2014). Moreover, VI might be used as a pre-treatment before food processing such as pre-canning, pre-freezing, pre-drying etc. Other food properties that can be modified by VI include reducing the water activity, changing food color, texture, taste, flavor, or acidity (Radziejewska-Kubzdela et al., 2014; Derossi et al., 2012).

Many types of solutions have been used in the VI process and showed a good effect on the fruit and vegetables properties. Some examples are:

- Cryoprotectants: which are used to reduce the amount of freezable water, and reduce the damage of the ice crystal in frozen fruit and vegetables (Zhao and Xie, 2004; Phoon et al., 2008).
- Antioxidants and antimicrobial substances: in order to prevent color deterioration, and microbial growth in the products (Zhao and Xie, 2004).

➢ Calcium lactate, used as texture enhancer. It has shown an improvement of texture quality of the strawberries and reduced the drip loss of frozen-thawed products (Zhao and Xie, 2004). Other studies reported that calcium lactate impregnated solution had a notable impact on the mechanical behaviour of eggplant and carrot (Gras et al., 2003).

Calcium is generally considered as one of the most important fortifiers in the functional food development. It is mainly founded in the dairy products, and considered as a critical compound in the diet. In the plant structure Ca^{+2} plays an important role to give a harder texture by forming bonds between pectin and cellular wall compounds. Thus, Introducing Ca^{+2} to the plant cellular matrix by using VI might modify the plant structure and mechanical properties (Gras et al., 2003)

➢ Sucrose provokes a decrease of water activity (aw), shortening of dehydration time. Sucrose impregnation has shown to improve the visual quality and decrease shrinkage of plum slices (Fante et al., 2011).

➢ Both of ascorbic acid and citric acid solutions have an effective inhibition of browning and softening of apple slices (Biegańska-Marecik R and Czapski J., 2007). Moreover, this

solutions increased the shelf life of pears for more than 20 days and prevented microbial growth (Radziejewska-Kubzdela et al., 2014a).

- γ aminobutyric acid (GABA) is a non-protein amino acid that is widely distributed in nature. Many studies have linked GABA to many physiological functions in animal's bodies such as neurotransmission, induction of hypotensive, diuretic effects, and tranquilizer effects. Its role, however, still not clear in plant tissues (Komatsuzaki et al., 2005).

  It has been reported that the GABA level in plant tissue is quite low, but increases under stress conditions such as mechanical excitation, darkness, acidification, water stress, drought, cytosolic acidification, and hypoxia (Oh et al., 2003). Other studies have shown that GABA levels in plant tissues have many roles such as: pH regulation, nitrogen storage, plant development, and defense factor against phytophagous insects (Oh et al., 2003).

  GABA levels in spinach leaves are nearly 414 nmole/g (DW), which it is quite high comparing to other types of uncooked vegetables such as potato, broccoli, kale, and squash (Oh et al., 2003).
- Maltodextrin has shown an improvement of the water and solute gain in sliced zucchini and reduce the hardness of the product (Elisabetta Occhino et al., 2011).
- Trehalose has shown an improvement of freezing tolerance of spinach leaves (Phoon et al., 2008).
- Chitosan- or casinate - based film - forming emulsions have shown an increase on the shelf life of pineapple slices (Radziejewska-Kubzdela et al., 2014a).

3.2.3 Metabolic consequences of VI

Most of the literature on VI are focused on the effect of vacuum pressure (by using various levels of pressure) or impregnated solutions on product structure after the impregnation process. Quite limited information is available about the metabolic consequences of VI.

Metabolic activity of the product might be enhanced following impregnation due to the modification of the product structure. This modification can be induced by the pressure changes, the impregnated molecules, and/or anaerobic stress. (Panarese et al., 2014). Previous studies reported that impregnated sucrose and trehelose may not completely remain in the extracellular space after 30 minutes of impregnation. Instead, a fraction of those compounds will be

incorporated into the cells (Panarese et al., 2014). Other study found that impregnating trehelose and sucrose showed a drastic increase on the metabolic activity of the spinach leaves. These molecules act as an energy reservoir when they are introduced into the plant cells, as they can be metabolized to provide energy, and this results in an increased metabolic activity. (Panarese et al., 2014). A new study made by our group showed that the gross metabolic activity of packed spinach leaves impregnated by 3 different substances (ascorbic acid, sucrose, calcium lactate) increased as compared to untreated controls (Yusof et al., 2017). The highest increase occurred when using sucrose, then followed by calcium lactate, and finally ascorbic acid.

3.3 Methods for assessing the metabolism of packed fruit and vegetables

3.3.1 Respiration rate

(Stoecker, 1998).

Suitable O_2 concentration around the packed commodities or in the storage environment is very important to maintain the aerobic respiration of commodities. The less O_2 concentration the less respiration rate of the commodities and the longer storage life. It was found that in most crops levels of 2 % to 3 % of O_2 is enough to give a beneficial effect on the reduction of the respiration rate and metabolic activity of the crops. On the other hand, the increase of CO_2 levels around commodities will prolong the shelf life and delay senescence. In contrast, some commodities are intolerant to high levels of CO_2, or even N_2, which might be used in modified atmosphere packaging (MAP) (Chakraverty and Singh, 2014).

3.3.2 Isothermal calorimetry

All processes, physical, chemical, biological etc. produce heat. Isothermal calorimetry can measure the heat production of specific processes, which might give a clear view about metabolism of microorganisms, respiration of tissues and even physical processes such as crystallization. The heat measurement in different processes will provide important information to understand different phenomena in diverse fields such as pharmaceutical industry, cement science and even defense laboratories. In food science, this technique is still new and has limited uses. The first use of a calorimetric measurement was made by Dubrunfaut in 1856, who studied the heat produced by wine during fermentation. Nowadays isothermal calorimeter is common specially in the

fundamental biology and studies focusing on microbiology and food sciences (Wadsö and Gómez Galindo, 2009).

The advantage of this technique is that it is multidisciplinary and versatile. It is a nondestructive technique that can measure the heat flow continuously during the experiment (Wadsö and Gómez Galindo, 2009). Disadvantages of this method are that it is a general method, which means that it provides the general heat for all processes simultaneously without details on the source of the produced heat.

4. Experimental overview

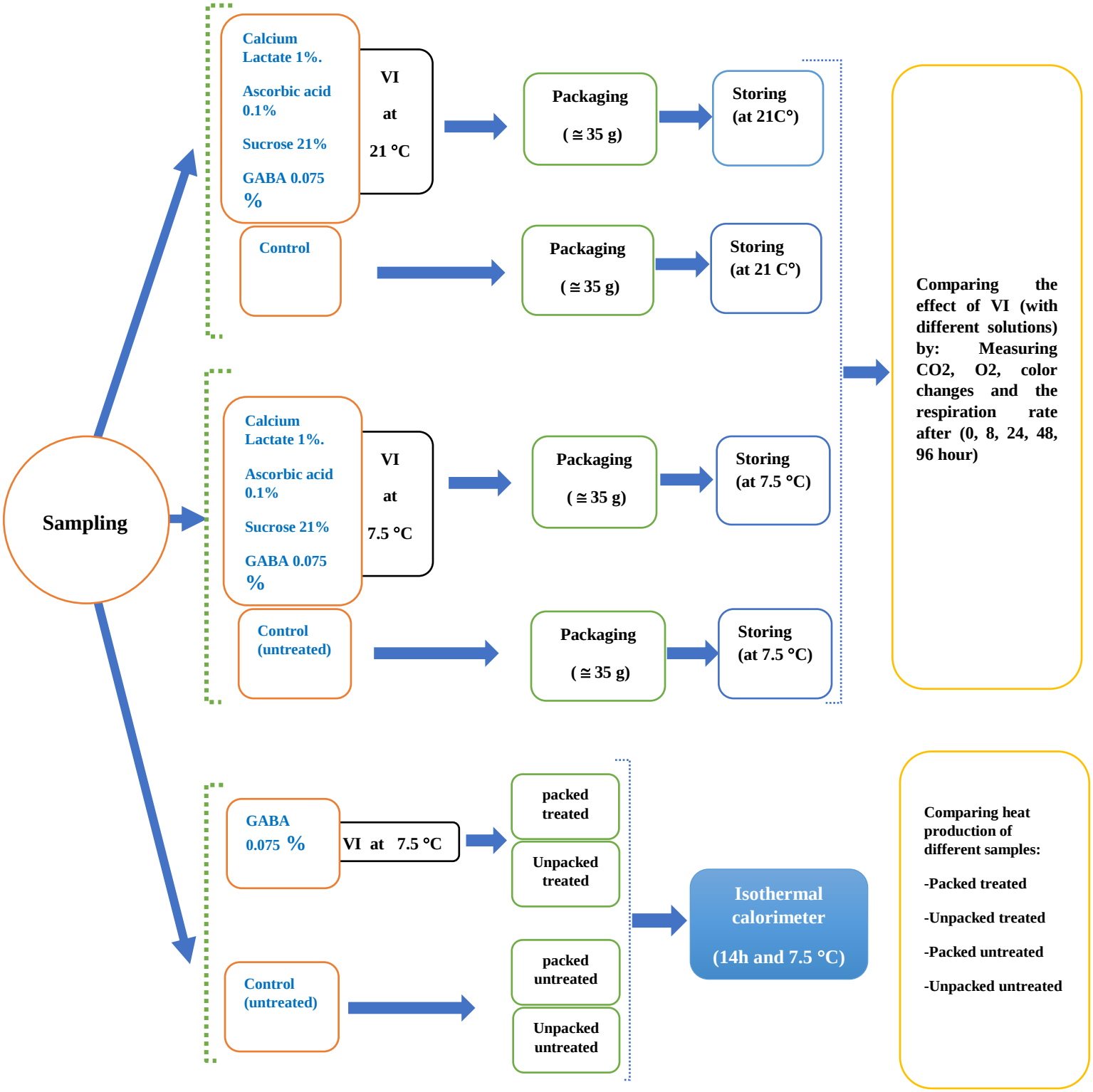


Figure 3. Diagram of the experimental procedure

5. Materials and Methods

5.1 Spinach leaves

Baby spinach leaves were grown in a greenhouse with 16 h of light at 20 °C during the daytime and 14 °C during the night. The greenhouse was provided with lamps giving 400-W Na and an initial light (Photosynthetic Photon Flux PPF) of 725 μmol/s. Trays (54 X 32 cm) were prepared and filled up with soil, which was fertilized with NPK (nitrogen, phosphorus, and potassium), containing 182, 91, and 194 g/m3 respectively. The soil pH was reduced to 5.5 - 6.5 by using dolomite and limestone.

After the soil was prepared and watered with 0.5 l/tray, the seeds were sowed 1.5 cm below the soil surface. There were 42 spinach plants grown in each tray. They were watered every second day with the same amount of water. The spinach leaves were harvested after 5 weeks, when the leaves dimensions were 7.0 ± 0.1 length and 3.0 ± 0.3 cm width at the center and 2.0 ± 0.1 cm of petiole.

Spinach leaves were harvested at 10 am every day. The spinach leaves were collected in a plastic bag and transported to the laboratory within 5 min. In the laboratory, some of the leaves were placed on the VI chamber to be impregnated and the rest kept at 7.5 °C to be used later. The time for the whole experiment (from harvesting to packing) did not exceed 3 hours from harvesting to packaging.

5.2 Impregnated substances

Different substances were used to investigate the effects of VI on packed spinach leaves:

Sucrose**:** An isotonic solution of sucrose was prepared. The concentration of the solution was determined by using different type of sucrose concentrations ranging from 0 to 24 % (w/v). Three spinach leaves were immersed in each solution. The variation in weight was recorded every hour until equilibrium.

Calcium lactate and Ascorbic acid: Solutions of 1 % (w/v) and 0.1 % (w/v) of calcium lactate and ascorbic acid respectively were used. These concentrations are the most common concentrations used for treatment of fruit and vegetables (Gómez Galindo and Yusof, 2015).

GABA: A solution of 0.075% (w/v) was used. This concentration was stablished based on the GABA concentration found in spinach leaves exposed to cold stress (Yoon et al., 2017).

5.3 Treatments

5.3.1 Vacuum impregnation

Spinach leaves (≅ 70 g) were placed in a beaker, immersed in different solutions and placed inside the VI chamber. To ensure that the leaves were completely immersed in the solution, a thin plastic net was placed on the surface of the beaker (Figure 4).

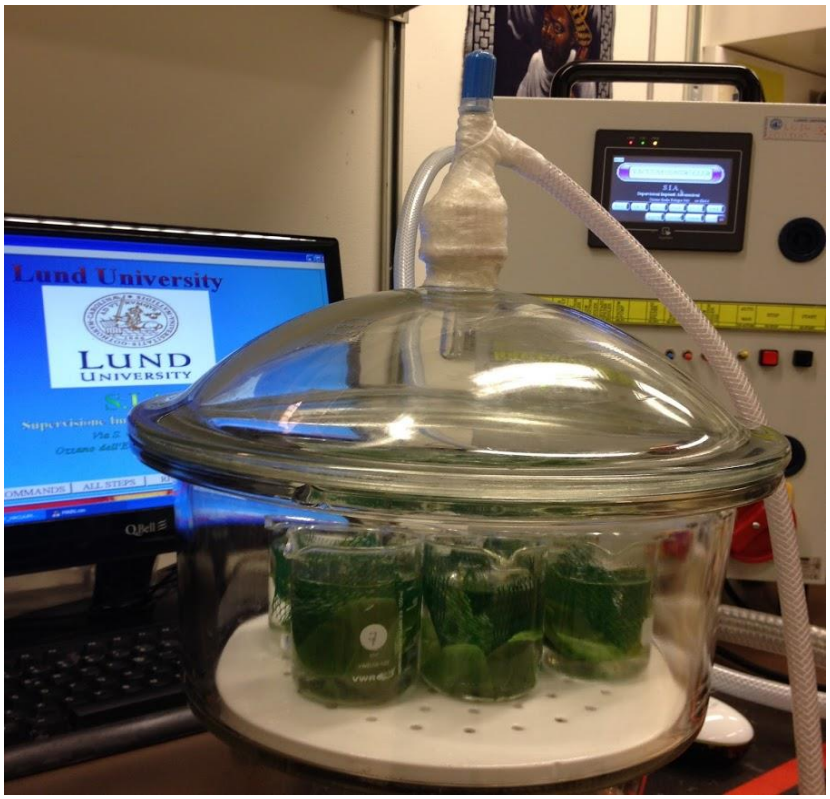


Figure 4 The experiment set up of VI

A Teflon tube was used to connect the VI chamber to a vacuum controller (SIA, Bologna, Italy). The SIA unit is controlling the pressure acting on the impregnating solution during the process. To achieve the complete process, a number of components were included in this system

such as : a pressure transmitter, vacuum actuators (valves and vacuum pump), a computer and a programmable logic controller device (PLC, Series 90-30, General Electric, Charlottesville, VA, USA) (Panarese et al., 2013).

VI was performed with the following protocol: The pressure decreased gradually from the atmospheric pressure (1000 mbar) to 150 mbar within 11 min. After the targeted final pressure 150 mbar was achieved, the pressure was kept for 1 min. The pressure was gradually increased to 1000 mbar within 7 mins and kept for 13 min. To complete the VI treatment this cycle was repeated. Every cycle took 32 min and the whole treatment 64 min.

After the impregnation was done, spinach leaves were removed from the chamber and gently blotted with a soft white tissue to make sure that no solution is still sticking on their surface. The spinach weight was taken again to determine the weight gain after the VI process. The VI experiments were performed at two temperatures: 21 °C and 7.5 °C.

5.3.2 Packing

Impregnated and non-impregnated leaves (control), approximately 35 g, were packed in bags with dimensions 18 x 18 cm and heat-sealed. The packaging material was obtained from Optifreeze AB. The material used was polypropylene with thickness of around 30 µm, and OTR (Oxygen transmission rate) of around 1300 cc/m^2/24hr/atm. This packaging material have perforations of 80 µm diameter and density of around 104 holes / m^2.

5.4 Analysis

5.4.1 Measuring atmosphere composition

Bags were stored at two different temperature 21 °C and 7.5 °C. To measure the O_2 and CO_2 concentration inside the bags, a PBI Dansensor model CheckMate 9900 (Cambridge, UK) was used. The device is provided with a needle or detector which is punched into the bags. The device absorbs the gases from the bags.

Measurements were done at 0, 2, 8, 24, 48 and 96 h during storage at both temperatures. Five packages were prepared for each time point and each temperature. After the measurement, the bag was discarded.

5.4.2 Color measurements

The spinach leaves color was measured by using a spectrophotometer CM-700d from Konica Minolta Sensing, Inc (Japan).

Color measurements were done for both treated (impregnated) and non-treated spinach leaves. 10 leaves were collected from each bag, and color was measured on their surfaces. The color measurement was done alongside with the gas measurements. The parameters L^*, a^*, and b^* were recorded.

5.4.3 Metabolic activity

An isothermal calorimetry device (Biocal 2000, Calmetrix Inc., USA), was used to measure the gross metabolic activity of impregnated and non-impregnated spinach leaves. The calorimeter was placed in a room at 21 °C but the internal temperature of the device was modified to 7.5 °C in order to simulate the conditions in the cold room. No fluctuations of the calorimeter´s internal temperature were recorded during 14h. A reference made of stainless-steel was used (with specific heat capacity 0.5 K/J). To get an accurate result, the reference should have approximately the same heat capacity as the sample. Before running the experiment, a baseline was recorded by using 74 g of stainless-steel and 10 g of water (the specific heat capacity of water is 42 K/J). Water samples were prepared in the cold room and then transferred into the calorimeter at room temperature as mentioned before. To prepare the water samples, water was filled into 125 ml plastic ampoules and left for 1 hour in the cold room before transferring them to the calorimeter (to make sure that their temperature was stabilized to 7.5 °C). After placing the sample in the calorimeter, few minutes were required for the device to get stable and to restore the exact temperature to 7.5°C. Calorimetry experiments were run for 14 h. The calorimeter device was connected to a computer which recorded the output of the heat flow (Voltage) every 60 s.

After obtaining the baseline, samples were prepared for measuring the metabolic activity. Samples were prepared in the cold room and left there for 1 h before being transferred to the calorimeter.

Four different experiments were performed. Spinach leaves were impregnated at 7.5 °C with GABA solution (0.075 % w/v). The packed samples were filled into small polypropylene plastics bags with isosceles triangle shape (base 25.5 cm, and height 18 cm). Then the packed or unpacked samples were placed into 125 ml ampules and closed tightly and left for 1 h at the cold room before

being transferred to the calorimeter. The sample weight was 7.1 g and the weight of the packaging material 1.3g. A small sheet of the packaging material was placed into the ampules next to the unpacked samples to guarantee that packed and unpacked samples have the same weight.

The corresponding thermal power (heat production rate) of the samples was calculated according to Equation (6).

$$P = \varepsilon \ \frac{(Vs - Vb)}{m} \qquad (6)$$

Where: P is the specific thermal power of the spinach sample (μW/g), ε is the calibration coefficient of the calorimeter (μW/μV), Vs the voltage signal from the calorimeter (μV), Vb the voltage recorded for the baseline (μW), and m is the mass of the sample (g).

5.4.4 Statistical analysis

Differences between treated and non-treated leaves were analyzed statistically by using ANOVA (single factor) at 95% confidence interval ($p < 0.05$). If a significant difference was found, Tucky´s HSD test was used to determine true differences. Excel (Microsoft Office 2016, Microsoft Cooperation, USA) was used for all statistical analysis.

6. Results and Discussion

6.1 Weight gain after impregnation with different substances

Table 1 shows that there are statistically significant differences ($p < 0.05$) in the weight gain of the leaves after impregnation with different substances. Among all the impregnating substances, spinach leaves impregnated with ascorbic acid shows the highest weight gain.

Table 1. Weight gain of the spinach leaves after impregnation with different substances. Reported are the average values with their standard deviation of 3 replicates. Values in a column followed by a different letter were significantly different at $p < 0.05$ according to Tukey-Kramer's test.

Impregnated substances	Weight gain (%)
Sucrose 21%	24.0 ± 2.3 a
Ascorbic acid 0.1 %	64.3 ± 2.9 b
Calcium lactate 1%	48.3 ± 1.9 c
GABA 0.075%	54% ± 2.1 d

6.2 Gas (O_2 and CO_2) composition in the bags

6.2.1 Comparison of the gas composition between the packed spinach leaves with different treatments and temperatures.

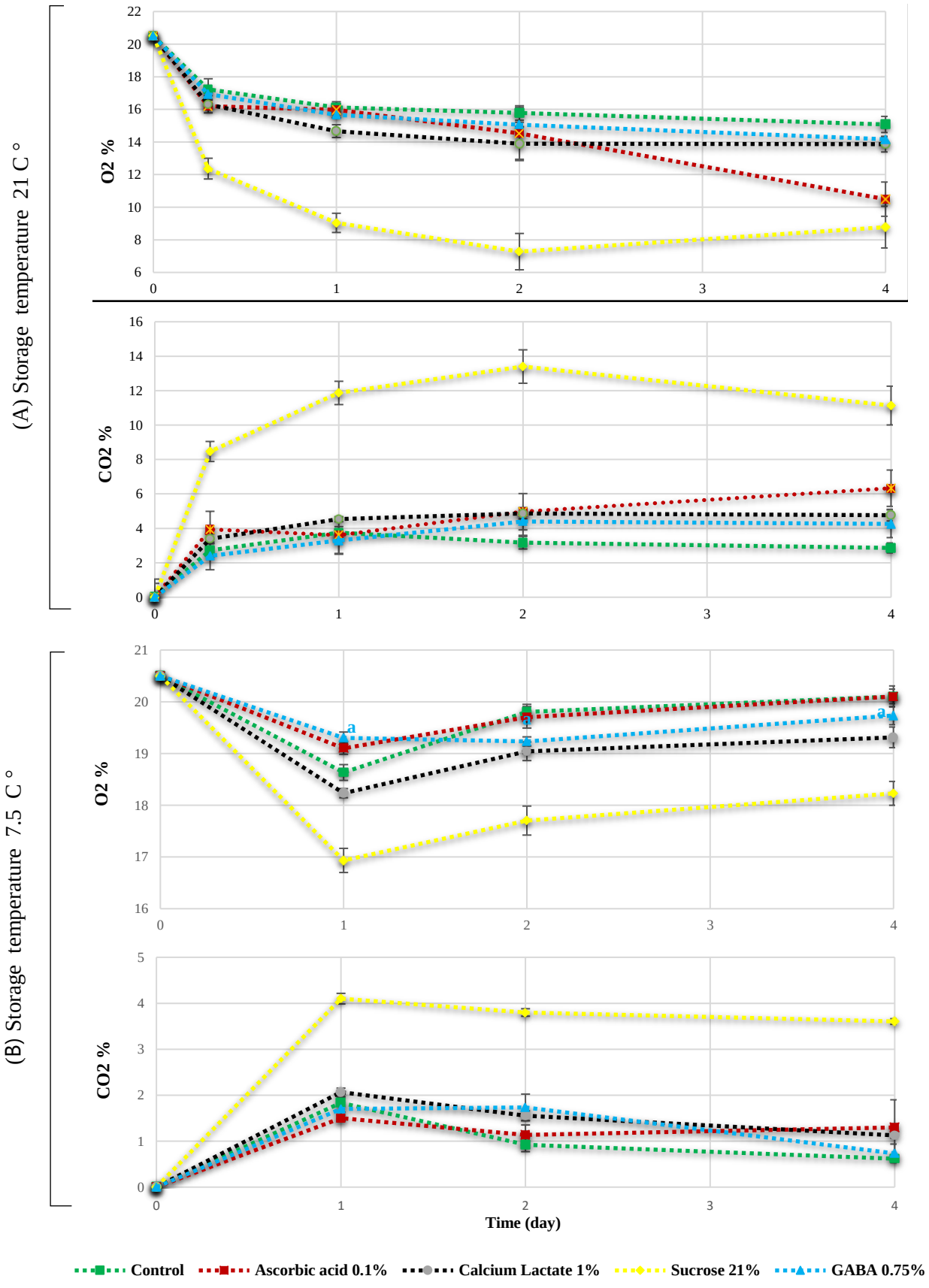

(A) Storage temperature 21 C °
O2 %
CO2 %
(B) Storage temperature 7.5 C °
O2 %
CO2 %
a
a
Time (day)
Control
Ascorbic acid 0.1%
Calcium Lactate 1%
Sucrose 21%
GABA 0.75%

Figure 5. Changes in the composition of oxygen and carbon dioxide of untreated baby spinach leaves and VI treated spinach leaves with different substances and stored at (A) 21 °C and (B) 7.5 °C. The first measurements (time 0) were taken 20 min after packing. In the Figure, average of 3 measurements are reported. Bars represent the STD.

Figure 5 and Table 3 show that all impregnated substances have different effects on the modification of the atmosphere around spinach leaves at both studied temperatures. Among all substances used, sucrose impregnated leaves show the highest O_2 consumption and CO_2 production throughout the storage period at both temperatures, followed by the calcium lactate impregnated leaves. Many studies have interpreted that the catabolic products of the impregnating sugars might be taken up by the cells through the cell membrane, which results in an enhanced metabolism of the spinach leaves (Dymek et al., 2016).

After 24 h, all the impregnated spinach leaves with different substances and at different storing temperature (21 and 7.5 °C) show a significant difference ($p < 0.05$) in consumption O_2 and production CO_2 comparing to the first day. Additional experiments were done at 21 °C and showed that most of this decrease occurs during the first 8 h of storage (Figure 5).

After 48 h of storing the samples, the decrease trend of O_2 consumption and the increase trend of CO_2 production continues slowly at 21 °C. While at 7.5 °C the same trends were reversed after 24 h.

There is no definitive interpretation for this phenomenon, but an assumption can be made that this happens due to the alteration caused by the respiration/metabolism rate. Storage of the leaves at a low temperature results in their reduced metabolic activity. The leaves continue to respire leading to a sharp decline in the O_2 concentration. Photosynthesis resumes once the leaves adjust to the cold conditions and the leaves start to release more O_2.

When the different treatments are compared to each other, it seems that all the impregnated substances (except sucrose) do not show any significant change on the modification of the packed spinach atmosphere after 24 h at both 21 and 7.5 °C. Significant differences between these substances start to appear after 96 h when storing at 21 °C. At 7.5 °C the significant difference between different impregnated substances appears after 24 h of storage. After 48 and 96 h of storage, all impregnated substances (except sucrose) do not show any significant difference on the composition of the atmosphere around the spinach leaves.

It was, however, observed that the ascorbic acid impregnated leaves deteriorated faster than the control and the leaves impregnated with other substances during storage at 21ºC. The fast increase of O_2 consumption of ascorbic acid impregnated leaves after 4 days of storage at 21 ºC may reflect signs of this deterioration (Yusof, 2017).

Only leaves impregnated with GABA and stored at 7.5 °C made no significant difference in the consumption of O_2 and production CO_2 between the 24^{th} and 48^{th} hour of storage.

Interestingly, GABA impregnated leaves show higher O_2 consumption compared with the control at 21 °C while it shows less O_2 consumption comparing to the control at 7.5 °C during the first 36 h after impregnation. These differences might be interpreted due to the cold stress exposure, which enhances GABA production on plant tissues in the first 36 hours of storing, and this production might decrease the respiration rate of plant tissues (Yoon et al., 2017).

Table 2. Comparison between the average of O_2 consumption and CO_2 production of packed spinach leaves impregnated with different substances and stored for 4 days at 21 °C (the comparison was done between the different substances at each time point).

Storage temperature (21 °C)										
	O_2					**CO_2**				
Time (h)	0.2	8	24	48	96	0.2	8	24	48	96
Control	20.50a	17.23a	16.13a	15.78a	15.08a	0.00a	2.33a	3.90a	4.03a	3.43a
Ascorbic Acid	20.50a	16.20b	15.97a	14.54a	10.49be	0.00a	3.93b	3.60a	4.97a	6.33a
Calcium Lactate	20.50a	16.30b	14.67a	13.90a	13.87ac	0.00a	3.40b	4.53a	4.87a	4.20a
Sucrose	20.50a	12.37c	9.04b	7.28b	8.78b	0.00a	8.47c	11.87b	13.40b	11.13b
GABA	20.50a	16.93ab	15.67a	13.07a	12.37ce	0.00a	2.40ab	4.30a	6.40a	6.27a

* A statistical comparison was done to compare the effect of different impregnated substances at each time. Results followed by different letters were significantly different at $p < 0.05$ according to Tukey-Kramer's test.

Table 3 shows a comparison between the average of O_2 consumption and CO_2 production within packed spinach leaves which impregnated by different substances and stored for 4 days at 7.5 °C (the comparison has done between the different substances at each time point).

Storage temperature (7.5 °C)								
	O_2				**CO_2**			
Time (h)	0.2	24	48	96	0.2	24	48	96
Control	20.50a	18.63ab	19.80a	20.10a	0.00a	2.07a	1.55a	1.13a
Ascorbic Acid	20.50a	19.10a	19.70a	20.10a	0.00a	1.50a	1.13a	1.30a
Calcium Lactate	20.50a	18.23b	19.04a	19.31a	0.00a	2.07a	1.55a	1.13a
Sucrose	20.50a	16.93c	17.70b	18.23b	0.00a	4.10b	3.80b	3.60b
GABA	20.50a	19.30a	19.23a	19.73a	0.00a	1.70a	1.73a	0.73a

***** A statistical comparison was done to compare the effect of different impregnated substances at each time. Results followed by different letters were significantly different at $p < 0.05$ according to Tukey-Kramer's test.

6.3 Color Changes:

Figure 6 shows that all impregnated and packed baby spinach leaves have a light green color with good appearance, except the impregnated leaves with ascorbic acid, where the deterioration started to be visually clear after 96 hours.

Samples store d at (21 C°) for 4 days

VI Ascorbic acid after 4 days (21 C°)

VI Calcium lactate after 4 days (21 C°)

VI sucrose after 4 days (21 C°)

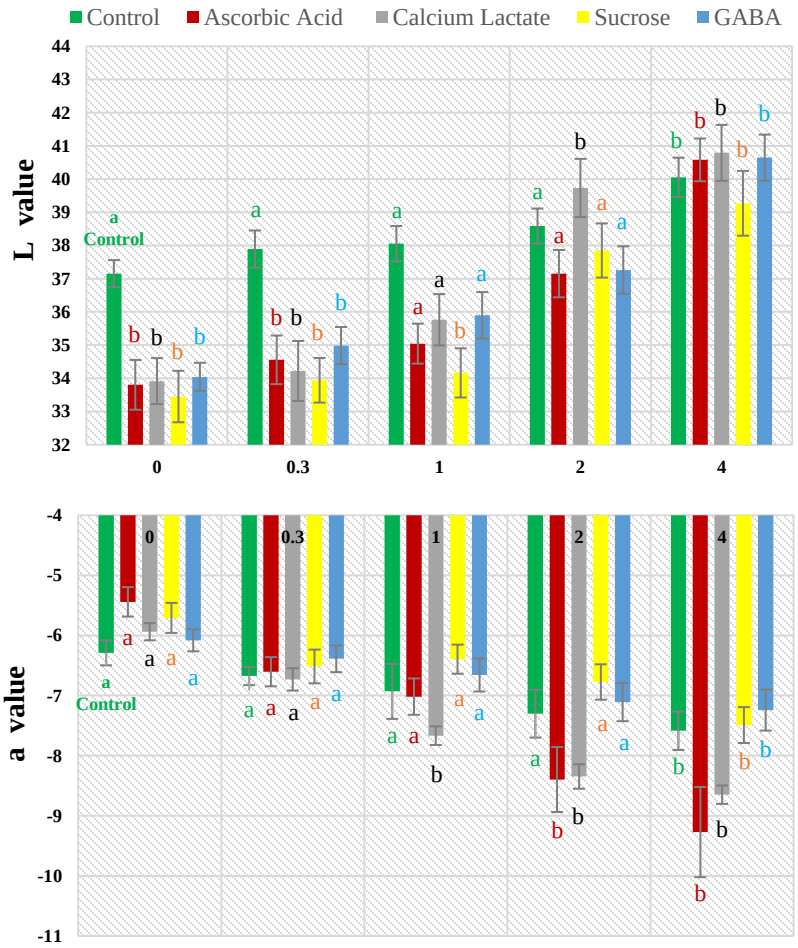
VI GABA after 4 days (21 C°)

Control after 4 days (21 C°)

Figure 6. Comparison between untreated and treated packed samples stored for 4 days at 21C°.

Figure 7 shows that all samples impregnated with different substances show a significant decrease for all color values (L^*, a^*, and b^*) in comparison with the control. After 24 h, values started to increase again to make no significant difference after 24 or 48 h. After 4 days the highest increase of all color values (L^*, a^*, and b^*) appears in leaves impregnated with ascorbic acid and

calcium lactate, while the lowest increase was detected with both leaves impregnated with sucrose and GABA. However, after 4 days of storage, all impregnated samples plus the untreated ones show a significant increase of all values as compared to the control (untreated one at 0 day).



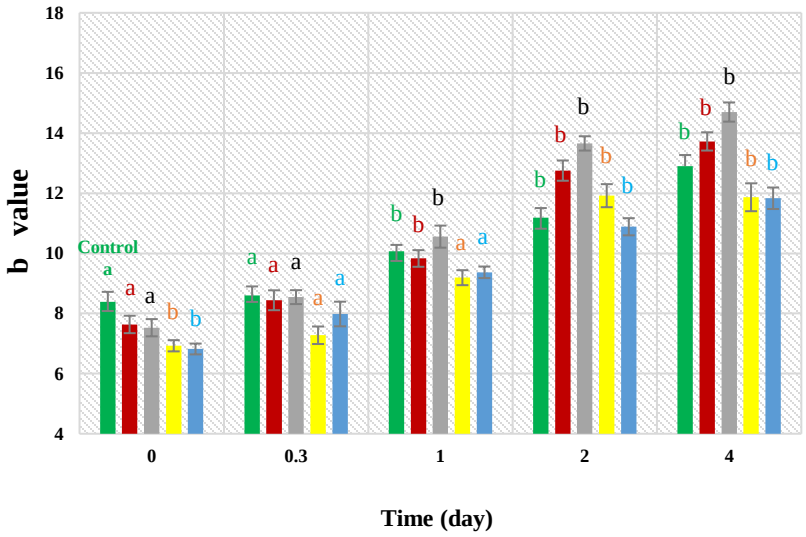


Figure 7. Changes of (L^*, a^*, and b^*) values of packed untreated spinach leaves and packed VI leaves, impregnated with different substances. Packages were stored at 21 °C for 4 days. Different letters above the error bars represent statistically significantly differences between treated samples and the control ($p < 0.05$).

Figure 8 shows that all impregnated spinach leaves (including the untreated ones) are still having an acceptable appearance after 8 days of storing at 7.5 C°. Both Figures 7, and 9 show that the samples treated and stored at both temperatures followed a similar trend in the color changes for the first 24 hours. There was a clear significant difference observed between the control and the samples impregnated with ascorbic acid and calcium lactate after days 6 and 8 respectively. While the leaves impregnated with GABA did not show any significant difference in comparison with the control from day 4 onwards.

Samples stored at (7.5 C°) for 8 days

VI Ascorbic acid after 8 days (7.5 C°)

VI Calcium lactate after 8 days (7.5 C°)

VI sucrose after 8 days (7.5 C°)

VI GABA after 8 days (7.5 C°)

Control after 8 days (7.5 C°)

Figure 8. Comparison between packed control and packed impregnated samples stored for 8 days at 7.5 °C.

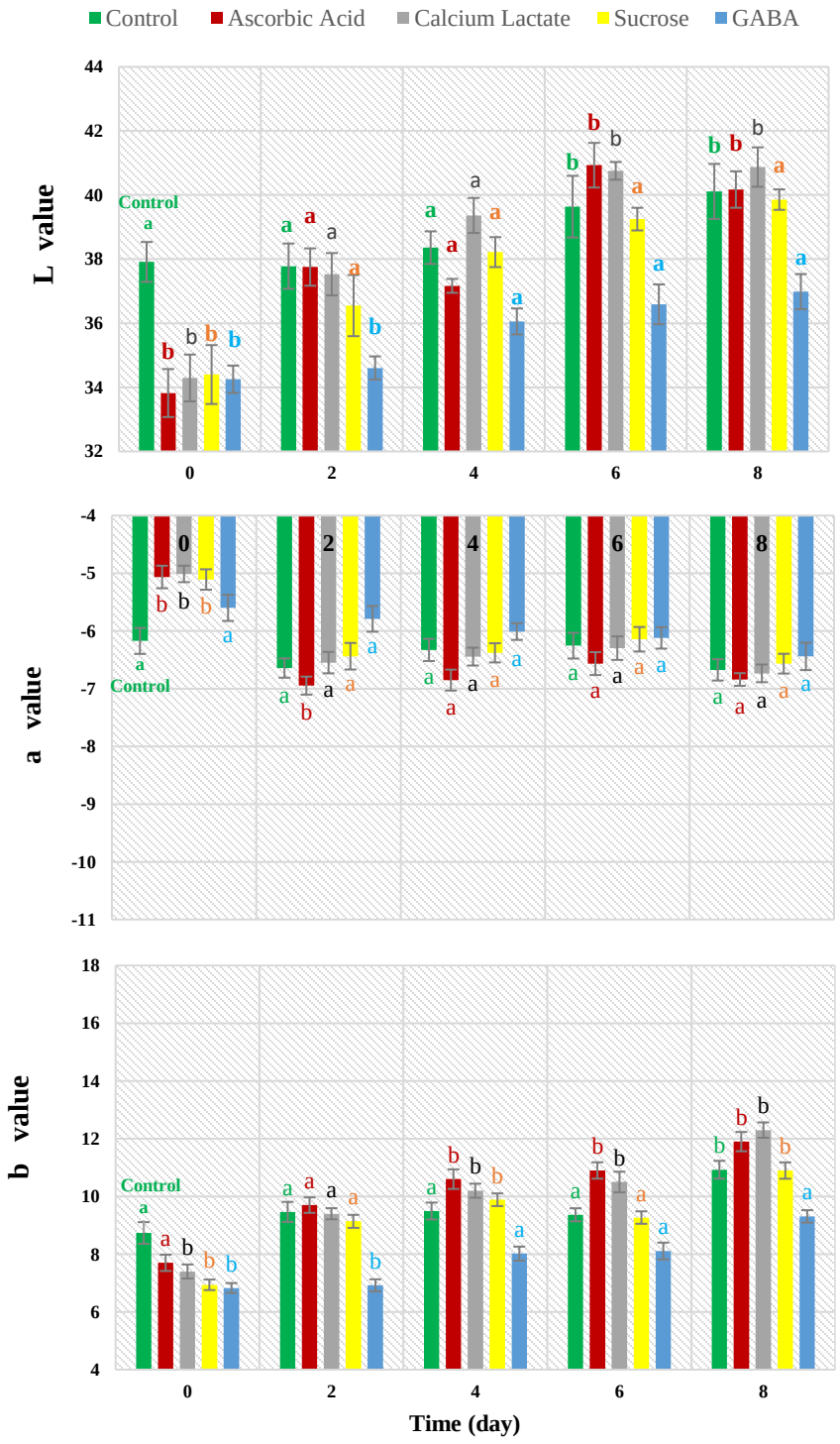
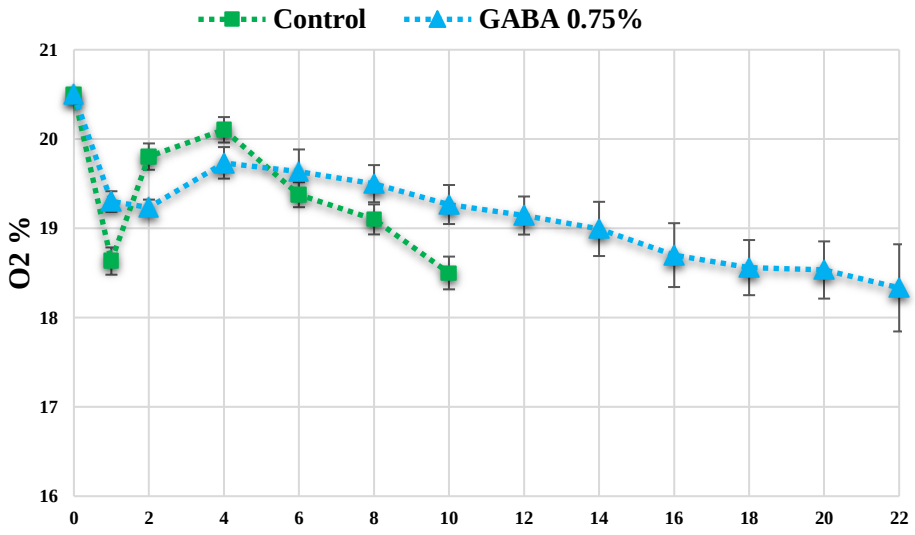


Figure 9. Changes of (L^*, a^*, and b^*) values in packed control and packed spinach impregnated with different substances and stored at 7.5 °C for 8 days. Different letters above the error bars represent statistically significantly differences between treated samples and the control ($p < 0.05$).

6.4 Effect of impregnation of GABA on the shelf life of packed spinach leaves at 7.5 °C

6.4.1 Atmosphere composition

Unlike experiments which were done at 21 °C, when the packages were stored at 7.5 °C, the O_2 concertation sharply decreases after the first 12 h in control samples (untreated packed spinach leaves) followed by an increase until the 4th day (Figure 10). After the 4th day of storage, the O_2 concentration started to decrease gradually to reach the lowest concentration after 10 days. GABA impregnated samples show less decrease in the first 12 hours of storage as compared to the control, and less increase between the second and 4th day of storage. After the 4th day, impregnated samples started to show a slight reduction of O_2 concentration, which continued till the 22th day. The experiment was finished after 22 days when the spinach leaves still showed acceptable visual characteristics.



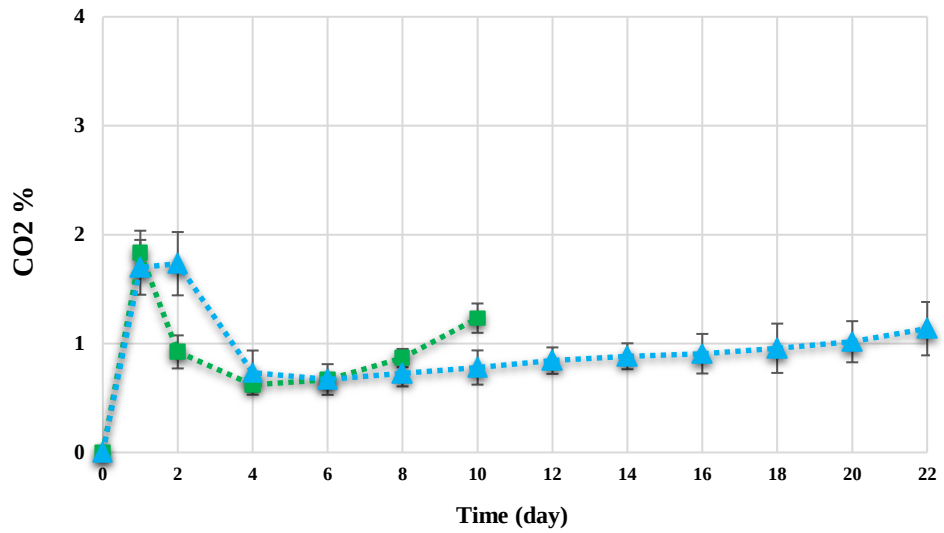


Figure 10. O_2 and CO_2 changes in packed control and packed spinach leaves impregnated with GABA 0.075 % and stored at 7.5 °C for 22 days. In the control samples, measurements did not continue after 10 days of storage due to visible signs of deterioration. Reported are average and STD of 3 measurements

Interestingly, the effect of impregnated GABA started to be visible after the 6th day of storage, where treated samples showed less O_2 consumption and higher CO_2 production than the control.

On the other hand, the O_2 consumption of the leaves with GABA kept increasing gradually till the end of the experiment, indicating that the effect of GABA was continuous throughout the period of observation.

6.4.2 Color changes at 7.5 °C

Figure 11 shows that GABA impregnated spinach leaves still have good green color with a good appearance after 10 days of storage. Untreated controls show less quality, as the leaves started to lose its cohesive strength and freshness (withered). The untreated packages were discarded at day 10 of the storage period.

Figure 12 shows that GABA impregnated packed spinach leaves still have good color and good appearance after 22 days of storage at 7.5 °C. While the normal shelf life of ready to eat vegetables including spinach leaves ranges between 7 to 14 days (Lara et al., 2013). The leaves color was

slightly turned to light green after 22 days of storage, but still acceptable from the consumer view of point.

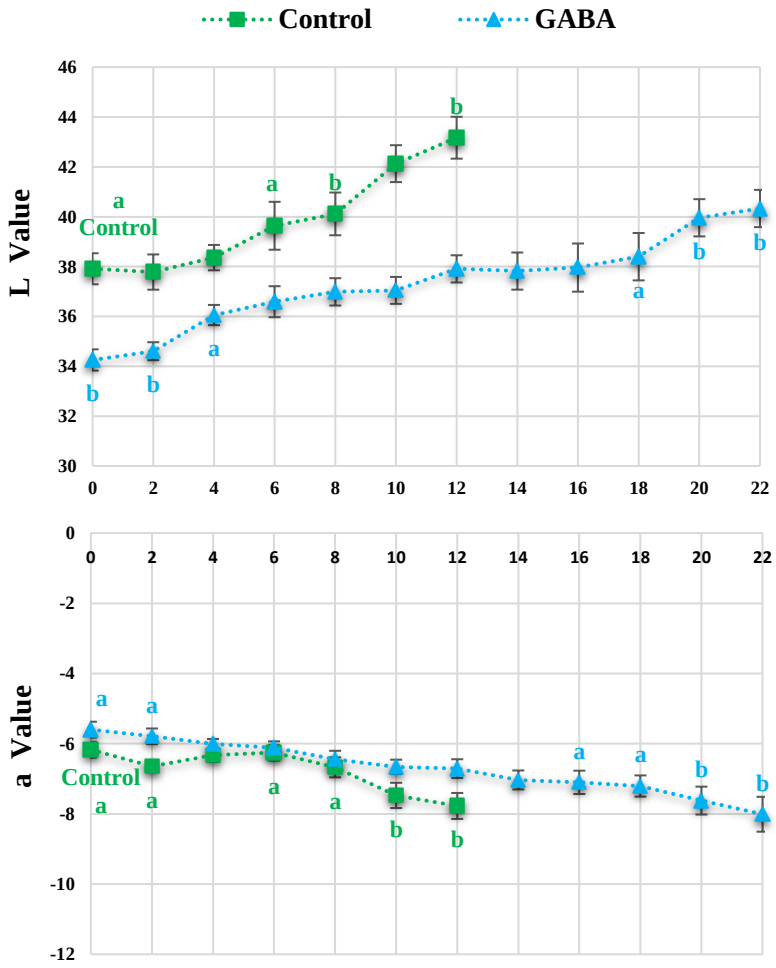
VI

Control

Figure 11. Comparison between packed control and packed impregnated spinach leaves with GABA 0.075 % and stored at 7.5 °C for 10 days.

Figure 12. Impregnated and packed spinach leaves using GABA 0.075 % and stored for 22 days at 7.5 °C.

Figure 13 reports that packed impregnated samples with GABA showed a significant decrease for the colour parameters L^*, and b^* in comparison with the control at day 0. While the a^* parameter showed significant increase after impregnation comparing to the control at day 0. During storage, both L^*, and b^* parameters increased gradually and restored the value of the control at day 0 after 12 and 8 days respectively. The value of the a^* parameter restored the initial value of the control sample after 8 days.



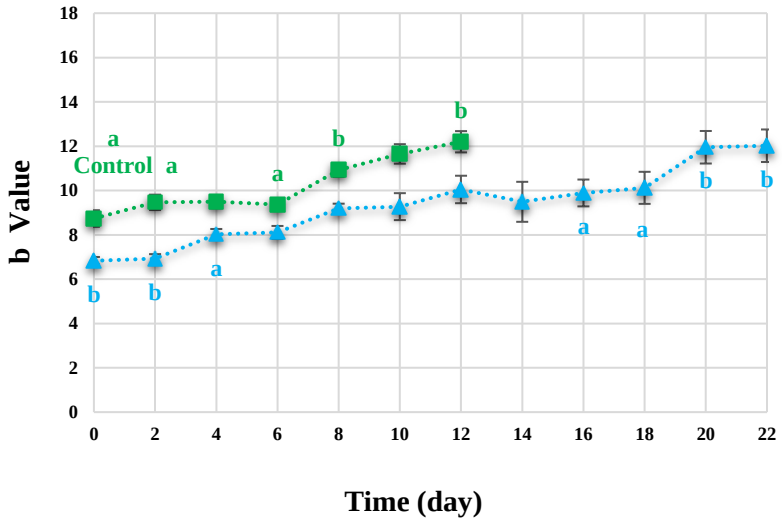


Figure 13. Changes of (L^*, a^*, and b^*) values in the untreated packed samples and packed samples impregnated with GABA 0.075 % and stored at 7.5 °C for 22 days.

6.5 Isothermal calorimetry measurements of heat production at 7.5 °C for the control (packed and unpacked) and impregnated spinach leaves (packed and unpacked) using GABA 0.075 %.

Figure 14 shows a comparison between the impregnated and non-impregnated leaves, packed and non-packed. Nearly 1 h was needed for the calorimeter to get a clear measurement of thermal power. It is obvious from Figure14 that the heat production of the spinach leaves has been affected by impregnating the leaves with GABA. The thermal power of the GABA impregnated leaves was lower than that of the control in both cases (packed treated and un-packed treated).

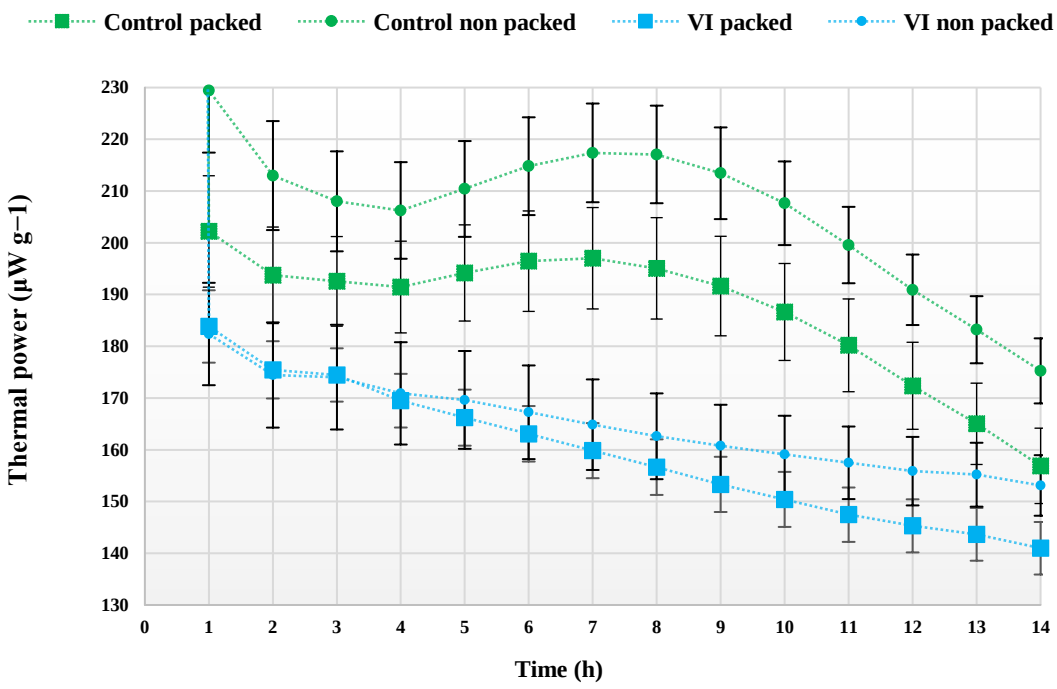


Figure 14. Calorimetric measurements of metabolic thermal power of packed and unpacked control and packed and unpacked impregnated leaves (treated with GABA 0.075 %) stored at 7.5 °C. Reported are average and STD of 3 measurements.

The result with packed (impregnated and non-impregnated/control) spinach is consistent with the result obtained by measuring O_2 and CO_2 concentrations at 7.5 °C. Figure 10 shows that the increase of O_2 concentration was lower in the impregnated, packed spinach leaves compared to non-impregnated packed leaves, at least in the first 14 hours.

An obvious increase of the heat production was clear in control samples (packed and unpacked) between the 4^{th} and the 10^{th} h. After this time point, the heat production gradually declines. GABA impregnated samples (packed and no packed) did not show any increase of heat production. The heat production started to decrease from the first hour gradually along the experiment period. This result undoubtedly shows the high effect of GABA on the metabolic activity of the spinach leaves during the short term after VI.

Further, both packed treated and packed untreated leaves show less heat production as compared to the unpacked leaves along the experimental period. In the VI treated leaves, the

obvious differences between packed and unpacked leaves appeared after 4 h. The lower heat production in packed leaves could be because of the atmosphere, where consumed O_2 and accumulated CO_2 resulted in reduction of their metabolic activity.

7. Conclusions

This study explored the effect of vacuum impregnating with different substances on color and respiration of packed spinach leaves at two temperatures. The following are the main results:

- The impregnation with sucrose, calcium lactate and ascorbic acid solutions affected the color of spinach leaves immediately after the process. Measurement by colorimeter showed a decrease in L^* and b^* values as well as an increase in a^* values regardless of the storage temperature.
- Among all samples, only the samples impregnated with sucrose showed statistically significant higher amount of O_2 consumption and CO_2 production compared to control.
- Impregnation with sucrose plays a key role for the atmosphere created inside the packed spinach during storage at both studied temperatures. Among all impregnated substances sucrose shows the highest O_2 consumption and CO_2 production.
- Impregnation with ascorbic acid leads to faster deterioration of the packed spinach leaves at 21 °C in comparison with leaves impregnated with the other substances.
- GABA impregnated spinach leaves stored at 7.5 °C showed an increase of the shelf life which exceeded 22 days, while the untreated control deteriorated after 10 days
- Measurement of the metabolic activity by isothermal calorimetry showed less heat production from the treated spinach with GABA comparing to the untreated control at 7.5 °C. The calorimetry studies showed higher heat production of the unpacked spinach comparing to the packed leaves at the same storage temperature.
- Measurement of heat production in packed leaves can be done by isothermal calorimetry. This parameter helps to understand the short-term metabolism of the packed leaves and may be used for testing the influence of different packaging materials on metabolism.

8. Future Work

This study has not looked at the effect of ascorbic acid, sucrose, and calcium lactate on the shelf life of the packed impregnated spinach leaves at 7. 5 °C. Therefore, further research is needed on shelf life and to compare the results with the result obtained using GABA.

More studies are needed to look at the effect of different concentrations of the impregnated GABA solution on the respiration and the metabolic activity of the packed spinach leaves.

Since GABA prolongs spinach shelf life for more than 22 days with slight colour alteration, it will be great if a new study can combine another substance with GABA to prolong the shelf life and reduce the colour alterations.

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