# Vacuum Impregnation of Spinach Tissue: Metabolic Consequences and their Potential Industrial Applications

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**DEPARTMENT OF FOOD TECHNOLOGY, ENGINEERING & NUTRITION | LUND UNIVERSITY**
**NOOR LIYANA YUSOF**

# Vacuum Impregnation of Spinach Tissue: Metabolic Consequences and their Potential Industrial Applications

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Engineering, LTH, Lund University, Sweden. To be defended in Lecture Hall B, at the Centre for Chemistry and Chemical Engineering (Kemicentrum) on Friday, 1st September 2017 at 10.15 a.m.

*Faculty opponent*

Dr Antonio Derossi, Department of Food Science, University of Foggia, Italy.

**Vacuum Impregnation of Spinach Tissue: Metabolic Consequences and their Potential Industrial Applications**

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VI has been extensively studied, particularly with regard to the modification of the physico-chemical properties and sensory attributes of food products. These studies include texture enhancement, enrichment of fruits and vegetables with probiotics or micro¬nutrients, the modification of sensory attributes, and the extension of shelf life by pH reduction. VI has also been widely used as a method of pre-treatment prior to, e.g., minimal processing, freezing, or drying of fruit and vegetables. However, little is known about the metabolic consequences of impregnating plant tissue with different substances using VI.

The short-term metabolic response of impregnating spinach leaves with different substances (calcium lactate, sucrose, citric acid and ascorbic acid) was investigated using isothermal calorimetry at 5 ºC, 2 h after VI treatment. The greatest increase in metabolic heat production was observed after impregnation with calcium lactate. This may be the result of metabolization of the lactate, as well as the mobilization of starch. The different impregnation solutes led to specific changes in the carbohydrate composition of the leaves during 4 days of storage, due to sugar metabolization, glucose-to-fructose inter¬conversion and starch mobilization in the plant tissue.

The effect on the nitrate content of spinach following impregnation with sucrose was also investigated. The results showed that a small amount of sucrose (5 g/100 ml) significantly decreased the nitrate concentration. Exogenously supplied sucrose reduced the nitrate concentration in the leaves by almost 70 % during 3 days of storage at 8 ºC, compared to non-impregnated leaves. Evidence showed that sucrose was metabolized during storage of the leaves, and that the reduction in nitrate was due to the use of sucrose as a substrate for the metabolization of stored nitrate and for respiration. Thus, VI could be beneficial in the food industry in reducing the nitrate content of spinach, leading to the improvement of nutritional characteristics.

The influence of impregnation with different substances on the metabolic activity of spinach leaves in modified atmosphere packaging (MAP) was investigated. The gross metabolic activity of the impregnated spinach leaves changed significantly, depending on the impregnation solute and treatment temperature. Sucrose induced the highest metabolic heat production at 21 ºC, whereas calcium lactate led to the highest metabolic activity at 5 ºC. The high metabolic activity of sucrose-impregnated leaves was reflected by the high oxygen consumption and carbon dioxide production measured in the packaged product stored at 21 ºC. However, this was not reflected by the changes in atmosphere inside the calcium lactate-impregnated, packaged products. The incongruity between calorimetric and atmospheric measurements may be the result of the different time scales of the measurements.

The results obtained in this work have helped provide a better understanding of how impregnation of spinach leaves affects their metabolic activity. The findings could be of importance in the food industry as they provide a better understanding of how spinach leaves could be metabolically affected by a certain type of compound, thus influencing specific quality characteristics and their respiration upon packaging.

Key words: Vacuum Impregnation, Metabolic Activity, Spinach Leaves, Modified Atmosphere Packaging

Signature                    Date   2017-06-01

# Vacuum Impregnation of Spinach Tissue: Metabolic Consequences and their Potential Industrial Applications

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*"in between goal is a thing called life that has to be lived and enjoyed"*

# Abstract

Vacuum impregnation (VI) is a unit operation that allows the introduction of solutions into the porous structure of plant tissues. The plant tissue is immersed in the solution of interest and is then subjected to partial vacuum, causing the removal of air from the tissue. When the atmospheric pressure is restored, the solution is drawn into the tissue, replacing the air. In this way, most of the air spaces are filled by the solution, although small air pockets may still remain.

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The influence of impregnation with different substances on the metabolic activity of spinach leaves in modified atmosphere packaging (MAP) was investigated. The gross metabolic activity of the impregnated spinach leaves changed significantly, depending on the impregnation solute and treatment temperature. Sucrose induced the highest metabolic heat production at 21 ºC, whereas calcium lactate led to the highest metabolic activity at 5 ºC. The high metabolic activity of sucrose-impregnated leaves was reflected by the high oxygen consumption and carbon dioxide production measured in the packaged product stored at 21 ºC. However, this was not reflected by the changes in atmosphere inside the calcium lactate-impregnated, packaged products. The incongruity between calorimetric and atmospheric measurements may be the result of the different time scales of the measurements.

The results obtained in this work have helped provide a better understanding of how impregnation of spinach leaves affects their metabolic activity. The findings could be of importance in the food industry as they provide a better understanding of how spinach leaves could be metabolically affected by a certain type of compound, thus influencing specific quality characteristics and their respiration upon packaging.

# Popular Scientific Summary

Some vegetables float in water due to the air contained in their structure. During vacuum impregnation (VI), vegetables are subjected to a slight vacuum to remove the air from the tissue so that it can be replaced with a solution containing a particular substance once the vacuum is released. VI is a commonly used method of introducing "foreign" molecules into plant tissues, and has been used extensively in the fruit and vegetable industry as a form of pre-treatment before other processing techniques such as drying or freezing. Among the substances that have been introduced into fruit and vegetables by VI are sugars, microbial preservatives, texture enhancers and compounds enhancing the nutritional composition of the product, such as vitamins.

The impregnation of plant tissue with various substances, aiming at improving certain quality characteristics, has been widely reported. However, the consequences of impregnation on the metabolism of plant tissues are not well understood. It is important to understand which substances increase the metabolic activity since the product shelf life is strongly dependent on this factor. The metabolic effects on spinach leaves after VI with substances commonly used in the food industry are in the subject of this thesis.

All physical, chemical and biological processes produce heat. This heat, which is produced by the metabolism of the fruit or vegetables, can be measured using a technique called isothermal calorimetry. In this work, the heat produced by baby spinach leaves after VI with sucrose, calcium lactate (texture enhancer), ascorbic acid (vitamin C) or citric acid (prevents changes in colour) was measured. The results showed that sucrose and calcium lactate caused rapid increases in the metabolic heat of the spinach leaves, while the other substances investigated showed no effect. The temperature during treatment and storage should also be taken into consideration, as they affect the metabolism after VI. The sugar (glucose, fructose, sucrose) and starch contents in the leaves were also affected by VI. Based on the results presented in this thesis, it was concluded that some substances used for the impregnation of spinach leaves are used by the cells in their metabolism. As a consequence of impregnation, starch reserves are used as an energy source.

Nitrate is found in spinach, and this may be harmful to human health, especially in infants. The results of this research show that the nitrate content was reduced by almost 70 % following impregnation with sucrose using VI. Experiments showed that sucrose was used as a substrate for both the metabolization of the nitrate and for respiration during cold storage of the leaves. Impregnating spinach leaves with a simple molecule, such as sucrose, could thus be used in the food industry to improve the nutritional quality of nitrate-containing vegetables.

Packaging is often used as a means of preservation, the aim of which is to retain attributes and quality similar to those of the fresh product. The effects on the respiration of packed spinach impregnated with different substances using VI was thus investigated. The changes in the atmospheric composition inside the packages (oxygen ($O_2$) consumed by the leaves, and carbon dioxide ($CO_2$) produced by the leaves) was determined during the storage of packaged spinach for four days at two different temperatures. The results showed that impregnation with sucrose significantly affected the $O_2$ consumption and $CO_2$ production of the spinach in the package, in contrast to the other substances studied (calcium lactate and ascorbic acid).

This study provides new insights into the response of metabolically active cells to the introduction of foreign molecules into spinach tissue. These results could be of importance in the food industry as they provide a better understanding of how spinach leaves could be metabolically affected by a certain type of compound, thus influencing specific quality characteristics and their respiration upon packaging.

# Ringkasan Saintifik Popular

Sesetengah sayur-sayuran terapung di dalam air disebabkan oleh udara yang terkandung di dalam strukturnya. Semasa proses impregnasi vakum (VI), sayur-sayuran ini telah diletakkan di bawah keadaan vakum bagi mengeluarkan udara yang terkandung di dalam tisu tumbuhan, lalu digantikan dengan cecair atau larutan yang mengandungi bahan dan fungsi tertentu apabila vakum dilepaskan. VI adalah salah satu kaedah yang biasa digunakan bagi memasukkan molekul "asing" ke dalam tisu tumbuhan, dan telah digunakan secara meluas dalam industri buah-buahan dan sayur-sayuran sebagai satu bentuk teknik awal dalam bidang pemprosesan makanan seperti pengeringan atau sejuk-beku. Antara bahan-bahan yang biasa dimasukkan ke dalam buah-buahan dan sayur-sayuran melalui VI adalah gula, pengawet makanan, penambah-baikan tekstur makanan dan sebatian yang dapat meningkatkan nutrisi produk, seperti vitamin.

Impregnasi tisu tumbuhan dengan pelbagai bahan bertujuan bagi meningkatkan sesetengah kualiti makanan telah dilaporkan secara meluas. Walau bagaimanapun, kesan daripada impregnasi ini terhadap metabolik tumbuhan itu sendiri tidak dapat difahami dengan baik. Pemahaman yang mendalam mengenai bahan manakah yang dapat meningkatkan aktiviti metabolik di dalam tisu tumbuhan adalah sangat penting kerana jangka hayat produk makanan sangatlah bergantung kepada faktor ini. Kesan metabolik yang berlaku di dalam sayur bayam selepas impregnasi dengan bahan-bahan yang biasa digunakan di dalam industri makanan menjadi subjek utama bagi thesis ini.

Semua proses fizikal, kimia dan biologi yang berlaku di dalam benda hidup mengeluarkan haba. Haba yang terhasil melalui proses metabolisme buah-buahan atau sayur-sayuran boleh diukur dengan menggunakan teknik yang dipanggil *"isothermal calorimetry"*. Dalam projek ini, haba yang dihasilkan oleh daun bayam selepas diimpregnasi dengan sukros, kalsium laktate (penambah-baikan tekstur), asid askorbik (vitamin C) atau asid sitrik (bagi mencegah perubahan warna) telah diukur. Hasil kajian menunjukkan bahawa impregnasi sukros dan kalsium laktate telah menyebabkan peningkatan haba metabolik yang tinggi di dalam daun bayam, manakala bahan-bahan lain tidak menunjukkan sebarang kesan. Suhu semasa pengendalian dan penyimpanan juga perlu diambil kira, kerana dua factor ini memberikan kesan kepada metabolisme selepas VI. Gula (glukosa, fruktosa, sukrosa) dan kandungan kanji di dalam daun juga turut terkesan disebabkan oleh

VI. Berdasarkan keputusan yang diperolehi di dalam tesis ini, kesimpulan dapat dibuat bahawa beberapa bahan yang digunakan bagi tujuan impregnasi ke dalam daun bayam telah digunakan oleh sel-sel bagi tujuan metabolisme tumbuhan. Kesan dari impregnasi ini, kanji terkumpul di dalam daun bayam ini juga telah digunakan sebagai sumber tenaga semasa aktiviti metabolik berlaku.

Nitrat yang terkandung di dalam daun bayam boleh memudaratkan kesihatan manusia, terutamanya bagi bayi. Keputusan dari kajian ini menunjukkan bahawa kandungan nitrat telah dapat dikurangkan sebanyak hampir 70% hasil dari impregnasi sukros menggunakan proses VI. Hasil kajian eksperimen menunjukkan bahawa sukros telah digunakan sebagai substrat untuk kedua-dua aktiviti iaitu metabolisme nitrat dan respirasi ketika bayam disimpan di dalam keadaan sejuk. Justeru, impregnasi daun bayam dengan bahan yang mudah didapati seperti sukros, boleh digunakan dalam industri makanan bagi meningkatkan kualiti makanan, khususnya sayur-sayuran yang mengandungi nitrat yang tinggi.

Pembungkusan makanan sering kali digunakan sebagai salah satu cara bagi memelihara dan mengekalkan sifat-sifat dan kualiti yang sama seperti makanan segar. Kesan terhadap respirasi daun bayam yang dibungkus dan diimpregnasi dengan bahan-bahan yang berbeza menggunakan VI telah dikaji. Perubahan komposisi atmosfera dalam bungkusan daun bayam (oksigen ($O_2$) yang digunakan dan karbon dioksida ($CO_2$) yang dihasilkan oleh daun) telah dikaji semasa bungkusan daun bayam disimpan selama empat hari di dalam dua suhu yang berbeza. Hasil kajian mendapati bahawa impregnasi dengan sukros telah memberi kesan yang ketara terhadap penggunaan $O_2$ dan pengeluaran $CO_2$ di dalam bungkusan bayam, berlainan dengan bayam yang diimpregnasi dengan bahan lain (kalsium laktate dan asid askorbik).

Kajian ini memberikan satu pencerahan yang baru mengenai respon dan tindak balas sel-sel metabolisme aktif yang berlaku di dalam tisu bayam apabila diimpregnasi dengan molekul asing. Hasil kajian ini dapat menjadi rujukan penting khususnya bagi industri makanan kerana ia memberikan pemahaman yang lebih baik bagaimana metabolisme daun bayam boleh terjejas dengan bahan-bahan yang biasa digunakan di dalam industri, lalu mempengaruhi ciri-ciri kualiti dan respirasi sayur-sayuran semasa dibungkus dan disimpan.

# List of Publications

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

I.  Gómez Galindo, F., **Yusof, N.L.** (2015)

    **New Insights into the Dynamics of Vacuum Impregnation of Plant Tissues and Its Metabolic Consequences**

    Journal of the Science of Food and Agriculture, 95, 1127-1130.

II. **Yusof, N.L.,** Wadsö L., Rasmusson A.G., Gómez Galindo, F. (2017)

    ***Influence of Vacuum Impregnation with Different Substances on the Metabolic* Heat Production and Sugar Metabolism of Spinach Leaves**

    Submitted for publication.

III. **Yusof, N.L.,** Rasmusson, A.G., Gómez Galindo, F. (2016)

    **Reduction of the Nitrate Content in Baby Spinach Leaves by Vacuum Impregnation with Sucrose**

    Food and Bioprocess Technology, 9, 1358-366.

IV. **Yusof, N.L.,** Husain, A., Wadsö L., Gómez Galindo, F. (2017)

    **Effect of Vacuum Impregnation of Baby Spinach Leaves on Package Gas Composition**

    Submitted for publication.

# The Author's Contributions to the Papers

    I.       The author collected all the relevant references, had critical discussions of their content with the co-author, and contributed to the organization of the information in the manuscript.

    II.     The author designed the isothermal calorimetry essays based on suggestions from the co-authors. The author performed all the chemical analyses, and took an active part in the discussions and writing the paper with minor contributions from the co-authors.

    III.    The author designed the study together with the co-authors, performed the experimental and theoretical work, evaluated the results and wrote the paper with minor contributions from the co-authors.

    IV.    The author designed the study and performed the experiments. The author evaluated the results in cooperation with the co-authors and wrote the paper.

# Contents

# 1. Introduction and Objectives

Vacuum impregnation (VI) is a mild form of treatment that can be used to introduce ingredients such as anti-browning agents, microbial preservatives and texture enhancers into the porous structure of fruit and vegetables. Large volumes of intercellular spaces in the plant tissue can be filled with a solution of the ingredient using VI, and it is generally used as a form of pre-treatment before, e.g., minimal processing, drying, or freezing (Chiralt et al., 1999).

VI has been studied extensively, particularly with regard to the modification of the physico-chemical properties and sensory attributes of food products (Betoret et al., 2003; Codoñer-Franch et al., 2013; Derossi, De Pilli, La Penna, & Severini, 2011; Derossi, De Pilli, & Severini, 2013); Fito et al., 2001; Gras, Vidal, Betoret, Chiralt, & Fito, 2003; Moreno, Bugueño, Velasco, Petzold & Tabilo-Munizaga, 2004; Radziejewska-Kubzdela, Biegańska-Marecik, & Kidoń; 2014). The impregnation process is, however, associated with several technological challenges due, not only to the heterogeneity and complexity of plant tissue structure, but also to the changes that can be expected in the structure and metabolism of the tissue when introducing a foreign molecule.

In the work described in this thesis, VI was applied to baby spinach leaves *(Spinacia oleracea)*. Spinach is a widely consumed leaf, which is rich in vitamins and minerals (Lucier, Allshouse, & Lin, 2004), although it contains high levels of  nitrates and the fresh, packed product has a limited shelf life. In the present work, VI was studied as a means of addressing these quality issues by improving our understanding of the consequences of the introduction of a foreign molecule into the tissue on cell metabolism.

The main aims of the research described in this PhD thesis were:

- to review the dynamics of VI and the state of the art regarding its metabolic consequences (**Paper I**),

- to investigate the metabolic responses of spinach leaves impregnated with substances commonly used in the food industry (**Paper II**),

- to study the influence of impregnation with sucrose on the nitrate content of spinach leaves (**Paper III**) and

- to describe the effect of VI on the atmosphere in modified atmosphere packaging of spinach leaves (**Paper IV**).

# 2. Overview of the Leaf 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 (Baumann & Henze, 1982; Khan & Vincent, 1990; Raven, 1996; Vincent, 1989). The orientation and structural geometry of the intercellular spaces in leaves play a fundamental role in efficient light capturing, and in facilitating liquid and gas transport in the plant tissue (Dražeta, Lang, Hall, Volz, & Jameson, 2004; Kuroki, Oshita, Sotome, Kawagoe, & Seo, 2004; Raven, 1996; Schotsmans, Verlinden, Lammertyn, & Nicolaï, 2004).

Figure 1 shows a microscopy image of the cross section of a spinach leaf, showing the epidermis, palisade mesophyll, spongy mesophyll, vein and air spaces. The epidermis is the outermost layer of cells located in the upper (adaxial) and lower (abaxial) parts of the leaf surface, consisting of flat, closely packed cells (Taiz, Zeiger, Møller, & Murphy, 2014). This is known as dermal tissue. In some leaves, the epidermis may be several layers thick, however, the epidermis in spinach leaves is usually only one cell layer thick (Evert, 2006). In woody plants the epidermis develops into a protective layer called the periderm, forming part of the bark (Biggs, 1985).

The main function of the epidermis is to prevent water loss from the plant tissue. A waxy layer known as the cuticle, produced by the epidermis, covers the leaf surface. The cuticle helps regulate gas exchange and protect the internal cells (Domínguez, Heredia-Guerrero, & Heredia, 2011; Kerstiens, 2006; Riederer & Schreiber, 2001). The epidermis contains stomata that are involved in gas exchange inside the cell. Two guard cells surround each stomata, regulating its opening and closing (Meidner, 1975).

The ground tissue of plants (i.e. neither dermal nor vascular tissue) consists of two different types: the palisade mesophyll and the spongy mesophyll (Warmbrodt & Van Der Woude, 1990). The palisade mesophyll, located close to the leaf surface, consists of rod-shaped cells that contain large numbers of chloroplasts that are used in photosynthesis. These cells are upright, elongated and tightly packed, in order to increase the available surface area for light absorption (Vogelmann & Evans, 2002). The cells of the spongy mesophyll are smaller than those of the palisade mesophyll, and are found in the lower part of the leaf. There are large air spaces between the

cells of the spongy mesophyll, which allow gas exchange (oxygen and carbon dioxide) between the leaf and the surroundings through the stomata during photosynthesis and respiration. In aquatic plants, the intercellular spaces in the spongy mesophyll help the leaves to float. About 30 % of the volume of spinach leaves is occupied by intercellular air spaces (Winter, Robinson, & Heldt, 1994).

The thickness of the leaf is strongly influenced by its internal anatomy, i.e., cell size and shape, density and the amount of intercellular air space in the mesophyll (Slaton & Smith, 2002). The mitochondria and endoplasmic reticulum of mesophyll cells are located near the chloroplasts in order to allow the efficient exchange of the intermediate metabolites of photosynthesis and respiration (Taiz et al., 2014).

The veins of leaves consist primarily of vascular tissue, and consist of the phloem and the xylem. Vascular tissue generally transports water and nutrients, and provides structural support for plant cells. The veins are surrounded by the parenchyma pith and collenchyma. The upper layer of the xylem transports water and minerals, while the phloem helps transport sugars produced in the leaves to other parts of the plant.
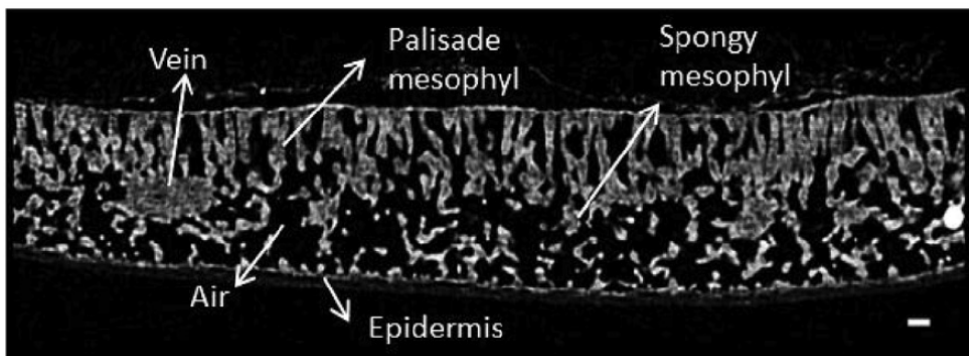


Figure 1. Image of a cross-section of a spinach leaf. Adapted from Figure 3A from Panarese et al. (2016) with kind permission from Elsevier.

The area around the spongy mesophyll is more easily impregnated than the area around the palisade mesophyll (Panarese et al., 2016). Most of the intercellular air spaces are located in the spongy mesophyll, and are rapidly and easily filled by the solution. The density of the stomata on the surface of the leaf is an important factor in the impregnation of the palisade mesophyll since the stomata provide access to the air-filled spaces.

# 3. Vacuum Impregnation

During VI, porous materials are immersed in solutions of different compositions and/or concentrations, and subjected to two-step pressure changes. In the first step, partial vacuum is applied to the solid–liquid system, and the gas inside the pores expands and leaves the pores until mechanical equilibrium is achieved. During the second step, when atmospheric pressure is restored, the residual gas in the pores is compressed and the external liquid flows into the pores, replacing the air (Fito, 1994; Fito, Andrés, Chiralt, & Pardo, 1996; Fito & Pastor, 1994; Tylewicz, Romani, Widell, & Gómez Galindo, 2013).

The dynamics of VI was reviewed in **Paper I**. According to the hydrodynamic model, the factors that play important roles in pore filling of plant tissues are the porosity of the sample, dimensional changes within the sample, and the ratio of the applied vacuum to the sum of the ambient pressure and Laplace pressure in the pores (Albors, Salvatori, Andrés, Chiralt, & Fito, 1996; Fito, 1994; Fito, Andrés, Chiralt, & Pardo, 1996; Tylewicz et al., 2012). The Laplace pressure depends on the wetting properties of the air space and on the shape and size of the pore. Therefore, it is not constant throughout the tissue. The impregnation process is also influenced by the deformation and expansion of the pores, which are related to the mechanical properties of the material (deformation–relaxation phenomena) (Fito et al., 1996; Fito & Pastor, 1994; Radziejewska-Kubzdela et al., 2014).

As a result of these factors, some tissues are more easily impregnated than others. Panarese, Dejmek, Rocculi, and Gómez Galindo (2013) showed that impregnation of apple tissue occurred as soon as the atmospheric pressure was restored, whereas in spinach leaves, the flow of liquid took place later. Schulze, Peth, Hubbermann, and Schwarz (2012) reported that VI resulted in a higher uptake of an isotonic solution in the inner apple cortex than in the outer part. The cause of the lower impregnation was attributed to smaller cells and lower intercellular space connectivity. Velickova et al. (2013) also reported lower impregnation of cryoprotectants in the outer tissue of strawberries, where the cells are small and the tissue is more compact than in the inner tissues of the fruit. It has also been shown that spinach tissue was not completely impregnated after the impregnation process was optimized with respect to the mass gain (Panarese et al., 2016), and the edge of the leaf was more difficult to impregnate.

The liquid phase infused into the material may have different properties, and VI can thus be used to change the composition and properties of plant tissues (Roza Biegańska-Marecik & Janusz Czapski, 2007; Chiralt et al., 2001; Derossi, De Pilli, & Severini, 2010; Radziejewska-Kubzdela, Biegańska-Marecik, & Kidoń, 2014). Impregnation of fruit and vegetables with ascorbic acid or citric acid solutions has been used extensively to inhibit enzymatic browning during storage and to reduce microbial growth (Róa Biegańska-Marecik & Janusz Czapski, 2007; Blanda et al., 2008; Shah & Nath, 2008; Yurttas, Moreira, & Castell-Perez, 2014). Impregnating fruit and vegetables, e.g. apples, pears, carrots and lettuce, with calcium lactate has been shown to improve their texture and structural properties (Alandes, Pérez-Munuera, Llorca, Quiles, & Hernando, 2009; Anino, Salvatori, & Alzamora, 2006; Mao et al., 2016; Rico et al., 2007). Impregnating blueberries with sucrose, one of the additives most commonly used in food processing, has been reported to shorten the dehydration time (Pallas, Pegg, & Kerr, 2013), and extend shelf life by reducing the water activity (Moreno et al., 2004; Chiralt, Escriche, & Serra, 2000).

Many studies have focused on the effects of the vacuum level, the structure and mechanical properties of the foodstuff on mass transfer during VI Carciofi, Prat, & Laurindo, 2012; Chiralt & Fito, 2003; Fito & Pastor, 1994; Laurindo, Stringari, Paes, & Carciofi, 2007; Mujica-Paz, Valdez-Fragoso, López-Malo, Palou, & Welti-Chanes, 2003; Paes, Stringari, & Laurindo, 2006), and the effects of the impregnating liquids on the structure and mechanical properties of the impregnated tissue (Guillemin, Degraeve, Noël, & Saurel, 2008; Guillemin et al., 2008). However, to the best of the author's knowledge, little is known about the metabolic consequences of VI. The effect on the cell metabolism may differ, depending on the type of molecule with which the tissue is impregnated. The state of the art regarding the metabolic consequences of impregnating plant tissue by VI is reviewed in **Paper I**, and the findings are summarized in later sections. However, the detailed metabolic consequences remain to be elucidated.

# 4. The Metabolic Consequences of VI

VI opens up a wide range of possibilities for introducing foreign molecules into plant tissues, which may induce metabolic changes. Interestingly, simple immersion of sliced potatoes in citric acid, ascorbic acid or L-cysteine for 3 min (during which partial impregnation of the intercellular space is expected) increased their metabolic heat production, as measured by isothermal calorimetry (Rocculi et al., 2007). Panarese et al. (2014) reported that impregnating spinach leaves with sugars using VI drastically increased the metabolic activity of the cells on short time scales. This increase was attributed to the metabolization of the impregnated substances. Dymek et al. (2016) also reported an increase in metabolic activity of spinach leaves when impregnated with trehalose. Trehalose is transported into the cells leading to the accumulation of trehalose-6-phosphate in the cells. This may have caused an increase in carbon utilization, increasing the metabolic activity in the tissue, involving mainly mitochondrial oxygen-consuming pathways.

## 4.1 New findings

**Paper II** describes the influence of VI with different substances on the metabolic heat production and sugar metabolism of baby spinach leaves. It was found that leaves impregnated with sucrose (0.6 mol/l) and calcium lactate (50 mmol/l) showed a drastic increase in metabolic heat production when measured at 5 ºC. However, leaves impregnated with either ascorbic acid (5.7 mmol/l) or citric acid (1.0 mmol/l), did not show any changes in their gross metabolic activity during measurements over 2 h (Figure 2).
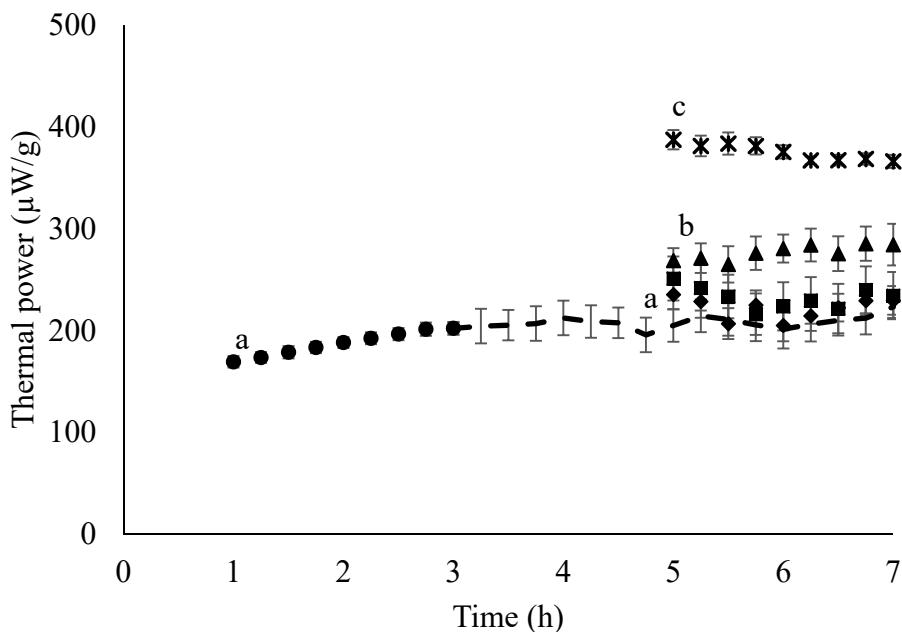
Figure 2. Calorimetric measurements of the metabolic thermal power of untreated spinach leaves (•) and spinach leaves subjected to VI with 50 mmol/l calcium lactate (pH 7.1) (x), 0.6 mol/l sucrose (pH 5.8) (▲), 5.7 mmol/l ascorbic acid (pH 3.2) (■) and 1.0 mmol/l citric acid (pH 3.2) (♦) at 5 ºC. In a separate experiment, the signal from the untreated leaves (dashed line) was recorded for six consecutive hours. Statistically significant differences between the different curves (p < 0.05) are indicated by different letters above the recorded thermal power curves. Bars represent the standard deviation of the mean of four replicate measurements (corresponds to Figure 2 in Paper II).

The long-term metabolic response following the impregnation of spinach leaves with different substances was further investigated by measuring the sugar and starch concentrations in the leaves. The results showed that different impregnation solutes led to different changes in the carbohydrate composition of the leaves during 4 days of storage at 5 ºC.

Increased metabolic activity may be the cause of the dramatic and sustained decrease in the concentration of impregnated sucrose and the drastic loss of starch after impregnation (Figures 3 and 5). However, the greatest increase in metabolic activity among the substances studied was detected after impregnation with calcium lactate. Externally supplied lactate has been shown to be rapidly metabolized by spinach leaves as well as leaves of other species of higher plants, such as lettuce and soybean (Betsche, 1983). However, the carbon supplied by the lactate represents only a small percentage of the carbon supplied by sucrose in the sucrose-impregnated leaves, but the resulting energy burst is much higher (Figure 2).

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Therefore, the calorimetry results suggest that the energy burst following impregnation with calcium lactate is the result of metabolic stimulation of the respiratory pathway by lactate as an effector, rather than as a substrate. The energy burst caused by impregnation with lactate may be caused by the mobilization of other carbon sources such as starch. The steady-state concentrations of fructose and glucose will be influenced by glucose-to-fructose interconversion and glucose and fructose metabolization (Figure 4).
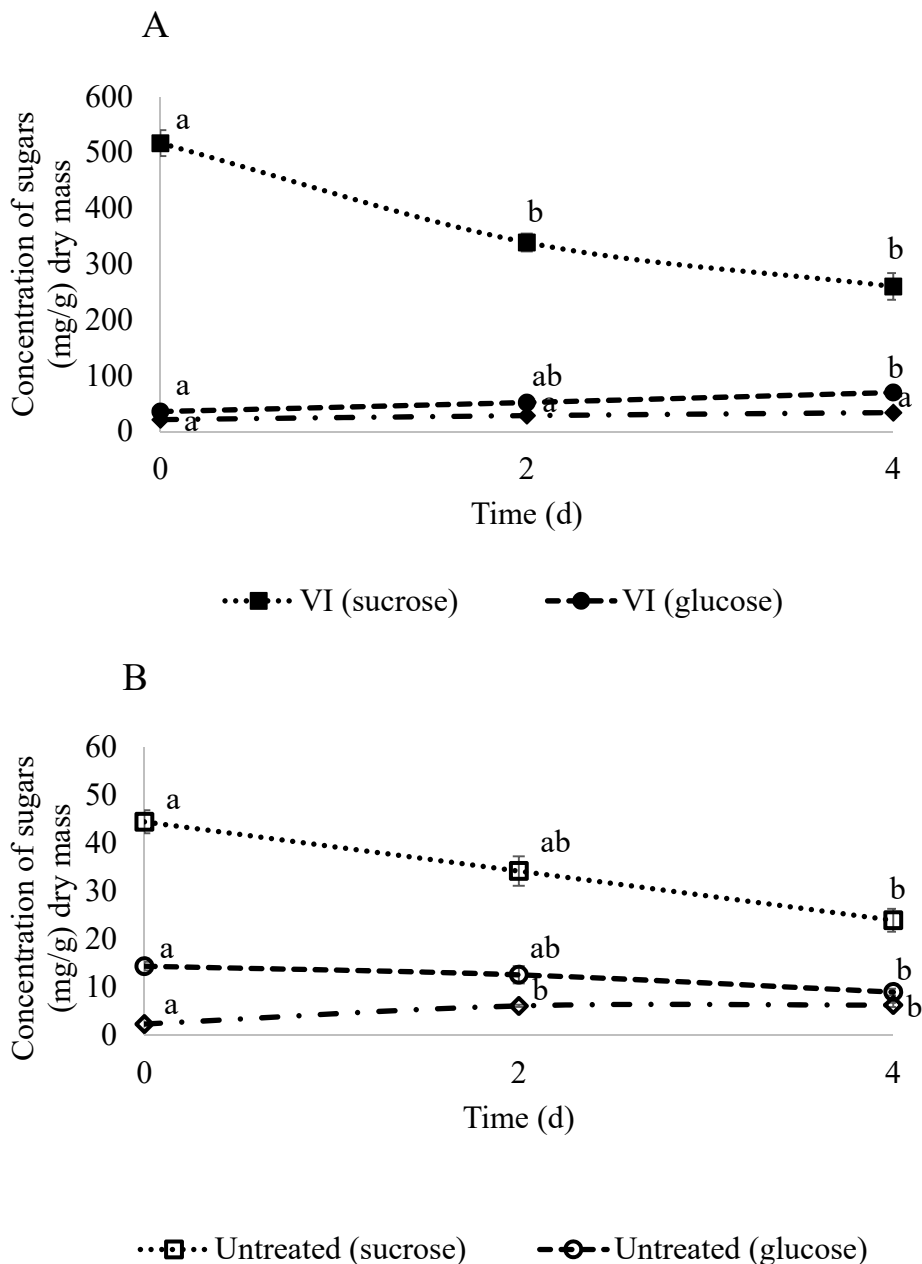
Figure 3. Concentration of sucrose, glucose and fructose in baby spinach leaves: (A) impregnated with 0.6 mol/l sucrose solution (pH 5.8) (filled symbols) and (B) untreated leaves (empty symbols), during a period of 4 days at 5 °C. The first data point for the VI-treated leaves represents the concentration of the sugars 15 min after VI. Statistically significant differences ($p < 0.05$) within each curve are indicated by different letters above the error bars. Bars represent the standard deviation of the mean of three replicate measurements (corresponds to Figure 3 in Paper II).
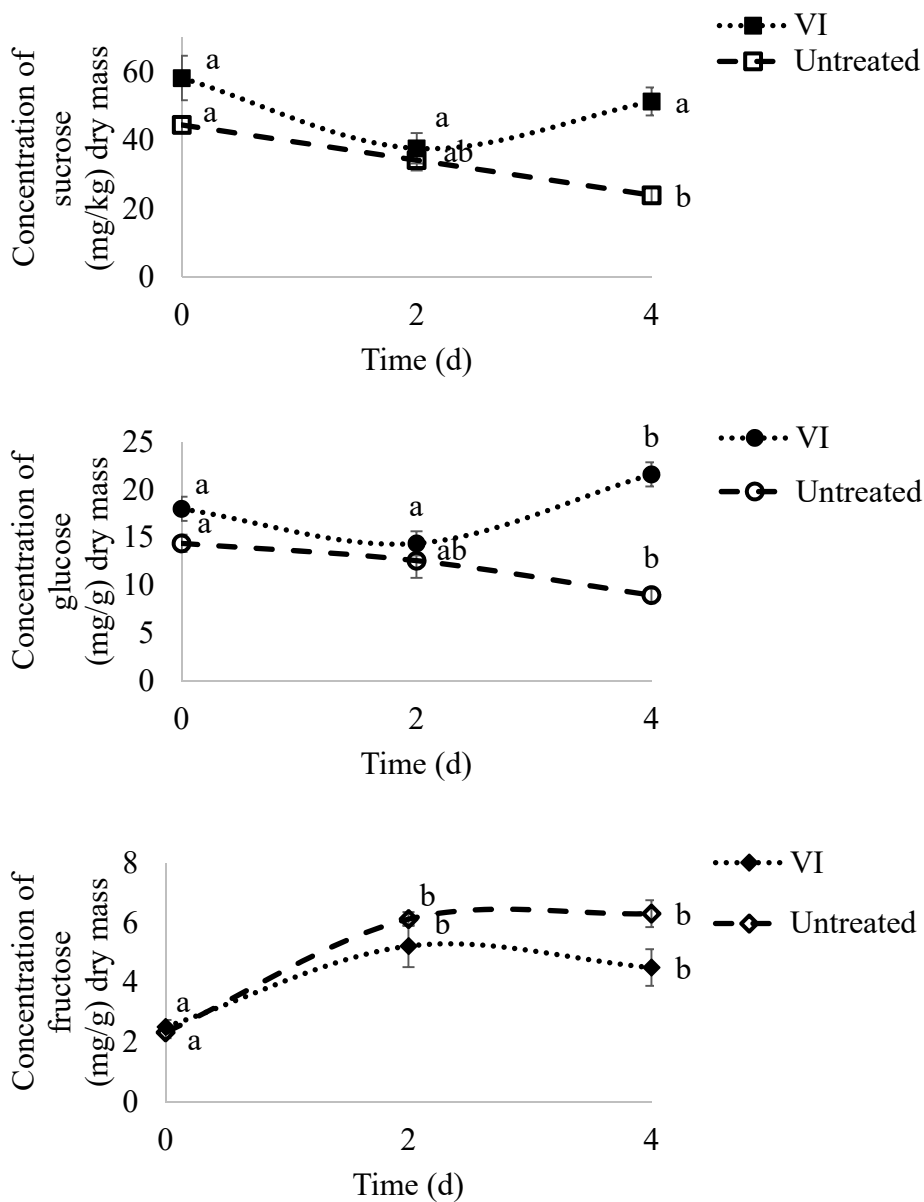
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Figure 4. Concentration of sucrose, glucose and fructose in baby spinach leaves impregnated with 50 mmol/l calcium lactate (filled symbols) and untreated leaves (empty symbols) over 4 days at 5 ºC. The first data point for the VI-treated leaves represents the concentration of the sugars 15 min after VI. Statistically significant differences ($p < 0.05$) within each curve are represented by different letters above the error bars. Bars represent the standard deviation of the mean of three replicate measurements (corresponds to Figure 4 in Paper II).
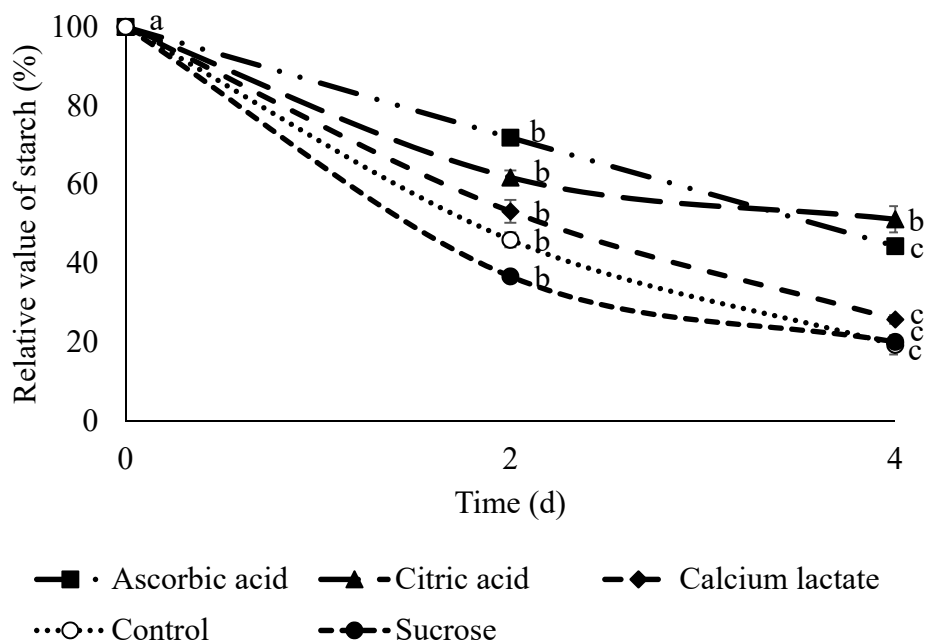
Figure 5. Relative concentration of starch in baby spinach leaves with an initial content of 0.393 ± 0.006 g/kg (DM). The leaves were not impregnated (control), or impregnated with 0.6 mol/l sucrose solution (pH 5.8), 50 mmol/l calcium lactate, 1.0 mmol/l citric acid (pH 3.2) or 5.7 mmol/l ascorbic acid (pH 3.2) during 4 days of storage at 5 ºC. Statistically significant differences ($p < 0.05$) within each curve are represented by different letters above the error bars. Bars represent the standard deviation of the mean of three replicate measurements (corresponds to Figure 7 in Paper II).

# 5. Potential Applications of VI-Induced Metabolic Changes

As pointed out above, VI affects the metabolically active cells in impregnated tissues. This chapter describes new findings describing how VI can influence a certain metabolic pathway in the impregnated leaves that influences their nitrate content. The influence on the respiration of packaged spinach impregnated with different molecules is also described.

## 5.1 Reduction of the nitrate content of spinach leaves

The presence of nitrates in foods is potentially harmful to human health. High concentrations of nitrates are associated with an increased risk of gastrointestinal cancer, and may lead to methemoglobinemia in infants. This condition is also known as blue baby syndrome, and is caused by the reduced ability of the blood cells to carry oxygen to the tissues, leading to difficulty in breathing, the risk of suffocation, or even death (Guay, 2009; Sanchez-Echaniz, Benito-Fernández, & Mintegui-Raso, 2001). Approximately 80 % of dietary nitrates are derived from the consumption of vegetables; other sources of nitrates include fruits and processed meats (Hord, Tang, & Bryan, 2009). Impregnating spinach with sucrose using VI drastically decreased the concentration of nitrates (**Paper III**). Impregnation with low amounts of sucrose (5 g/100 ml) could potentially be used in the food industry to reduce the high nitrate content in spinach leaves (around 5 mmol/kg DM) to improve their quality. Exogenously supplied sucrose by VI reduced the nitrate concentration of the leaves by almost 70 % during 72 h of storage at 8 ºC, compared to non-impregnated leaves (Figure 6). An initial decrease in nitrates was observed already within 12 h of storage.

Exogenously supplied sucrose was found to be metabolized during the storage period (Figure 7). This may be the result of the use of sucrose as a substrate for both the metabolization of stored nitrate and for respiration.

Plants have been equipped with a sensing system to control the uptake of nitrates from the soil (Imsande & Touraine, 1994; Krouk, Crawford, Coruzzi, & Tsay, 2010). According to Sheen, Zhou, and Jang (1999), sucrose can act as a signalling molecule that controls the expression of the genes regulating the nitrate assimilation pathway. Morcuende, Krapp, Hurry, and Stitt (1998) also reported that sucrose plays a major role in increasing the nitrate reductase (NR) activity, mimicking the induction of light in the transcription of the NR involved in the nitrate assimilation pathway (Figure 8). In **Paper III**, evidence is provided showing that the supply of sucrose by VI can replace the role played by light, as the amount of nitrate was reduced during storage in the dark. Jang and Sheen (1994) and Cheng, Acedo, Cristinsin, and Conkling (1992) also reported that the expression of the nitrate reductase gene could be achieved by sugar treatment in the dark.
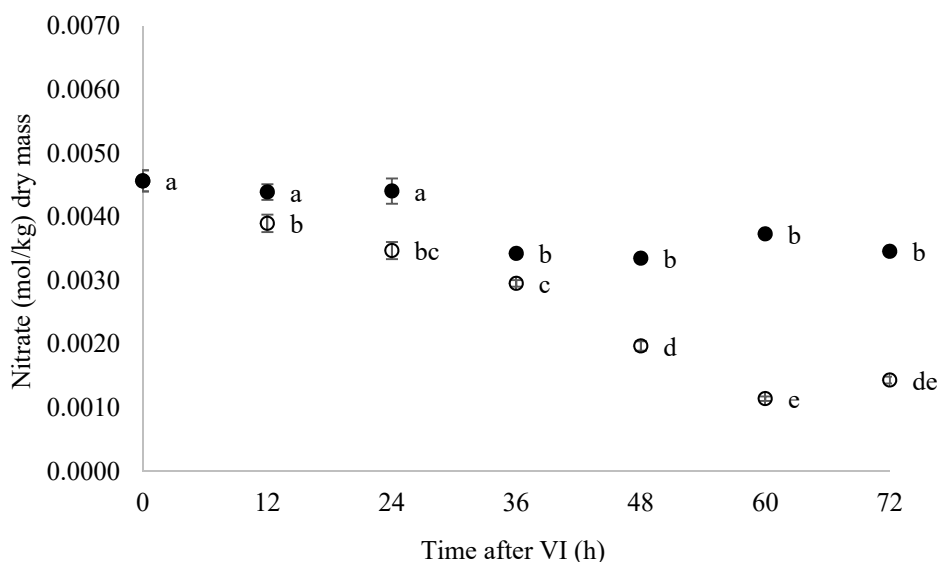


Figure 6. Change in nitrate concentration in baby spinach leaves during storage at 8 °C for up to 72 h. Leaves were either non-impregnated (filled circles) or vacuum impregnated with sucrose solution (empty circles). Statistically significant differences are indicated by different letters above the error bars. Bars represent the standard deviation of the mean of three replicate measurements (corresponds to Figure 2A in Paper III).
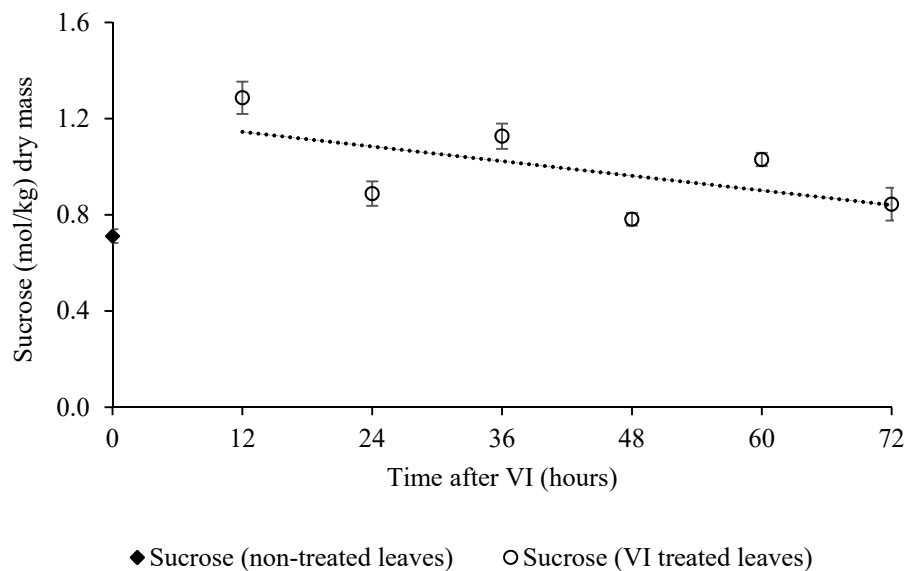
Figure 7. Change in the sucrose concentration in baby spinach leaves during storage at 8 ºC for up to 72 h. Leaves were either non-impregnated (filled circles) or vacuum impregnated with sucrose solution (empty circles). Bars represent the standard deviation of the mean of three replicate measurements (corresponds to Figure 2B in Paper III).

Figure 8. Relationship between the carbon metabolism and nitrate assimilation pathways. The supply of carbon leads to the induction of nitrate reductase activity and the subsequent metabolization of nitrate in plant tissue (Taiz et al., 2014). The abbreviations for the enzyme involves are: nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamine oxoglutarate aminotransferase (GOGAT) and amino transferase (AT) (corresponds to Figure 1 in Paper III).

# 5.2 Effect of impregnation on gas composition in packaging

Consumer demand for fresh fruit and vegetables coupled with the demand for convenience has triggered the necessity of minimally processed products. Minimally processed fruit and vegetables retain attributes and quality similar to those of the fresh produce (Alzamora, López-Malo, & Tapia, 2000). MAP is often used to complement refrigeration in order to ensure long storage life and high quality (Ahvenainen, 1996; Barry-Ryan & O'Beirne, 1997). Deterioration is usually caused by microbial spoilage, physiological ageing, biochemical changes and loss of nutritional quality. Several techniques have been developed through the years with the aim of maintaining the fresh-like characteristics of packed vegetables for a longer period on the shelf. These methods have targeted the growth of microorganisms that would potentially cause deterioration of the product, or the metabolic activity of the product in order to influence the respiration rate (see review by (Ma, Zhang, Bhandari, & Gao, 2017). **Paper IV** describes a study of the application of VI of baby spinach using different substances as a means of influencing the metabolic activity of the leaves during MAP.

The spinach leaves were impregnated with substances that are commonly used in the food industry, e.g. sucrose, calcium lactate and ascorbic acid. The leaves were then packed in micro-perforated polypropylene bags and stored at two different temperatures, 5 ºC and 21 ºC, for 4 days. Calorimetric measurements of metabolic activity after impregnation were also performed at both temperatures. The results showed that VI causes significant changes in the gross metabolic activity of the impregnated spinach leaves, depending on the type of substance used and the treatment temperature. Sucrose led to the highest metabolic heat production at 21 ºC, whereas spinach impregnated with calcium lactate exhibited the highest metabolic activity at 5 ºC. A significant increase in gross metabolic activity was observed in ascorbic acid-impregnated leaves stored at 21 ºC, but not at 5 ºC (Figure 9).
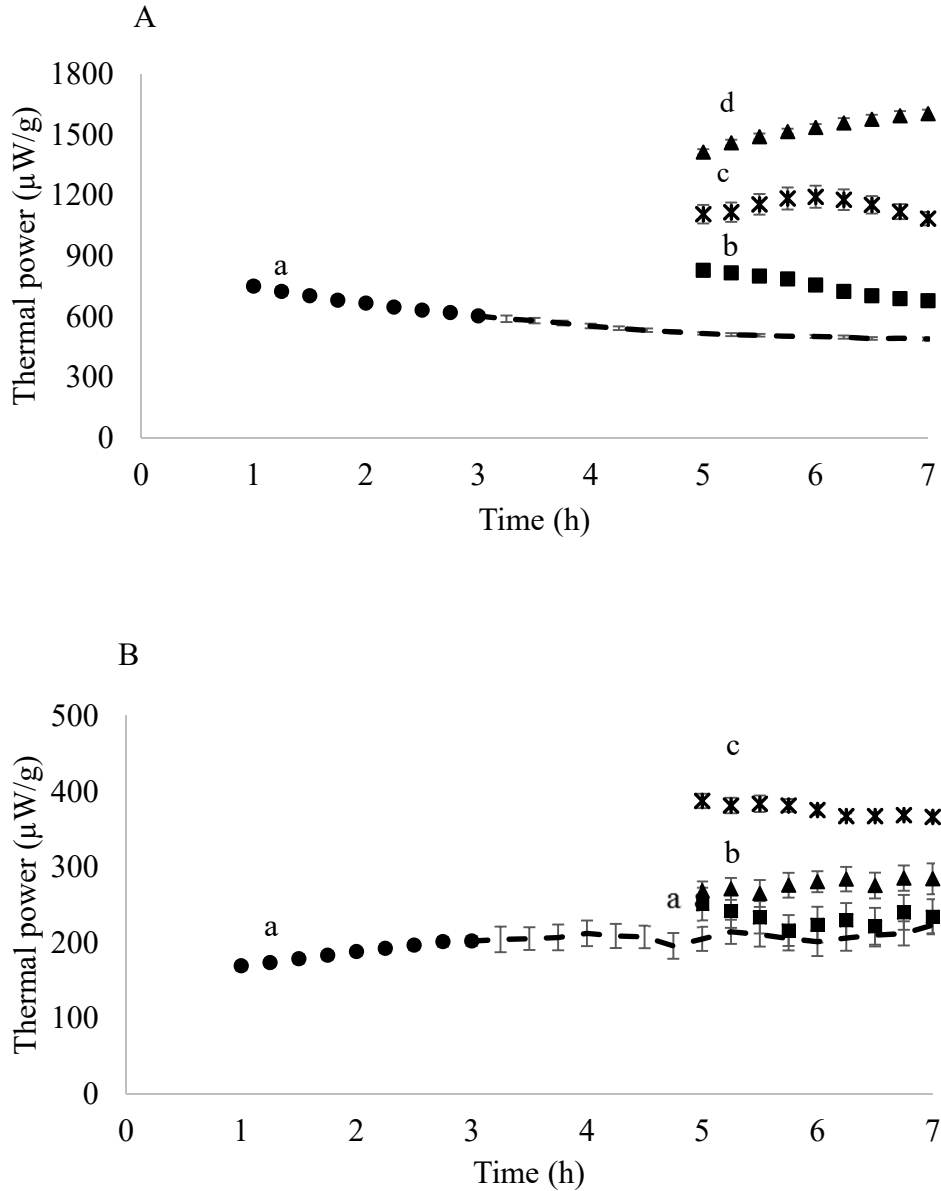
Figure 9. Calorimetric measurements of the metabolic thermal power of untreated spinach leaves (●) and spinach leaves treated with VI: 0.6 mol/l sucrose (pH 5.8) (▲), 50 mmol/l calcium lactate (pH 7.1) (x) and 5.7 mmol/l ascorbic acid (pH 3.2) (■) at 21 ºC (A), and 5 ºC (B). In a separate experiment, the signal from the untreated spinach leaves (dashed line) was recorded for six consecutive hours. Statistically significant differences between the different curves ($p < 0.05$) are indicated by different letters above the recorded thermal power curves. Bars represent ± 1 standard error of the mean of four replicates measurements (corresponds to Figure 1 in Paper IV).

Impregnation with sucrose had a significant influence on the atmosphere inside the MAP during storage of the spinach at both temperatures studied (Figure 10). The high metabolic activity of the spinach leaves resulting from impregnation with sucrose reflected the high oxygen consumption and carbon dioxide production measured in the packaged product stored at 21 ºC. However, at 5 ºC, lactate led to the highest gross metabolic activity, but this result was not reflected by the change in atmosphere inside the packaging. The discrepancy between the calorimetric and the atmospheric findings may be the result of the different time scales of the measurements. Calorimetric measurements were only made for a few hours after impregnation, whereas the first measurement of atmospheric composition in the packaging was made after 24 h. The preliminary results presented in **Paper IV** may be beneficial to the food industry for gaining a better understanding of how the metabolic activity of impregnated leaves affects the atmosphere inside the packages. These studies should, in the future, be complemented with determination of shelf life where correlations between the respiration of the packed, impregnated product and quality during storage may be established.
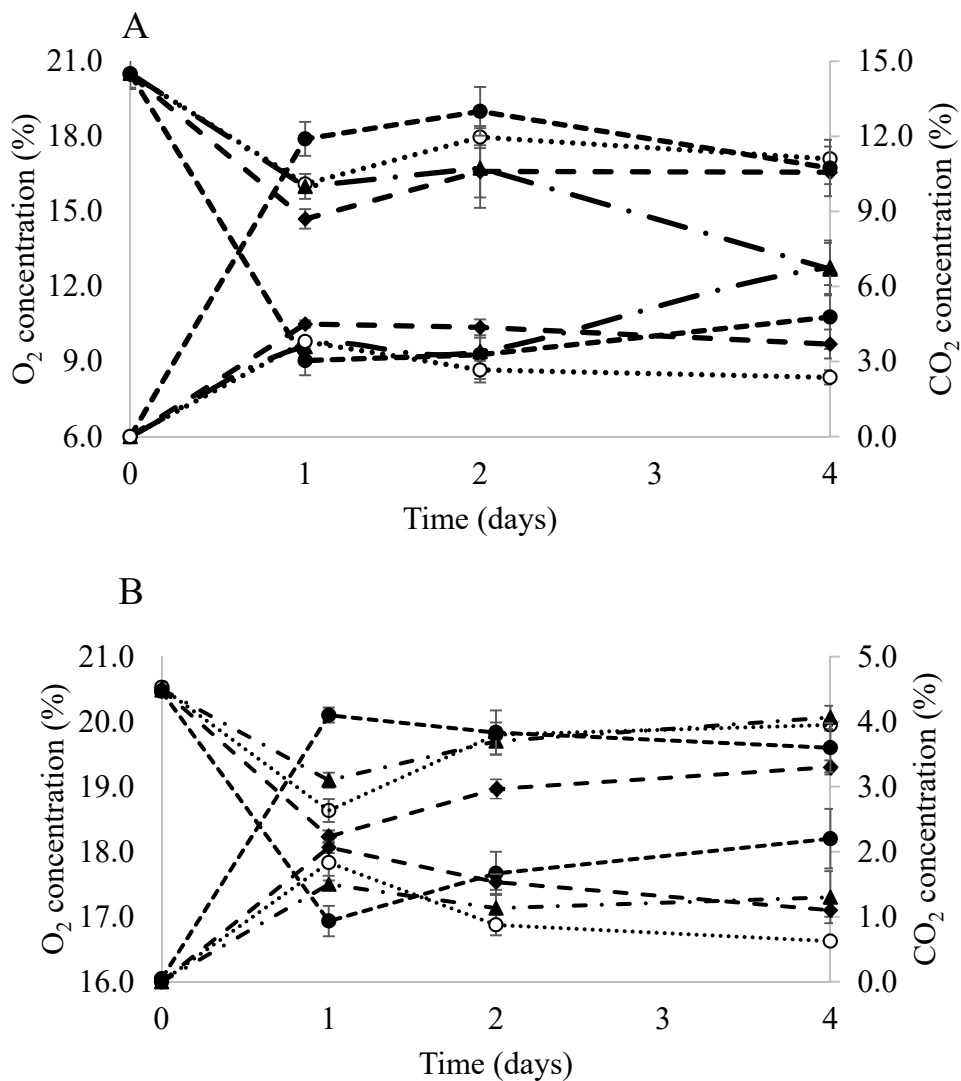
Figure 10. Changes in the proportions of oxygen (primary axis) and carbon dioxide (secondary axis) in MAP of untreated baby spinach leaves (o) and baby spinach leaves treated with VI: 0.6 mol/l sucrose solution (pH 5.8) (●), 50 mmol/l calcium lactate (pH 7.1) (♦) and 5.7 mmol/l ascorbic acid (pH 3.2) (▲), when stored at 21 ºC (A) and 5 ºC (B) for 4 days. Curves starting at 21 % indicate $O_2$ and curves starting at 0 % indicate $CO_2$. Bars represent ± 1 standard error of the mean of three replicates measurements (corresponds to Figure 2 in Paper IV).

# 6. Conclusions

The results presented in this thesis reveal the influence of VI on metabolic response in spinach leaves, depending on the type of impregnation substance in the extracellular space and the treatment temperature. These VI-induced metabolic changes took place within hours and days after treatment. The most important conclusions are presented below.

- VI of plant tissues affects not only the mass transfer in, functionality and structure of plants, but also the metabolism of metabolically active cells in the impregnated tissue (**Paper I**).

- Introducing different substances into baby spinach leaves by VI resulted in different effects on the short-term gross metabolic activity, measured with isothermal calorimetry at 5 ºC and 21 ºC. Sucrose and calcium lactate resulted in rapid increases in the gross metabolic activity of the leaves, while ascorbic acid caused an increase only at 21 ºC (**Papers II** and **IV**).

- The changes in sugar and starch concentrations were dependent on the impregnation substance. The total sugar concentration is likely to be the result of the balance between the metabolization of sugars and the mobilization of starch. The concentration of starch in the impregnated leaves decreased during several days of cold storage in the dark. The observed effects of impregnation with different substances are likely to be the consequence of their metabolization by the cells in the tissue (**Paper II**).

- Sucrose supplied by VI significantly reduced the nitrate content in baby spinach leaves. Sucrose was used as a substrate to metabolize the stored nitrate during cold storage in the dark. This method could potentially be useful in the food industry to reduce the high nitrate content in spinach leaves, and thus improve their nutritional quality (**Paper III**).

- Impregnation with sucrose plays a major role in the atmosphere created inside modified atmosphere packaged spinach during storage at 5 ºC and 21 ºC. Among the substances investigated, sucrose-impregnated leaves showed the highest $O_2$ consumption and $CO_2$ production (**Paper IV**).

# 7. Future outlook

The work described in this thesis sheds new light on the metabolic consequences of VI. However, a number of important areas need to be studied in the future.

- Detailed studies should be carried out on the effects of impregnation on the shelf life of baby spinach leaves. These could include microbiological studies and investigations on the changes in the quality of packed spinach leaves, for example, electrolyte leakage as an indicator of membrane deterioration, colour and chlorophyll changes. The overall quality of the impregnated leaves should be investigated by sensory evaluation studies.

- Further investigation is needed on changes in other important nutritional factors such as ascorbic acid. The use of redox markers may provide information on the change in ascorbate, and/or the ascorbate-to-dehydroascorbate ratio.

- Metabolic profiling (metabolomics) of impregnated tissues should be carried out in order to obtain a general overview of the metabolic pathways that may be affected by VI.

- Studies on the dynamics of the impregnation process in the tissue could be performed using X-ray microtomography. *In situ* visualization of the impregnation of plant tissue by a solution in real time may be possible with 4D microCT technology.

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# Paper I

# Mini-review

# New insights into the dynamics of vacuum impregnation of plant tissues and its metabolic consequences

## Federico Gómez Galindo* and Noor Liyana Yusof

## Abstract

The complex and highly interconnected intercellular air spaces of plant tissues occupied by gas or native liquid has offered the possibility for impregnation with a wide range of compounds. In food processing, the development of vacuum impregnation has allowed a controlled way to introduce these compounds to the tissue structure aiming at modifying structural, nutritional, and/or functional properties as well as improving the processability of fruits and vegetables. In the last 10 years, more than 100 research articles have been published on the topic and significant insights had been gained including improved understanding of mechanisms for mass transfer as well as the development of new, fascinating industrial applications. In the recent years, our knowledge on these aspects has increased by bringing new exploration technologies for studying the impregnation of porous materials and plant cell physiology approaches to bear on the topic. The aim of this paper is to highlight some of these exciting advances.
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**Keywords:** mass transfer; GASMAS; metabolic consequences; X-ray tomography

## INTRODUCTION

In a vacuum impregnation (VI) process, porous products are immersed in a solution of different compositions and/or concentrations and subjected to a two-step pressure change.[1] In the first step (vacuum step), the product is immersed in a solution and exposed to sub-atmospheric pressure. During this step, the gas of the product pores is expanded and partially flows out until mechanical equilibrium is achieved. When the atmospheric pressure (impregnation step) is restored, the residual air in the pores compresses and the external liquid flows into the pores due to the action of a hydrodynamic mechanism (HDM), described and modelled by Fito[2] and Fito and Pastor.[3] The term 'pulsed vacuum' could be used when the samples are kept inside the solution at atmospheric pressure for a long time after the initial application of a vacuum.[4,5]

The filling of the pores is affected by several variables, which may be regarded as external and internal of foods.[6] External variables comprise the vacuum pressure,[7,8] the time of the vacuum and impregnation steps,[6,9] the viscosity, temperature and osmotic pressure of the external solution.[10,11] Internal variables are the capillary pressure, which depends on pore size and distribution, surface tension of the liquid, and wetting angle between the liquid and the pore walls.[12] As discussed later in this paper, the properties of the intracellular air spaces including the porosity fraction and pore connectivity are key variables in the vacuum impregnation process. Pressure changes may also promote deformation of the product due to the visco-elastic properties of the solid matrix (deformation–relaxation phenomena).[13,14] Other internal variables to consider are the size and shape of the samples[15] as

well as the cut direction of the tissue samples[16] and the extent of cell damage caused by the knife.[9]

Once the external liquid of a certain composition and properties has penetrated into the extracellular space of the tissue, it may affect its structure and mechanical properties[17,18] as well as its composition and/or functional properties, leading to potential industrial applications widely studied in the literature over the years. Applications, extensively studied in the literature, may include pretreatment before drying such as for osmotic dehydration,[2,3,19−21] impregnating cryoprotectants aiming at improving the freezing tolerance of the impregnated tissue,[22−24] the development of compositionally formulated products for extending their shelf life,[25,26] improving the pH reduction of vegetables,[4,5] texture enhancement[18,27−29] or developing nutritionally fortified products.[15,30−34] These applications have been covered in review articles elsewhere.[6,10]

This review focuses primarily on the newest findings targeting research needs on the mechanisms involved during impregnation as well as its consequences for the metabolically active cells in the tissue that is impregnated. Research focused on improving our understanding of the impregnation process dynamics as well as how the impregnated substances may affect the cells in the tissue

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are key issues regarding process optimisation, quality, composition and shelf life.

## THE DYNAMICS OF VACUUM IMPREGNATION

According to the HDM model (a model for the equilibrium state in VI processes in porous foods), the extent of pore filling depends on accessible sample porosity, sample dimensional changes and the ratio of applied vacuum to the sum of ambient pressure and Laplace pressure in pores.[2,3,13] The Laplace pressure depends on the wetting properties of the air space and on pore size and form and, therefore, is not constant through the tissue.

The complicated network of highly interconnected intercellular air spaces consisting of tortuous paths and clusters contributes to both anisotropy and heterogeneity of the tissue. Tylewicz et al.[11] suggested that the flow of the impregnated liquid in the tissue is strongly influenced by the topology and geometry of this network. Using gas in scattering media absorption spectroscopy (GASMAS), these authors found that apples in which air was not totally exhausted during impregnation keep an internal reduced pressure which rises slowly towards ambient temperature over a time scale of hours after the operation is terminated. This finding suggests that, in the case of apple parenchyma, the interconnected air spaces expose at least in part an essentially hydrophobic surface and the Laplace pressure term in Fito's model can be locally negative. The liquid flow will be arrested if the liquid interface arrives to a pore so narrow that the driving pressure would not overcome the capillary pressure. Therefore, pressure equilibration can only be achieved either by gas diffusion in gas phase or by gradual wetting of the pores.

During vacuum impregnation, the liquid will convectively penetrate into the pores at a time scale given by pore sizes, the driving pressure difference and liquid viscosity. Fito and Chiralt[35] calculated that the time scale for liquid flooding of apple air space is of the order of 1 s for a low viscosity liquid. This time scale was confirmed by the *in situ* time lapse microscopy results published by Panarese et al.[7] where the impregnation of apple tissue was seen as soon as the restoration of the atmospheric pressure was started. These microscopic observations were also performed in spinach leaves, where impregnation occurred later. The difference between the impregnation of both materials was attributed to their different microstructure, pore size and wetting angle.

Panarese et al.[7] found an interesting discrepancy from the HDM model. In the HDM model, when the system is exposed to the lowest working pressure for a certain period of time, an equilibrium condition is reached and impregnation of pores by the solution occurs. However, microscopic observations during impregnation could not confirm this prediction; instead, impregnation was only detectable during the restoration of atmospheric pressure. As pointed out by the authors, the HDM model assumes that the impregnated solution wets the pores. However, it is possible that the wetting angle of the pores is high because the pores in plant tissues have evolved to allow the diffusion of oxygen and carbon dioxide, which is much faster in gas than in water and, therefore, a high wetting angle could avoid the plant from being 'drowned'.

The above-mentioned observations on the dynamics of VI strongly suggest that impregnation might not be homogeneous in the heterogeneous matrix of a plant tissue sample due to differences on pore size distribution, morphology and porosity. These differences in impregnation have been confirmed by high resolution X-ray microtomography (μCT). Schulze at al.[34] reported

that VI resulted in a higher isotonic solution uptake in the inner apple cortex than in the outer part. Smaller cells and lower intercellular spaces connectivity were attributed to be the cause of the lower impregnation results. Velickova et al.[24] also reported lower impregnation of cryoprotectants in the outer tissue of strawberries where the cells are small and the tissue more compact than in the inner tissues of the fruit. The influence of tissue heterogeneity on impregnation was also observed in spinach leaves in our own observations using μCT (unpublished results), where the edge of the leaf was more difficult to impregnate.

## METABOLIC CONSEQUENCES

Very little attention has been paid to the consequences of VI on the metabolism of the impregnated tissue. This is an important aspect because the product shelf-life is strongly dependent on the tissue metabolic activity. Metabolic consequences of VI might be the consequence of structural modifications provoked by the pressure changes, the impregnated molecules and/or anaerobic stress. The onset of anaerobic metabolism in sucrose-impregnated strawberries was demonstrated after 24 h of storage at 10°C[36] but could not be detected on sucrose-impregnated persimmon 7 days after VI.[37] These results might indicate that the onset of anaerobiosis, at the tested time scales, might depend on the extent of impregnation of the porous structure, which according to μCT observations, is not complete. Schulze et al.[34] did not detect full impregnation of apples and this result was supported by our own measurements on spinach leaves (unpublished) where less that 5% of the leaf tissue reminded unfilled and with visible 'air pockets'. It was this remaining air in the tissue that was still allowing the spinach leaf to continue with photosynthetic activity, although in reduced rates.[34]

There is increasing evidence indicating that when sugars such as sucrose and trehalose are impregnated into the extracellular space by VI, they will be taken up and metabolised by the cells. However, the amount of the impregnated sugars that are metabolised is still unknown. Tylewicz et al.[11] suggested endocytosis as a transport mechanism that might be involved in the uptake of sucrose and trehalose into the cells of impregnated apples. Recent metabolic observations made by Panarese et al.[38] using isothermal calorimetry supported the results reported by Voitsekhovskaya et al.[39] that sucrose loaded into the apoplast could be co-transported with protons into the symplast, as well as the results published by Möller and Beck[40] concluding that exogenously administrated sucrose to *Utrica dioica* was taken up and metabolised by cells rather than cleaved in the apoplast before the uptake. Metabolisation of sucrose may have also caused the drastic increase of gross metabolic activity of impregnated spinach of about 100% reported by Panarese et al.[38] Interestingly, a similar level of metabolic increase was reported after impregnating the leaves with trehalose which might also be taken up by the cells through non-specific transporters. It has been shown, for example, that feeding trehalose to *Arabidopsis thaliana* seedlings could affect sucrose transport, with high levels of trehalose displacing sucrose at the sucrose transporters.[41]

The findings concerning metabolisation of trehalose gain high interest in the light of the cryoprotection application of VI reported by Phoon et al.,[22] where VI with trehalose followed by the application of pulsed electric fields (PEF) significantly improved the freezing tolerance of spinach leaves. This cryoprotection effect was attributed to the uptake of trehalose by the cells. Metabolic consequences of this uptake, such as the possible accumulation of

trehalose-6-phosphate (a powerful signal molecule in plants), are the subject of a current investigation of our research group.

## CONCLUSIONS AND FUTURE TRENDS

Non-destructive structural research techniques such as GASMAS and μCT have improved our understanding of the role of the complex heterogeneous microstructure of porous plant tissues on the impregnation procedure, especially concerning the gradual wetting of the tissue air space influenced by the wetting angle, pore size and possible changes of volume in the impregnated tissue. The next challenge is to use these types of technique not only for making observations after the impregnation has finished but also during impregnation. These measurements would require more sophisticated, expensive setups but would contribute with knowledge that should be incorporated into the existing mathematical models for optimisation of the VI procedure.

Findings pointing to the direction of the metabolisation of the impregnated molecules open a wide range of questions and possibilities. The recent literature, reviewed here, has presented evidence that commonly impregnated substances such as sugars affect the metabolic activity of the cells within short time scales after impregnation. Detailed metabolic consequences, however, still remain to be understood. Metabolic effects might also be true for the impregnation of other substances such as ascorbic acid or L-cysteine, as suggested by the results reported by Rocculi et al.[42] where simple immersion of potato slices in these compounds provoked an increase on their gross metabolic activity and depletion of reducing sugars.

What are the consequences of impregnating any other molecule that has been reported in the literature? This question should remind researchers that vacuum impregnation of plant tissues is not only about mass transfer, functionality and structure but also how we affect the metabolically active cells in the impregnated tissue. The answer to this question may open possibilities for the induction of specific metabolic responses leading to specific quality characteristics.

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**1130**

Paper II

# Influence of Impregnated Substances on the Metabolic Activity and Sugar Metabolism of Spinach Leaves

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**Abstract**

# Abstract

Vacuum impregnation (VI) has been widely used as pre-treatment prior to e.g. minimal processing, freezing, or drying of fruit and vegetables. Most of the investigations have focused on the applicability of VI to modify physico-chemical, sensory, and nutritive characteristics. However, little attention has been paid to the metabolic consequences of impregnating different substances into the plant tissue. This study explores short and long term metabolic responses of baby spinach leaves at 5 °C after impregnation with several substances that are commonly used in food industry e.g. sucrose, calcium lactate, citric acid and ascorbic acid. Short term metabolic response of impregnated spinach leaves was measured with isothermal calorimetry for 7 h. Results demonstrated that leaves impregnated with calcium lactate and sucrose showed a drastic increase in metabolic heat production, but no change was recorded in leaves impregnated with solutions of ascorbic acid or citric acid. Long term metabolic responses were evaluated by measuring sugars and starch. The different impregnation solutes provoked specific changes in the carbohydrate composition during cold storage and the concentrations at each time point are likely to be the result of mobilization of starch, which sharply decreased during storage, and solute-specific differences in metabolization and interconversion of sugars.

# 1. Introduction

Vacuum impregnation (VI) is a unit operation that allows the introduction of solutions into the porous structures of fruit and vegetables. It has been widely used as a pre-treatment method prior to e.g. minimal processing, freezing, or drying of fruit and vegetables (Chiralt et al., 1999). During VI, porous materials are immersed in solutions of different compositions and/or concentrations, and subjected to two step pressure changes. The first step occurs when vacuum is applied to the solid-liquid system, the gas inside the pores expands and the native liquid flows out until mechanical equilibrium is achieved. The second step occurs when the atmospheric pressure is restored, the residual gas in the pores is compressed and the external liquid flows into the pores, replacing the air (Tylewicz, Romani, Widell, & Gómez Galindo, 2013) . Therefore, VI is a controlled way to access the intercellular space and introduce different compounds that modify the structural, functional, or nutritional properties of plant tissues, depending on the type of molecules impregnated (Chiralt et al., 1999).

Application of VI has been extensively studied, particularly with regard to the modification of the physico-chemical properties and sensory attributes of food products (Betoret et al., 2003; Codoñer-Franch et al., 2013; Derossi, De Pilli, & Severini, 2010; Fito et al., 2001; Gras, Vidal, Betoret, Chiralt, & Fito, 2003; Moreno, Bugueño, Velasco, Petzold, & Tabilo-Munizaga, 2004). A significant interest for VI has been received from the food industry since it has the potential to improve a number of issues related to food production and quality. Impregnation of fruit and vegetables with ascorbic acid or citric acid solutions with concentrations ranging from 10 mg/L to 20 g/L has been extensively used in order to inhibit enzymatic browning during storage and reduce microbial growth (Bieganska-Marecik & Czapski, 2007; Blanda et al., 2008; Radziejewska-Kubzdela, Czapski, & Czaczyk, 2007; Shah & Nath, 2008; Yurttas, Moreira, & Castell-Perez, 2014). Similar VI of calcium lactate at concentrations ranging from 0.5 g/L to 2.5 g/L has been shown to improve the texture of minimally processed apples, pears, carrots and lettuce (Alandes, Pérez-Munuera, Llorca, Quiles, & Hernando, 2009; Anino, Salvatori, & Alzamora, 2006; Martín-Diana et al., 2006; Rico et al., 2007), and to enhance the rigidity and brittleness in carrot and eggplant when impregnated together with sucrose (Gras et al., 2003). Luna-Guzmán and Barrett (2000) showed that dipping fresh cut cantaloupe cylinders in calcium lactate solution significantly increased the firmness throughout cold storage. VI with sucrose is normally used to extend shelf life by reducing the water activity (Moreno et al.,

2004; Moreno, Chiralt, Escriche, & Serra, 2000; Perez-Cabrera, Chafer, Chiralt, & Gonzalez-Martinez, 2011), as well as to shorten dehydration time (Pallas, Pegg, & Kerr, 2013).

To the best of our knowledge, little attention has been paid to the metabolic response of the plant tissue once different substances are impregnated into the structure. However, recent findings pointing to the direction of the metabolisation of the impregnated molecules open a wide range of questions and possibilities. Panarese et al. (2014) presented evidence that substances that are commonly used for VI, such as sugars affect the metabolic activity of the cells within short time scales after the treatment. Detailed metabolic consequences, however, still remain to be understood. Metabolic effects might also be true for the impregnation of other substances such as ascorbic acid, as suggested by the results reported by Rocculi et al. (2007) where simple immersion of potato slices in this compound provoked an increase on their gross metabolic activity and depletion of reducing sugars. As pointed out by Gómez Galindo and Yusof (2014), VI of plant tissues is not only about mass transfer, quality attributes and structure but also how the metabolically active cells in the impregnated tissue may be affected.

The main purpose of this study was to explore the metabolic consequences of impregnating different substances, commonly used in the food industry such as sucrose, calcium lactate, citric acid and ascorbic acid, into baby spinach leaves. Short term metabolic response was evaluated by measuring the metabolic heat production using isothermal calorimetry. Calorimetrically measured heat production rates are proportional to metabolic activity (Wadsö & Gómez Galindo, 2009). Long term metabolic response was evaluated by starch and sugar analysis during four days of cold storage at 5 ºC.

# 2. Material and Methods

## 2.1 Plant Material

Baby spinach leaves (*Spinacia oleracea* cv. Misano F1) were grown in a greenhouse with 16 h of light at 20 °C during day and night times. The greenhouse lamps were 400 W metal halide lamps with a photosynthethic photon flux of 100 μmol/m$^2$/s. Two seeds were sowed 1.5 cm below the soil surface and 4.0 cm apart from each other. The dimension of plants growing tray was 54 cm x 32 cm and there were 42 spinach plants grown in each tray. The spinach trays were watered every second day. Leaves from five weeks old spinach were harvested. At the time of harvesting, the length of each leaf blade was $7.0 \pm 0.1$ cm with $2.0 \pm 0.1$ cm petiole and the width at the center of the leaf was $3.0 \pm 0.3$ cm. Leaves were harvested at 10 am, which was 4 h after the start of the light period and only the non-shaded leaves were used. Leaves were harvested randomly from plants located in 5 trays, placed into sealed plastic bags and transported to the laboratory within 10 min. For each experimental replication, the harvesting of the leaves was done from 5 new trays where the plants were 5 weeks old.

## 2.2 Solutions

An isotonic sucrose solution 0.6 mol/l (pH 5.8) in equilibrium with the spinach leaves was designed with respect to the cell sap. The isotonic solution concentration was determined by immersing three spinach leaves in a series of solutions with different concentrations. The variation of tissue weight was recorded every hour until equilibrium. Ascorbic acid and citric acid solutions were prepared at 5.7 mmol/l (pH 3.2) and 1.0 mmol/l (pH 3.2) respectively. Calcium lactate solution was prepared at 50 mmol/l (pH 7.1). These concentrations were chosen based on the most commonly used concentrations for vacuum impregnation (VI) of fruits and vegetables (Bieganska-Marecik & Czapski, 2007; Radziejewska-Kubzdela et al., 2014; Yurttas et al., 2014). The chosen concentrations did not noticeably change the taste of the leaves immediately after VI. The impregnating solutions were kept at 5 ºC before the VI treatment took place.

## 2.3 Vacuum Impregnation

Four leaves ($3.8 \pm 0.1$ g) were submerged in the solutions and immediately subjected to VI, which was carried out in a chamber connected to a vacuum controller (SIA, Bologna, Italy) and a vacuum pump, as described by Panarese, Dejmek, Rocculi, and Galindo (2013). The chamber was covered with aluminum foil to keep dark condition during VI. The setup was placed in a temperature controlled room so that the impregnation was carried out at $5.0 \pm 0.1$ ºC.

Based on preliminary experiments, to establish maximum weight gain and avoid tissue damage, a protocol with a minimum absolute pressure of 150 mbar was chosen. The chosen pressure profile ensured that the cell viability was maintained (cell viability after VI was verified by vital staining with fluorescein diacetate (FDA) as described by Phoon, Gómez Galindo, Vicente, and Dejmek (2008)). During the first phase of VI, the pressure was gradually decreased from 1000 mbar to 150 mbar in 11 min and was kept at 150 mbar for 1 min. During the second phase, vacuum was released and the pressure progressively increased to atmospheric pressure during 7 min and was kept at atmospheric pressure for 13 min. The total treatment time was 32 min and this cycle was repeated twice. After VI, the excess solution on the surface of the spinach leaves was removed with tissue paper and the leaves were immediately transferred to calorimetry ampoules.

## 2.4 Short term metabolic response: isothermal calorimetry measurements of heat production

The calorimetric measurements were performed with a two-channel isothermal calorimeter (Biocal 2000, Calmetrix Inc., USA), where each calorimeter is equipped with its own reference cell. The reference cell was made up of aluminum (91.0 g). The primary output from the heat flow sensors in the calorimeter (voltage) was recorded every minute by a computer. The corresponding thermal powers (heat production rates) were calculated according to Eq. 1.

$$P = \varepsilon \frac{(Vs - Vbl)}{m} \qquad \text{(Eq. 1)}$$

Where $P$ is the specific thermal power of the spinach sample ($\mu W \, g^{-1}$), $\varepsilon$ is the calibration coefficient of the calorimeter ($\mu W \mu V^{-1}$), $V_s$ the voltage signal from the calorimeter ($\mu V$), $V_{bl}$

the voltage recorded for the baseline (µW), and $m$ is the mass of the sample (g). Baselines were recorded before every new measurement.

Isothermal calorimetry measurements were performed at 5.0 ºC. No condensation or temperature fluctuations were registered in the equipment as it was placed in the cold room at $5.0 \pm 0.1$ ºC. Eight leaves ($7.8 \pm 0.1$ g) were placed in a 1.1 L closed plastic container with wet tissue on the bottom for 3 h at $5.0 \pm 0.1$ ºC in the darkness. After this incubation period, the leaves were subjected to two treatments, described below. Four replications were made for each of the impregnated solutions and the control.

i) The leaves were placed into sealed 125 ml plastic ampoules which were placed in the calorimeter. After the initial disturbance, the signal was recorded for two hours. They were then removed from the ampoule and VI was applied. The leaves were subsequently placed back in the ampoule and the calorimeter. After the initial disturbance, the signal was recorded for two more hours.

ii) Untreated leaves (control) were placed in the ampoule and, after the initial disturbance, the signal was recorded for two hours. They were removed from the ampoule for 64 min but VI was not applied; instead, the leaves were placed in a container with saturated atmosphere. The leaves were subsequently placed back in the ampoule and the calorimeter. After the initial disturbance, the signal was recorded for two more hours.

In a separate experiment, the signal from the untreated leaves was recorded for six consecutive hours.

## 2.5 Long term metabolic effects: sugar, starch and colour analysis

The untreated and impregnated spinach leaves were placed in 1.1 L closed plastic containers with wet tissue on the bottom for 4 days at $5.0 \pm 0.1$ ºC in darkness. During these 4 days of cold storage, there were no visual signs of senescence or microbial degradation in the leaves. Samples were taken 15 min, 2 days and 4 days after impregnation for sugar and colour analysis. Starch was analyzed 2 days and 4 days after impregnation. For all analysis, each of the three replications was done by repeating the procedures from the harvesting of the leaves. Three measurements were performed for each time point in each replication.

For sugar analysis, the extraction was done according to the method described by Toledo, Ueda, Imahori, and Ayaki (2003). Of freeze dried spinach tissue, 0.30 g was placed into a reflux tube containing a boiling solution of 20 ml of 99 % ethanol for 10 min and later cooled under

running water. After this, 50 ml 80 % ethanol solution was added, and the extract was evaporated in vacuum at 50 °C. The extracts of spinach tissue and ethanol solution were filtered and centrifuged at 13 000 g for 25 min. The supernatant was analyzed enzymatically for sucrose, D-glucose and D-fructose by using the Megazyme K-SUFRG 06/14 Assay Procedure (Megazyme, Megazyme International, Ireland). The absorbance of the blank and samples was measured at 340 nm using a Varian Cary® 50UV–Vis spectrophotometer (Varian Inc., Santa Clara, CA, USA).

The total starch analysis was carried out according to the method described by Buysse and Merckx (1993) with certain modifications. Of freeze dried spinach tissue, 0.10 g was hydrolyzed with 100 ml of 1 M sulfuric acid at 100 °C for 3 h, with occasional stirring. Of the hydrolyzed sample solution, 1.0 ml was pipetted into a test tube and 1.0 ml of miliQ water, 0.05 ml of 80 % phenol and 5 ml of concentrated sulfuric acid were added. Tubes were vortexed and incubated at 25 °C in a water bath for 10 min. The absorbance of the blank and samples was measured at 490 nm using a Varian Cary® 50UV–Vis spectrophotometer (Varian Inc., Santa Clara, CA, USA). The standard curve was prepared by diluting a glucose stock solution (20 g/L) in deionized water to obtain solutions with concentrations ranging from 0 to 500 mg/L. The concentrations were determined against the glucose standard curve and the starch content of the samples were estimated by the glucose equivalent, multiplying the concentration with a 0.9 factor (Nielsen, 2010). Knowing that before hydrolysis the spinach samples have a mixture of starch and sugars, a separate experiment was designed to evaluate the influence of those sugars on the phenol reaction after acid hydrolysis. Essay tubes were prepared by adding the concentrations of sucrose, glucose, fructose measured enzymatically on each sample and the amount of starch measured after acid hydrolysis of each of the spinach samples (potato starch was used). Other tubes were prepared by adding the same concentrations of sucrose, glucose and fructose as in the first tubes but without starch. All the tubes were subjected to acid hydrolysis and phenol reaction as described above. The difference in absorbance was used to calculate the amount of starch in the samples against the glucose standard curve. For every time point after VI, the concentration of the starch was expressed as relative to the levels at the time of treatment. At least three measurements were performed for each time point.

Color measurements were performed using a spectrophotometer (model CM-700d, Minolta Corporation, Japan) in a room at 5.0 ± 0.1 ºC. The L*, a*, b* values of the spinach leaves were recorded before VI, 15 min, 2 days, and 4 days after VI with each of the studied solutions. Measurements were done on three different points of a leaf. Ten leaves per time point and three

replicates were used for the measurements. The color of the untreated leaves was also measured at the same time points (control).

## 2.6 Statistical Analysis

The statistical significance ($p < 0.05$) of the treatments was tested by means of one-way analysis of variance (ANOVA) using Excel (Microsoft Office, Redmond, WA USA). The Tukey-Kramer multiple comparison test was used to evaluate true differences in treatment means.

# 3. Results

3.1 Short term metabolic responses: Effects of different impregnating solutions on metabolic heat production at 5 ºC

Typical raw calorimetric data are shown in Figure 1. The calorimetric signal was disturbed each time the ampoule was placed in the calorimeter. The signal returned to the recording range after about 1 h.
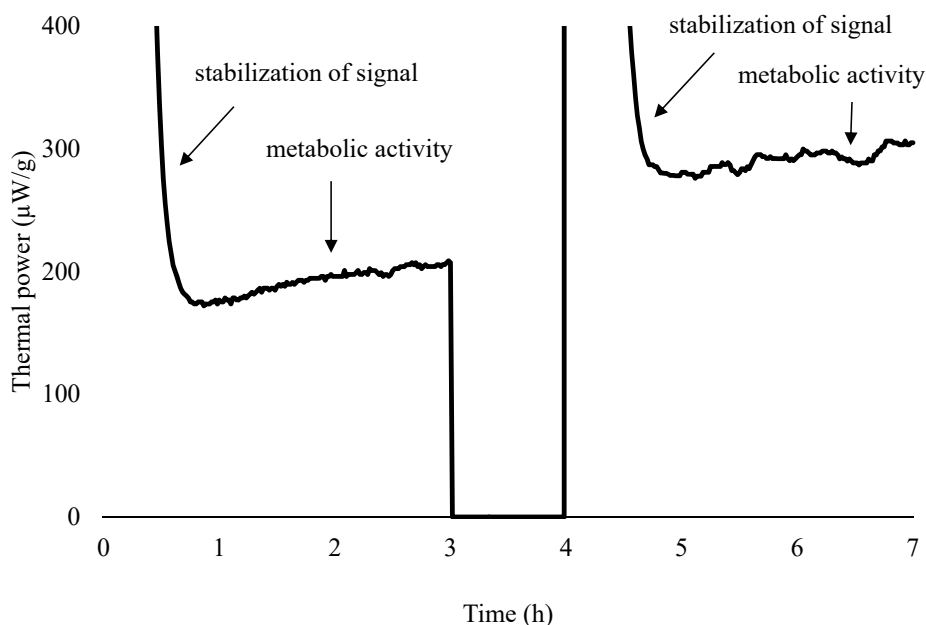


Figure 1. An example of the raw calorimetric data obtained using the following measurement sequence. The leaves were placed in the ampoule for 3 h. The first 1 h was needed to stabilize the signal and the signal from the metabolic activity of the sample was recorded for the next 2 h. The leaves were then removed from the ampoule for 1 h for the VI. The leaves were subsequently placed back in the ampoule and the signal was recorded. Again, the first 1 h is the stabilization of the signal and the metabolic activity of the impregnated sample was recorded for the next 2 h.

The results from the calorimetric measurements on spinach leaves when impregnated with sucrose, calcium lactate, citric acid or ascorbic acid solutions are reported in Figure 2. In this figure, to facilitate the comparison between the treatments, the signals during the 1 h stabilization periods are not shown, and the continuous measurement of the control sample was

10

added (dashed line). Although a transient change on thermal power shorter than 2 h cannot be excluded, a doubling of the thermal power was observed when the leaves were impregnated with calcium lactate solution; an increase in thermal power was also seen for the sucrose solution. No change in thermal power was detected when leaves were impregnated with ascorbic acid or citric acid solutions.
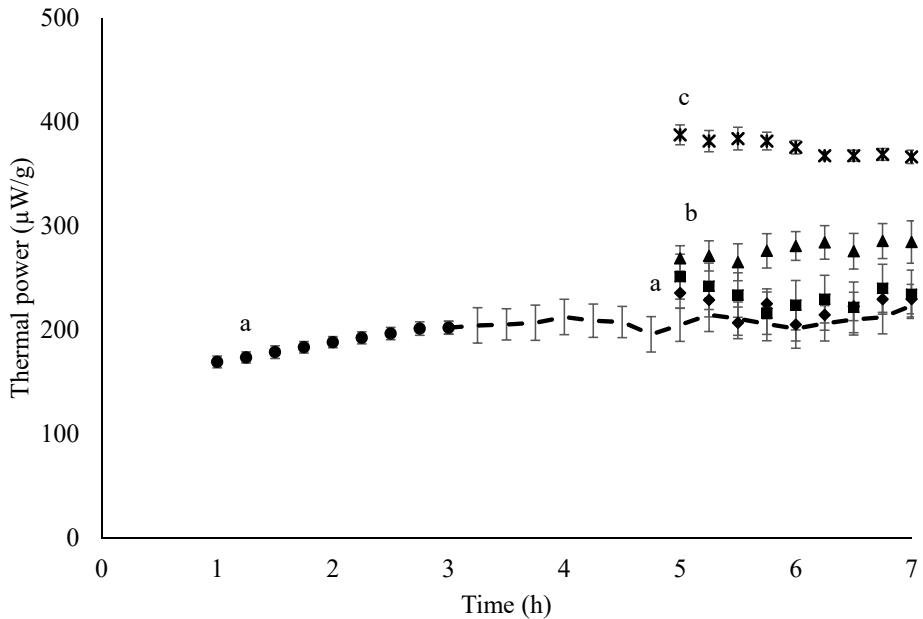


Figure 2. Calorimetric measurements of metabolic thermal power of untreated (•) and VI treated spinach leaves with 50 mmol/l calcium lactate (pH 7.1) **(x)**, 0.6 mol/l sucrose (pH 5.8) (▲), 5.7 mmol/l ascorbic acid (pH 3.2) (■) and 1.0 mmol/l citric acid (pH 3.2) (♦) at 5 °C. In a separate experiment, the signal from the untreated leaves (dashed line) was recorded for six consecutive hours. Statistically significant difference of the different curves (p < 0.05) is represented by different letters above the recorded thermal power curves. Bars represent ± S.E.

## 3.2 Long term metabolic responses: Effects of different impregnating solutes on the concentration of sugars during storage at 5 °C

The effect of impregnating different solutions into spinach leaves during 4 days of cold storage is shown in Figures 3 to 6. Figure 3A shows that the impregnated sucrose is being metabolized at a high rate during the storage period, reaching a final concentration of 260 mg/g (DM) at the end of the storage time. This shows that about 50 % of the exogenously fed sucrose has been metabolized during 4 days of storage. For glucose, the concentration increased

significantly from 36 mg/g (DM) to 70 mg/g (DM) during cold storage, whereas for fructose, no change was observed.

Figure 3B shows the concentration of sucrose, glucose and fructose in the untreated leaves. Over the storage period, sucrose and glucose were significantly decreased, reaching a final concentration of 24 mg/g (DM) and 9 mg/g (DM) respectively. A significant increase of fructose was observed at the end of the storage period, with final concentration of 6 mg/g (DM).
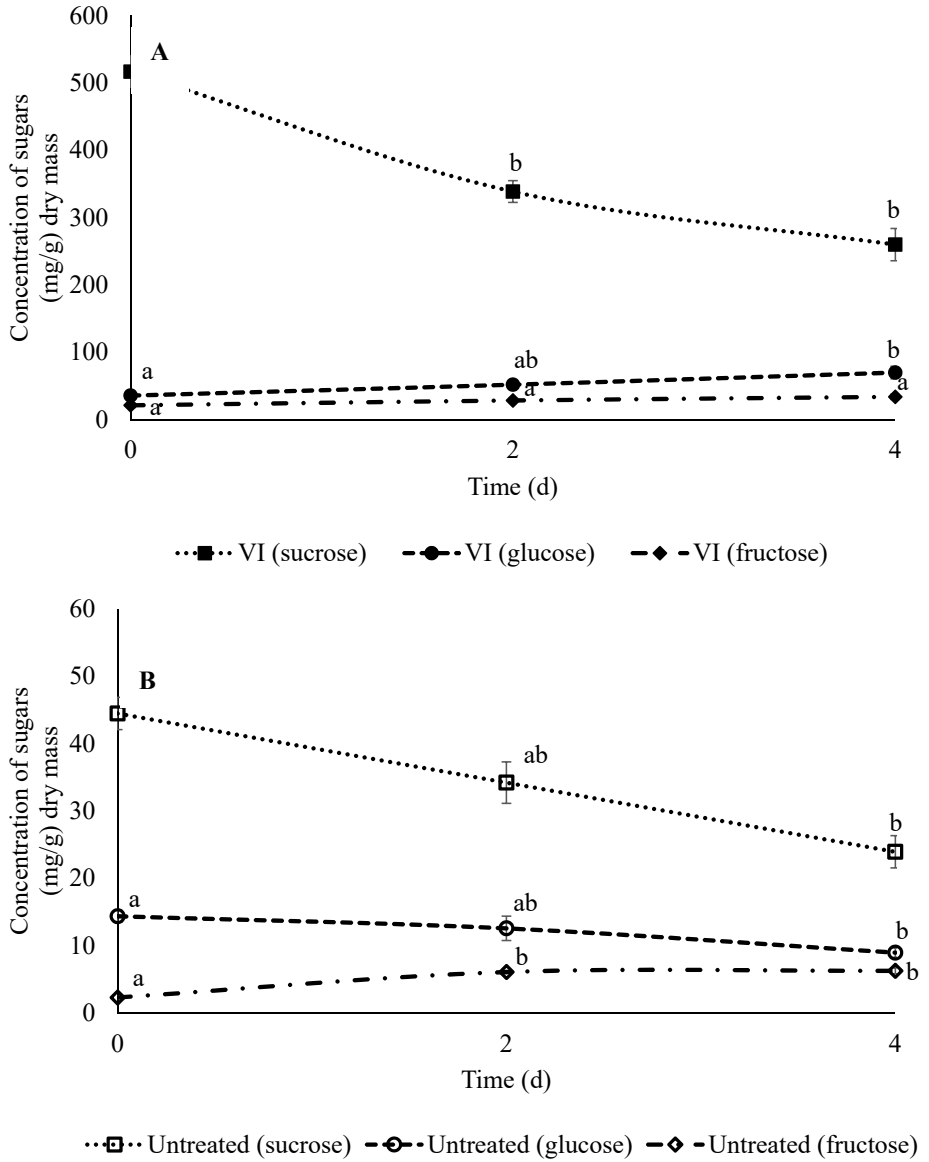
Figure 3. Concentration of sucrose, glucose, and fructose in leaves (A) impregnated with 0.6 mol/l sucrose solution (pH 5.8) (filled symbols) and (B) untreated leaves (empty symbols) during 4 days at 5 ºC. The first point of the VI treated leaves represent the concentration of the sugars 15 min after VI. Statistically significant difference (p < 0.05) within each curve is represented by different letters above the error bars. Bars represent ± S.E.

Figure 4 shows the changes of sucrose, glucose and fructose in leaves impregnated with calcium lactate during the studied storage period in comparison with the control. For the impregnated samples, a significant increase in glucose was detected 15 min after treatment and throughout storage period whereas for fructose, the concentration increased after 2 days of storage. In contrast, in the control samples, sucrose and glucose concentrations showed a steady decrease over the storage period. The concentration of fructose in the calcium lactate impregnated sample increased to a lower extent in comparison with the control leaves.

When leaves were impregnated with citric acid (Figure 5), the concentration of sucrose over the storage period was declining in a way that is comparable to the control. For the VI samples, the concentration of glucose remained unchanged over the storage period, whereas a steady decrease was observed in the control. The concentration of fructose increased over four days of storage.

When leaves were impregnated with ascorbic acid (Figures 6), the concentration of sucrose was unchanged during 4 days of storage. In contrast, there was a steady decrease in sucrose content in the control. For the impregnated leaves, the concentration of glucose was significantly increased after 4 days, in contrast to the steady decrease observed in the control. The changes in the concentration of fructose in the impregnated leaves were comparable to those of the control samples. However, the concentration increased to a higher extent in the impregnated samples.

Figure 4. Concentration of sucrose, glucose, and fructose in baby spinach leaves impregnated with 50 mmol/l calcium lactate (filled symbols) and untreated leaves (empty symbols) during 4 days at 5 ºC. The first point for the VI treated leaves represents the concentration of the sugars 15 min after VI. Statistically significant difference ($p < 0.05$) within each curve is represented by different letters above the error bars. Bars represent ± S.E.
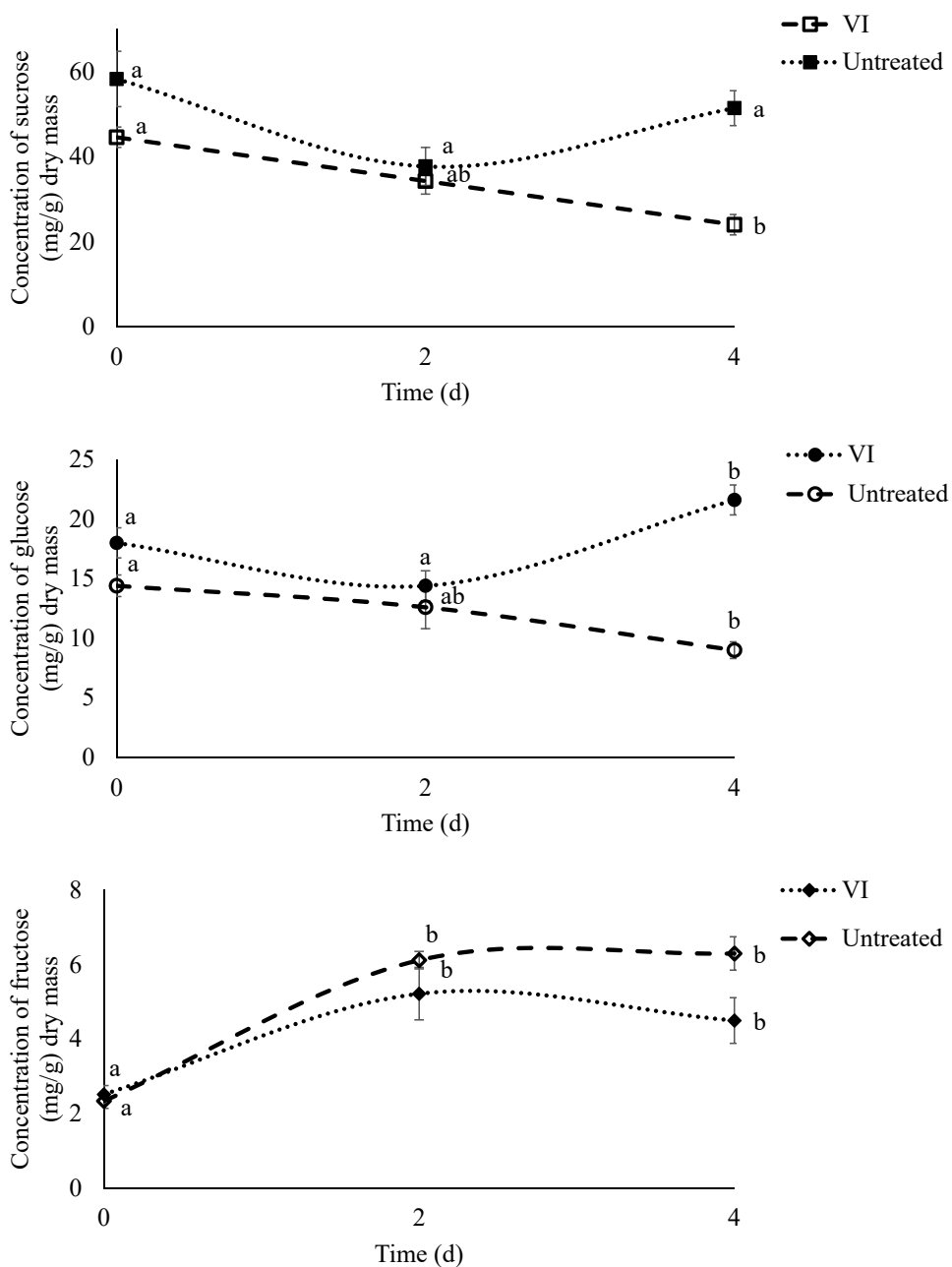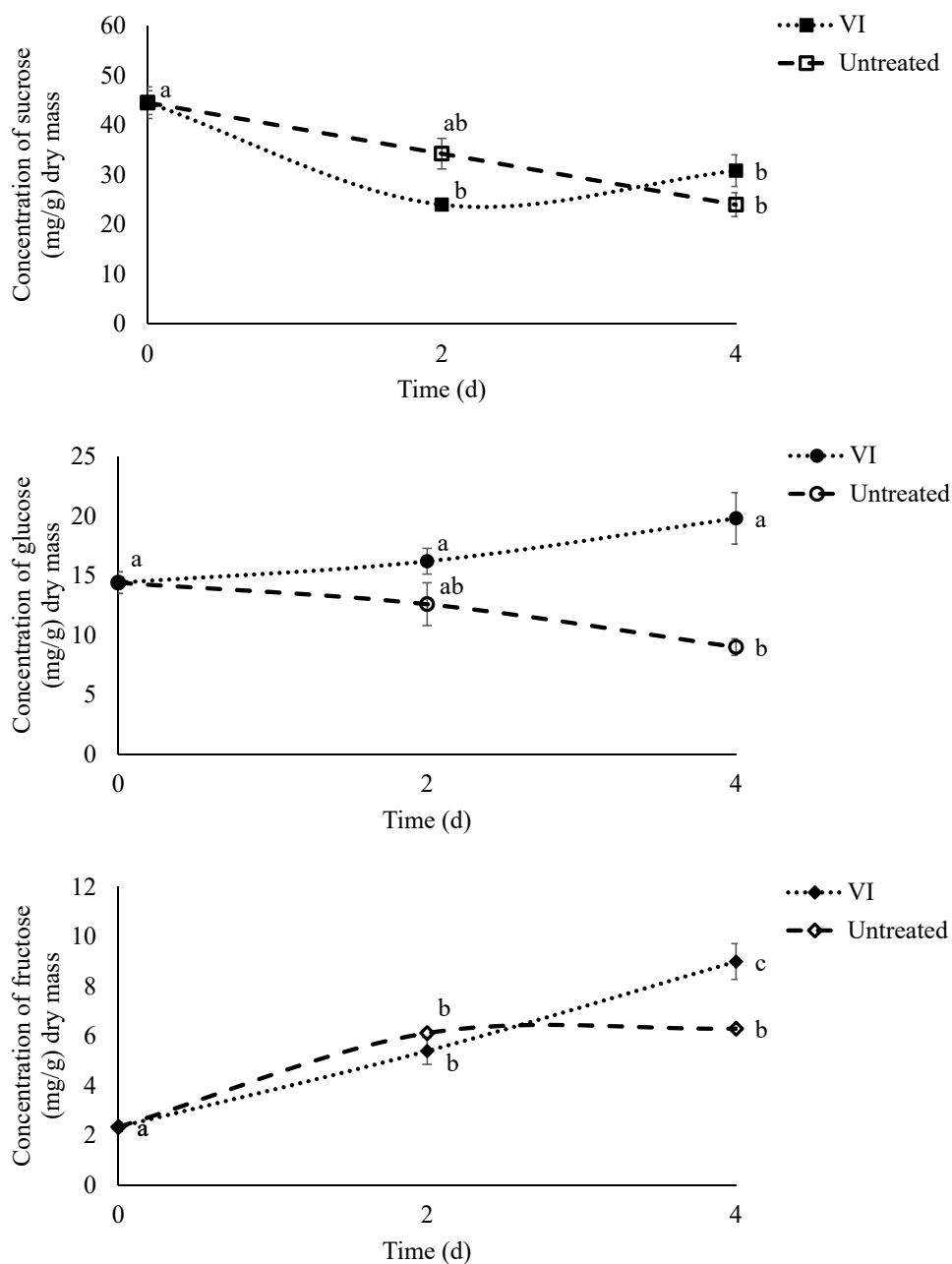
Figure 5. Concentration of sucrose, glucose, and fructose in baby spinach leaves impregnated with 1.0 mmol/l citric acid (pH 3.2) (filled symbols) and untreated leaves (empty symbols) during 4 days at 5 ºC. The first point of the VI treated leaves represent the concentration of the sugars 15 min after VI. Statistically significant difference ($p < 0.05$) within each curve is represented by different letters above the error bars. Bars represent ± S.E.

Figure 6. Concentration of sucrose, glucose, and fructose in baby spinach leaves impregnated with 5.7 mmol/l ascorbic acid (pH 3.2) (filled symbols) and untreated leaves (empty symbols) during 4 days at 5 ºC. The first point of the VI treated leaves represent the concentration of the sugars 15 min after VI. Statistically significant difference ($p < 0.05$) within each curve is represented by different letters above the error bars. Bars represent ± S.E.
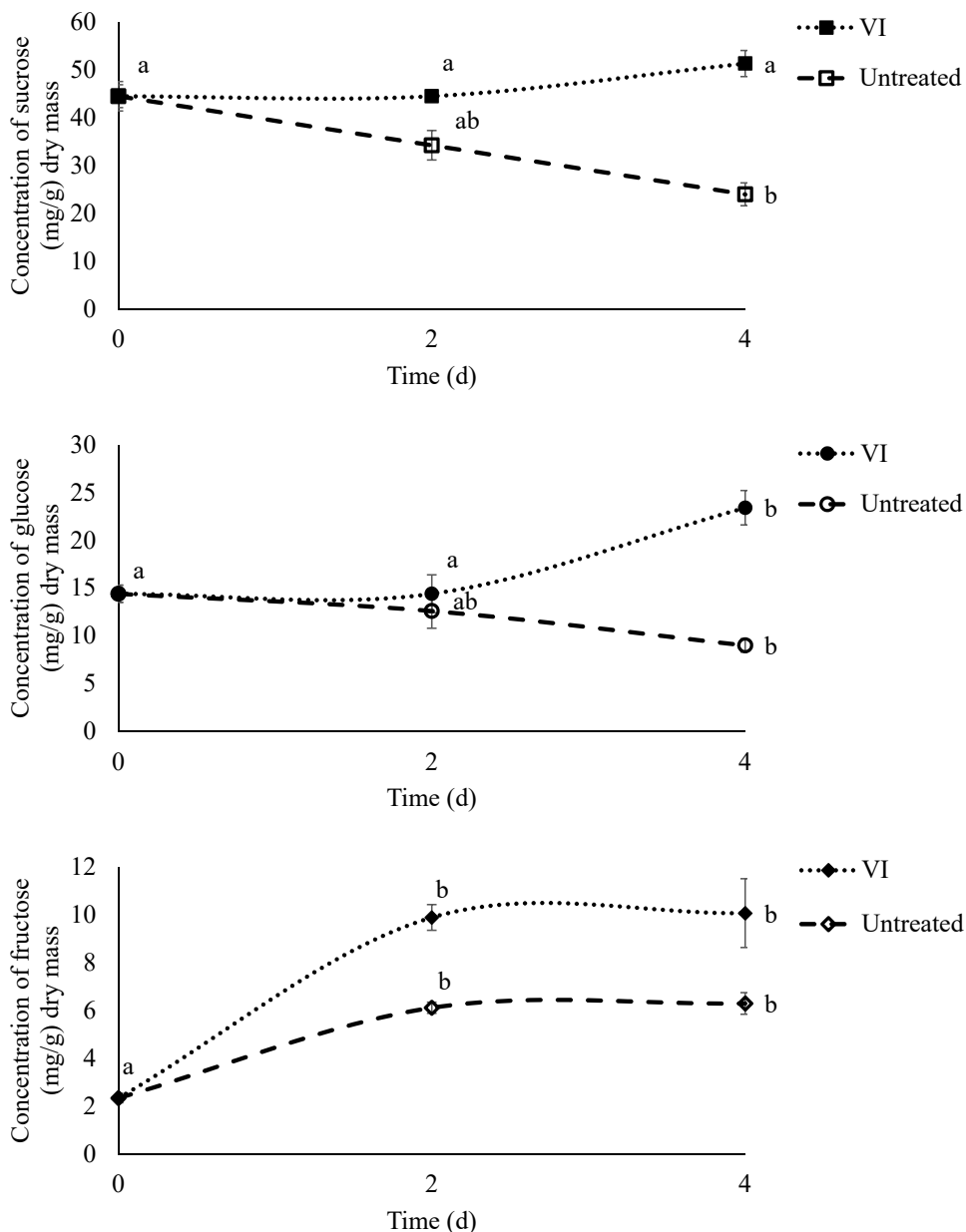
## 3.3 Long term metabolic responses: Effects of different impregnating solutes on starch concentration during storage at 5 ºC

To account for an observed high variability in the initial concentration of starch in the leaves used, the concentration of starch during storage after impregnation with solutions of sucrose, calcium lactate, citric acid and ascorbic acid is reported relative to the start concentration in Figure 7. There was a steady decrease of the starch in the control as well as all impregnated leaves. Among all substances, sucrose-impregnated leaves showed the most drastic decrease after 2 days of storage, with a 63 % starch reduction. In citric acid-impregnated leaves, the concentration of starch decreased about 40 % during the first 2 days, but did not decrease much more between days 2 and 4.
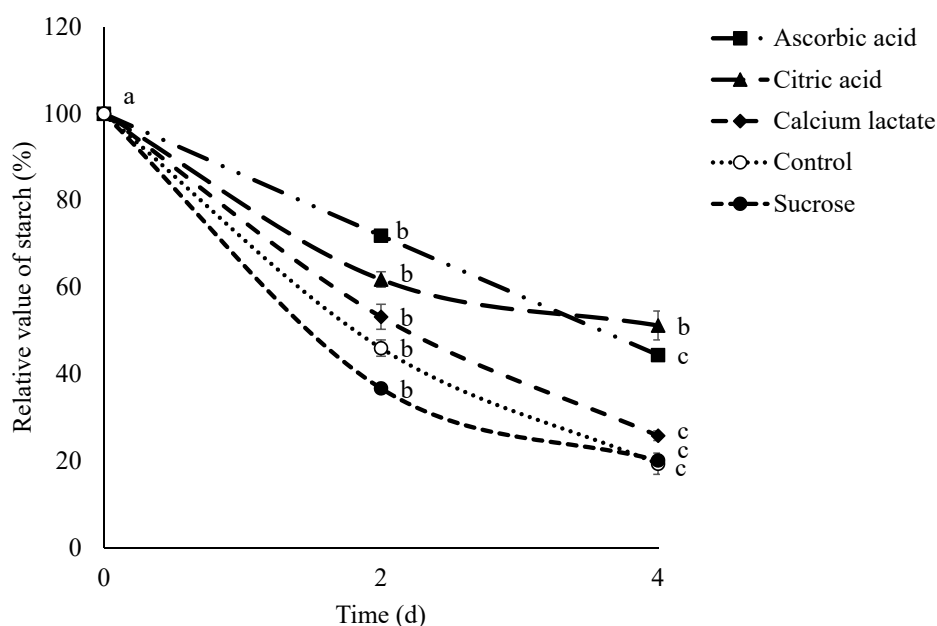


Figure 7. Relative concentration of starch in baby spinach leaves with an initial content of 0.393 ± 0.006 g/kg (DM). The leaves were not impregnated (control), impregnated with sucrose solution 0.6 mol/l (pH 5.8), 50 mmol/l calcium lactate, 1.0 mmol/l citric acid (pH 3.2) and 5.7 mmol/l ascorbic acid (pH 3.2) during 4 days at 5 ºC. Statistically significant difference ($p < 0.05$) within each curve is represented by different letters above the error bars. Bars represent ± S.E.

3.4 Long term quality changes: Effects of different impregnating solutes on leaf color during storage at 5 ºC

Figures 8A-C show the changes of color in spinach leaves impregnated with sucrose, calcium lactate, citric acid or ascorbic acid solution during the studied period in comparison with the non-impregnated leaves. Shortly after VI, the impregnated leaves show darker color (lower L* value) as compared to the control and the greenness of the leaves (a*) significantly increased in all impregnated leaves. The values for yellowness (b*) was generally lower than in the untreated sample, but changes were not significant. After 2 days of storage, the color parameters of the impregnated leaves had reverted back to their original color and impregnated samples and control were similar regarding the measured parameters.
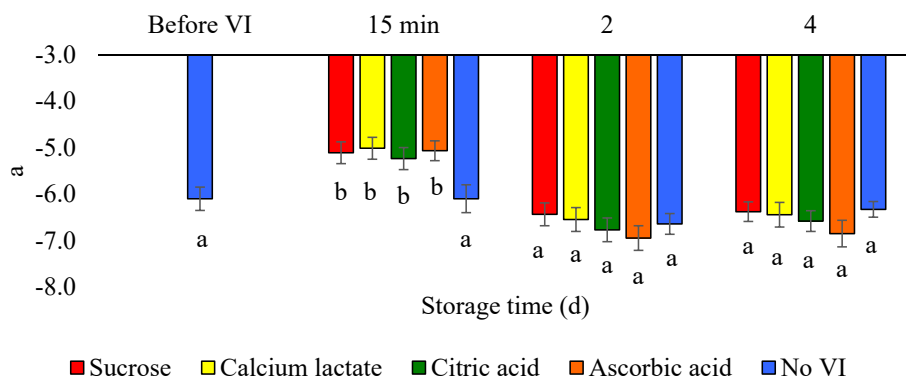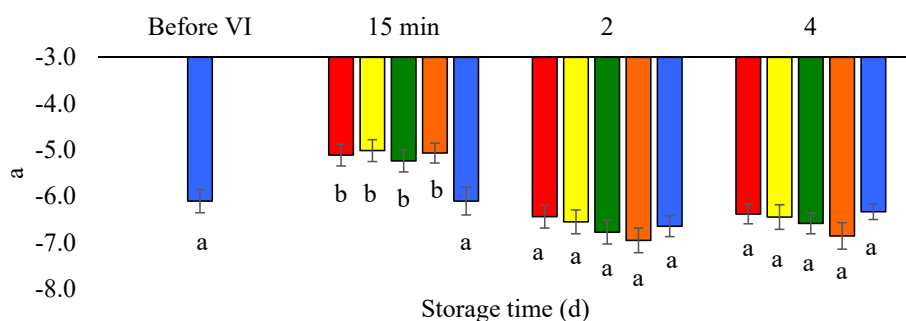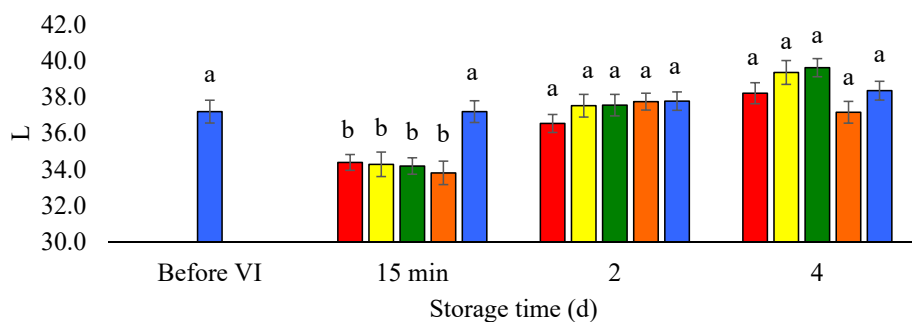
Figure 8. Effect of different impregnating solutions on color changes during storage at 5 ºC. Spinach leaves were either untreated or impregnated with sucrose, calcium lactate, citric acid or ascorbic acid solutions. The leaves were stored for 4 days. Color parameters: (A) L* (from 0 black to 100 white), (b) a* (from –a* green to +a* red), (C) b* (from –b* blue to +b* yellow). Values represent means ± SEM of three replicates. Statistically significant difference (p < 0.05) is represented by different letters above the error bars. Bars represent ± S.E.

# 4. Discussion

A deeper understanding of the metabolic consequences of impregnating different molecules into the structure of a plant tissue is of key importance for product development, since the product composition and shelf-life is strongly dependent on the tissue metabolic activity. In this study, we provide evidence that the different substances impregnated into baby spinach leaves have different effects on the short-term gross metabolic activity of the leaves and provoke different changes to their carbohydrate pools. During storage in the dark, the starch decreases drastically (clearly shown in Figure 7), which was expected as, in nature, starch is mobilized during the night (Zeeman, Smith, & Smith, 2007).

In this study, calorimetric measurements provide evidence of a significant increase in spinach leaf gross metabolism as a consequence of impregnation with sucrose (Figure 2), which is consistent with previous observations by our group (Panarese et al., 2014). In contrast, the gross metabolic activity of the control remains practically unchanged after the first 3 h of measurement. According to Panarese et al. (2014), the sucrose-induced increase might be caused by the metabolization of the impregnated sucrose. Sucrose might be taken up and metabolized by the cells. Sucrose loaded into the apoplast can be co-transported with protons into the symplast, driven by the plasma membrane ATPase, which pumps protons into the apoplast (Voitsekhovskaya et al., 2002). The sucrose can then be stored in the vacuole or directly metabolized into glucose and fructose derivates, before entering respiratory or biosynthetic pathways. The increased metabolic activity observed by isothermal calorimetry may be the cause of the dramatic and sustained decrease in the concentration of the impregnated sucrose and the drastic loss of starch after the impregnation (Figures 3 and 7). The total concentration of the sugars may thus be the final result of the mobilization of starch and the metabolization of sugars occurring during the storage period, whereas the relative changes observed between glucose and fructose concentrations should reflect also interconversions within sugar metabolism.

The gross metabolic activity of the baby spinach leaves increased drastically after impregnation with calcium lactate, accounting to the largest increase in metabolic activity among the studied substances. Externally fed lactate has shown to be rapidly metabolized by spinach leaves and leaves of other species of higher plants such as lettuce and soybean (Betsche, 1983). This lactate metabolization was shown to occur in the light and the dark. A fast, mitochondrial lactate metabolism has also been reported in potato tubers (Paventi, Pizzuto,

Chieppa, & Passarella, 2007). However, the carbon supplied with the lactate represents only a small percentage of the carbon supplied with the sucrose in the sucrose impregnated leaves, but the resulting energy burst is much higher (Figure 2). Therefore, the calorimetry results (Figure 2) suggest that the energy burst caused by the impregnation of calcium lactate is the result of a metabolic stimulation of the respiratory pathway by lactate as an effector rather than as a substrate. The energy burst caused by lactate impregnation may be caused by the mobilization of other carbon sources such as starch. During storage in the dark, mobilization of starch would explain the significant increase in fructose 2 days after impregnation and in glucose 4 days after impregnation. As, in the darkness, glucose is easier metabolized by the leaves than fructose (Sagishima, Kubota, & Ashihara, 1989), the overall effect was an earlier detection of the increase of fructose. However, the steady state concentrations of fructose and glucose will be influenced by glucose to fructose interconversion and glucose and fructose metabolization.

The impregnation of citric and ascorbic acid into the leaves did not provoke an increase of their gross metabolic activity, which is an evaluation of short term metabolic responses (Figure 2). Interestingly, the effect of the impregnation with these acids is only noticeable through the sugar and starch composition analysis, days after the impregnation (Figures 5 and 6). Upon VI with these solutions (at pH 3.2), the leaf's apoplastic pH might have been considerably affected, which may have provoked changes in respiration (Lambers et al., 1998) and/or the alteration of gene expression patterns as reported by Lager et al., 2010, although this study was performed at higher pH values (4.5 to 6.0). As with the other impregnated substances, the measured concentrations of sugars will be the final result of the balance between sugar metabolization, starch mobilization and sugar interconversions, differentially affected by the impregnated solutes.

# 5. Conclusions

This study explores metabolic responses of baby spinach tissue that follows the application of VI with commonly used substances for impregnation of fruit and vegetables. The following are the main results:

i.  Introducing different substances into baby spinach leaves by vacuum impregnation provoked different effects on the short term gross metabolic activity measured with isothermal calorimetry. Sucrose and calcium lactate provoked rapid increases in the gross metabolic activity of the leaves while no change was detected after impregnation of citric or ascorbic acid.

ii.  Different changes in sugar composition (sucrose, glucose and fructose) were detected, depending on the impregnated substance. The total sugar concentrations at each time point is likely the result of the balance between metabolization of the sugars and mobilization of starch, the concentration of which decreases in the impregnated leaves during 4 days of cold storage in darkness.

iii.  The detected effects of the impregnation with different substances are likely to be a consequence of their metabolization by the cells in the tissue.

# Acknowledgements

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# Paper III

CrossMark

ORIGINAL PAPER

# Reduction of the Nitrate Content in Baby Spinach Leaves by Vacuum Impregnation with Sucrose

Noor Liyana Yusof[1,2] · Allan G. Rasmusson[3] · Federico Gómez Galindo[1]

**Abstract** Vacuum impregnation (VI) has been widely utilized as a pre-treatment method prior to, e.g., the minimal processing, freezing, or drying of foods. In many cases, VI has been used to focus on the enrichment of fruits and vegetables with probiotics or micronutrients, texture enhancement, the modification of the sensory attributes, and extension of shelf life by pH reduction. However, little attention has been paid to the exploration of the metabolic consequences of VI that could lead to changes in the quality characteristics of plant tissues. Since nitrate has long been discussed as a compound that is harmful to human health, the aim of this investigation was to reduce the nitrate content in spinach leaves by feeding sucrose into the tissue using VI. The leaves, either non-treated or treated with VI, were stored under saturated humidity conditions and in darkness at 8 °C, for up to 72 h. VI-treated leaves showed a remarkable reduction in nitrate content as compared to the non-treated samples. Upon storage, sucrose was reduced, indicating that this sugar had been respired and had induced metabolization of the stored nitrate. The nitrite content of the treated leaves was unaffected, proving that this toxic compound was not accumulated in the baby spinach leaves upon external sucrose feeding.

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## Introduction

Vacuum impregnation (VI) is a unit operation in which porous materials are immersed in solutions of different compositions and/or concentrations and subjected to two-step pressure changes. The first step occurs when vacuum is applied to the solid–liquid system. During this step, the gas inside the pores expands and flows out until mechanical equilibrium is achieved. The second step occurs when the atmospheric pressure is restored, in which the residual gas in the pores is compressed and the external liquid flows into the pores, replacing the air. VI is, therefore, a controlled way to access the intercellular space and introduce different compounds that improve the structural, functional, or nutritional properties of plant tissues, depending on the type of molecules impregnated (Chiralt et al. 1999; Radziejewska-Kubzdela et al. 2014). However, to the best of our knowledge, little is known about the metabolic consequences that the impregnated substances may provoke in the cells. As remarked by Gómez Galindo and Yusof (2015), findings pointing to the direction of the metabolization of the impregnated molecules open a wide range of questions and possibilities.

Plants are multicellular organisms composed of different cells with specialized functions and equipped with several complex metabolic pathways that play major roles in the plants' development and maintenance. Energy is generated from photosynthesis, a process that converts light and carbon dioxide into sugar and water. The sugar is then used to build all the molecules that form the plant, including proteins, lipids, cellulose, and other macromolecules. To power these metabolic conversions, the sugar is mobilized in the cytosol and enters the metabolic pathways of glycolysis, pentose phosphate

pathway, citric acid cycle, and oxidative phosphorylation, as well as activates genes that modulate cell activities (Cheng et al. 1992; Farrar et al. 2000; Foyer et al. 2000). In glycolysis, sugar is partly oxidized via hexose phosphate and triose phosphate. These molecules can also be directed into the pentose phosphate pathway and oxidized into ribulose-5-phosphate and carbon dioxide (Kruger and von Schaewen 2003). Pyruvate, the end product of glycolysis, enters the second stage of the respiration pathway, namely, the citric acid cycle, which is located in the mitochondrial matrix. The citric acid cycle degrades pyruvate to $CO_2$ and extracts the redox energy for driving the oxidative phosphorylation. However, the 2-oxoglutarate from the citric acid cycle is required as a carbon acceptor for ammonium assimilation (Hodges 2002).

When high nitrogen is sensed, the plants stop the nitrate uptake (Coruzzi and Zhou 2001). The development of this sensing system is important for monitoring the changes in nitrogen level. High nitrate uptake from the soil leads to nitrate accumulation in plants. To regulate the nitrate assimilation pathway, sugar plays a major role in activating the nitrate reductase (NR) gene expression and enzymes (Morcuende et al. 1998; Sheen et al. 1999; Kaiser and Huber 2001). As shown in Fig. 1, this pathway involves several enzymes such as NR,

nitrite reductase (NiR), glutamine synthetase (GS), and glutamine oxoglutarate aminotransferase (GOGAT), which reduce nitrate to nitrite, ammonium, and glutamate, respectively (Coruzzi and Zhou 2001; Stitt et al. 2002; Nunes-Nesi et al. 2010). Morcuende et al. (1998) reported that the rates of nitrate assimilation, 2-oxoglutarate, and amino acid synthesis were increased by feeding sucrose into detached tobacco leaves.

When there is a high concentration of nitrate in the soil, especially after fertilization, the absorption of nitrate by the roots is greatly elevated and may exceed the plants' ability to assimilate the nitrogen, leading to accumulation and plants with high nitrate content. The effect of nitrate on public health has been a matter of concern for the past few years, and consumers' intake of leafy vegetables containing a high amount of nitrate is treated as a serious issue (Pennington 1998; Pannala et al. 2003; Santamaria 2006; Savino et al. 2006; Anjana and Iqbal 2007; Hord et al. 2009). Nitrate is harmful to human health, especially infants. Nitrosamine formation during processing is carcinogenic and could result in methemoglobinemia, also known as blue baby syndrome, which is caused by the decreased ability of the blood cells to carry oxygen to the tissues, leading to difficulty in breathing, the risk of suffocation, or even death (Sanchez-Echaniz et al.
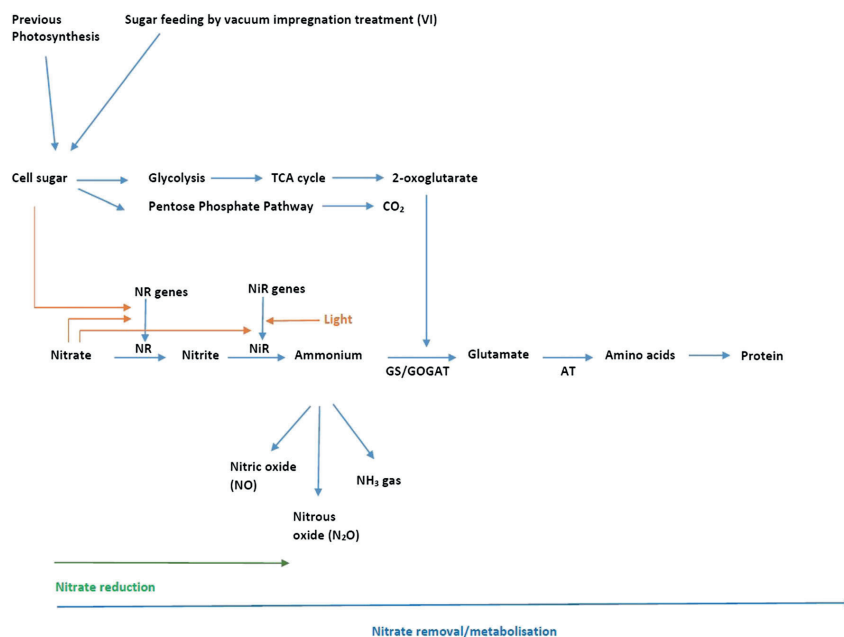


**Fig. 1** Relationships between the carbon metabolism and nitrate assimilation pathways. The supply of carbon leads to the induction of nitrate reductase activity and the subsequent metabolization of the nitrate content in plant tissue (Taiz et al. 2014). The abbreviations of the enzymes involved are listed as *NR* nitrate reductase, *NiR* nitrite reductase, *GS* glutamine synthetase, *GOGAT* glutamine oxoglutarate aminotransferase, and *AT* amino transferase

2001; Guay 2009; Correia et al. 2010). According to EFSA (2008), the acceptable daily intake (ADI) for nitrate is equivalent to 222 mg/kg per day for a 60-kg adult. As spinach is high with nitrate and its content can reach up to 3000 mg/kg (fresh weight), consuming 100 g of the spinach leaves per day would exceed the ADI level, contributing to nitrate toxicity.

In this study, we suggest that exogenous administration of sucrose using VI reduces the concentration of nitrate and present evidence that the metabolization of the impregnated sucrose directly influences metabolic pathways affecting nitrogen metabolism. This finding has the potential to reduce the concentration of this anti-nutritional factor in baby spinach leaves.

## Materials and Methods

### Plant Material

Baby spinach leaves (*Spinacia oleracea* cv. Misano F1) were grown in a greenhouse with 16 h of light at 20 °C during daytime and 14 °C during nighttime. The greenhouse lamps were 400-W Na lamps with an initial light (PPF) of 725 μmol/ s. Seeds were sowed 1.5 cm below the soil surface and 4.0 cm apart from each other. The soil was fertilized with NPK fertilizer containing 182, 91, and 194 g/m$^3$ of nitrogen, phosphorus, and potassium, respectively. The soil has been limed with both dolomite and limestone to get a pH of 5.5–6.5. The spinach trays were watered daily. Leaves from 5-week-old spinach were harvested. The length of each leaf blade was $7.0 \pm 0.1$ with $2.0 \pm 0.1$ cm petiole, and the width at the center of the leaf was $3.0 \pm 0.3$ cm. Leaves were harvested at 9 am, which was 3 h after the start of the light period, and only the non-shaded leaves were used. The leaves were placed into sealed plastic bags and transported to the laboratory in less than 5 min. In the laboratory, the plastic bags were stored at 8 °C and the leaves were subjected to VI treatment within 3 h after harvesting.

### Impregnating Solution

An isotonic sucrose solution in equilibrium with the spinach leaves was designed with respect to the cell sap. The isotonic solution concentration was determined by immersing three spinach leaves in a series of solutions with different concentrations, ranging from 0 to 24 % (*w/v*). The variation of tissue weight was recorded every hour until equilibrium. VI was carried out at room temperature ($20 \pm 2$ °C) in a chamber connected to a vacuum controller (SIA, Bologna, Italy) and a vacuum pump, as described by Panarese et al. (2013).

### Vacuum Impregnation

Based on preliminary experiments, to establish maximum weight gain and avoid tissue damage, a protocol with a minimum absolute pressure of 150 mbar was chosen. The chosen pressure profile ensured that the cell viability was maintained (cell viability after VI was verified by vital staining with fluorescein diacetate (FDA) as described by Phoon et al. (2008)). The following two parameters were set for each pressure step: duration (min) and absolute pressure value (mbar). During the first phase of VI, the pressure was gradually decreased from 1000 to 150 mbar in 11 min and was kept at 150 mbar for 1 min. During the second phase, vacuum was released and the pressure progressively increased to atmospheric pressure during 7 min and was kept at atmospheric pressure for 13 min. The total treatment time was 32 min. This cycle was repeated twice. After VI, the excess sugar solution on the surface of the spinach leaves was removed with tissue paper and the weight gain of each leaf was recorded.

### Chemical Analysis

#### Sample Preparation

Impregnated and non-impregnated leaves were placed in a closed container with saturated humidity and left in darkness at 8 °C for 12, 24, 36, 48, 60, and 72 h. Additional time points at 18, 27, 33, and 42 h were added for the soluble protein analysis, and the leaves were treated the same as for the previous time points. The non-impregnated leaves were immersed in the impregnating sucrose solution (21 % *w/v*) for 64 min without subjecting them to VI. For the analysis of nitrate and nitrite, the impregnated and non-impregnated leaves were cut into small pieces and ground to a fine powder using liquid nitrogen and a mortar and pestle. The powder was then dried in an oven (Termaks AS, Norway) overnight at 70 °C before chemical analysis. For the sugar and soluble protein analyses, the spinach leaves were freeze-dried. Freeze-drying was carried out using a laboratory freeze-dryer (Hetosicc freeze-dryer CD 12, Birkerod, Denmark) for 3 days. After drying, the leaves were ground to a fine powder with mortar and pestle.

#### Nitrate

Nitrate was determined as described by Cataldo et al. (1975). Of dried powder, 0.1 g was suspended in 10 ml deionized water; the suspension was incubated at 45 °C for 1 h and vortex-mixed. The homogenate was centrifuged at 5000$g$ for 20 min. Of supernatant, 0.2 ml was pipetted into 50 ml of an Erlenmeyer flask, mixed thoroughly with 0.8 ml of 5 % salicylic acid in concentrated H$_2$SO$_4$. The mixture was left for 20 min at room temperature. Of 2 N NaOH, 19 ml was added slowly to raise the pH above 12. The absorbance of the blank and samples was measured at 410 nm using a Varian Cary® 50 UV–Vis spectrophotometer. The standard curve was prepared by diluting the nitrate standard for ion

chromatography (Sigma-Aldrich) in deionized water to obtain solutions with concentrations ranging from 0 to 300 µg/ml. The nitrate content was expressed as mol per kilogram of dry mass. At least three measurements were taken for each time point after VI.

### Soluble Sugars

The extraction was done according to the method described by Toledo et al. (2003). Of freeze-dried spinach tissue, 0.50 g was placed into a reflux tube containing a boiling solution of 20 ml of 99 % ethanol for 10 min and later cooled under running water. Of 80 % ethanol solution, 50 ml was added, and the extract was evaporated in vacuum at 50 °C. The extracts of spinach tissue and ethanol solution were filtered and centrifuged at 13,000$g$ for 25 min. The supernatant was analyzed enzymatically for sucrose, D-fructose, and D-glucose using the Megazyme K-SUFRG 06/14 Assay Procedure (Megazyme, Megazyme International, Ireland). Three measurements were taken for each time point after VI.

### Nitrite

The extraction was done according to the method described by Chang et al. (2013). Of dried leaf spinach, 2 g was mixed in 5 ml of 0.02 M sodium tetraborate ($Na_2B_4O_7.10H_2O$; Sigma-Aldrich) solution before adding 100 ml of hot water at 80 °C. The mixture was vortex-mixed and placed in a boiling water bath for 15 min. Two milliliter of 0.25 M potassium hexacyanoferrate (II) ($K_4Fe(CN)_6.3H_2O$; Sigma-Aldrich) solution and 2 ml of 1 M zinc acetate dehydrate ($Zn(CH_3CO_2)_2.2H_2O$; Sigma-Aldrich) solutions were added. The solution was vortex-mixed and left to cool down to room temperature. The solution was transferred to a 200-ml volumetric flask, and distilled water was added to complete the volume. The solution was mixed and filtered through 0.45-µm-size cellulose nitrate filter paper. Ten milliliter of the sample was pipetted into a 50-ml volumetric flask before 20 ml of distilled water, 5 ml of 0.1 M sulfon-amide ($C_6H_8N_2O_2S$), and 3 ml of 0.1 M hydrochloric acid (HCl) were added. The solution was then vortex-mixed and the bottle was wrapped in aluminum foil to protect the solution from the light and stored at room temperature for 5 min. One milliliter of 0.04 M N-1-naphtyl-ethylenediamine ($C_{10}H_7NHCH_2CH_2NH_2.2HCl$) solution was added, allowed to set at room temperature for 3 min, and distilled water was added to complete a volume of 50 ml. The absorbance of blank and samples was measured at 538 nm within 15 min. The standard curve was prepared by diluting sodium nitrite (Sigma-Aldrich) in deionized water to obtain solutions with concentrations ranging from 0.0 to 2.0 µg/ml. The

nitrite content was expressed as mol per kilogram of dry mass. Three measurements were taken for each time point after VI.

### Soluble Protein

The extraction was carried out according to the method described by Martínez-García et al. (1999). Of freeze-dried powder, 0.05 g was placed in tubes and mixed with 5 ml buffer (125 mM Tris–HCl (Merck; pH 8.8), 1 % ($w/v$) sodium dode-cyl sulfate (SDS; VWR Brand), 10 % ($v/v$) glycerol, and 50 mM sodium metabisulfite ($Na_2S_2O_5$; Merck). The mixture was homogenized using Ultra Turrax for 30 s in an ice bath and taken to room temperature (20 °C). The homogenate was centrifuged at 13,000$g$ for 10 min, and the supernatant was saved. Of the supernatant, 25 µl was pipetted and 25 µl of compatibility reagent stock solution (provided by the BCA Kit) was added, and the tubes were vortex-mixed. The tubes were incubated in a water bath at 37 °C for 15 min. One milliliter of working reagent, obtained by mixing 50 parts of BCA Reagent A with one part of BCA Reagent B (provided by the BCA Kit), was added to the tubes, and the solution was mixed thoroughly with vortex. The tubes were incubated at 37 °C for 30 min and cooled down to room temperature for 10 min. The absorbance of blank and samples was measured at 562 nm within 10 min. Bovine serum albumin (BSA) standard solution was diluted to obtain solutions of concentration ranging from 0 to 2000 µg/ml. The protein content was expressed as milligram per kilogram of dry mass. Three measurements were taken for each time point after VI.

### Statistical Analysis

The statistical significance ($p < 0.05$) of the treatments was tested by means of one-way analysis of variance (ANOVA) using Excel (Microsoft Office, Redmond, WA USA). The Tukey-Kramer multiple comparison test was used to evaluate true differences in treatment means.

## Results

### Effect of VI on the Nitrate and Sucrose Contents of Baby Spinach Leaves

The mass gain after VI was 23.3 % ± 3.4. Figure 2a shows the change in nitrate content with time after VI with 21 % ($w/v$) sucrose solution in the baby spinach leaves. A drastic decrease in the nitrate content of sucrose-fed spinach leaves can be observed by its 70 % reduction 72 h after VI, while the leaves that were immersed in the sucrose solution without VI showed only 24 % reduction after storage for 72 h (filled circle). A significant reduction of nitrate content was observed in the

**Fig. 2** Change of nitrate (**a**) and sucrose (**b**) contents in baby spinach leaves during storage at 8 °C up to 72 h. Leaves were either non-impregnated (*filled circle*) or vacuum impregnated with sucrose solution (*empty circles*). Statistical significance is represented by *different letters above the error bars*
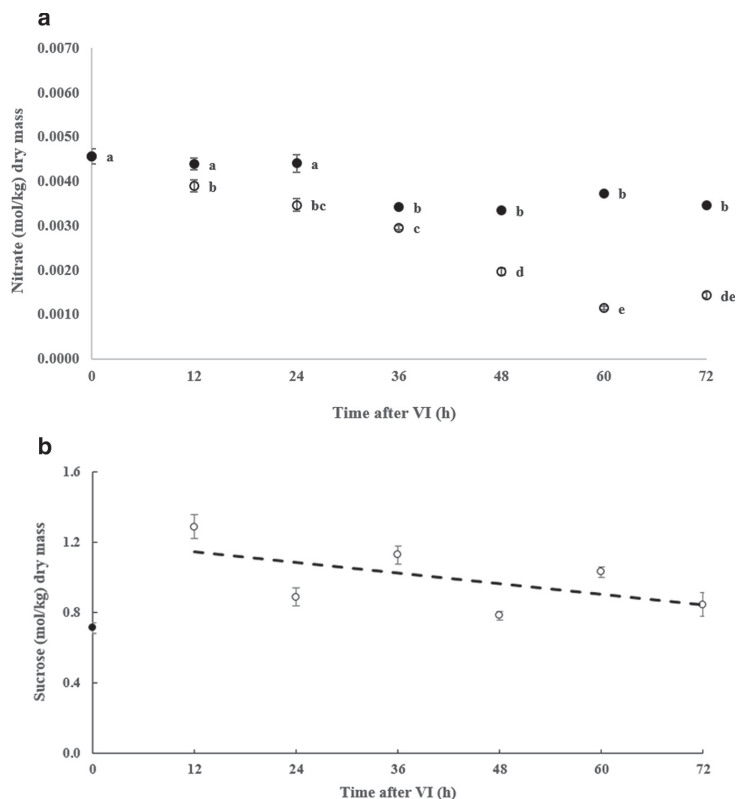


impregnated leaves between 0 and 12 h after VI, whereas no significant reduction was observed in the non-impregnated leaves. Later in time, the nitrate content was sharply reduced for the VI-treated leaves. The change in sucrose content with time after VI is shown in Fig. 2b. It is apparent that the sucrose content has increased up to 1.3 mol/kg dry mass when measured 12 h after VI relative to its initial concentration of 0.7 mol/kg dry mass for the non-impregnated leaves. However, upon storage, the sucrose decreased significantly over time.

### Effect of VI on the Glucose and Fructose Contents of Baby Spinach Leaves

Figure 3a, b shows the changes in glucose and fructose contents with time after impregnation with sucrose, respectively. The glucose content for the non-impregnated spinach leaves was 0.29 mol/kg dry mass, and there was no significant difference between 0 and 12 h after

VI. However, the glucose content significantly increased throughout the storage time. The fructose content for non-impregnated spinach leaves was 0.11 mol/kg dry mass and increased up to 0.18 mol/kg dry mass when measured 12 h after VI. However, throughout the storage time, the fructose decreased.

### Effect of VI on the Nitrite and Soluble Protein of Baby Spinach Leaves

Figure 4 shows the influence of VI with sucrose solution on the nitrite content in baby spinach leaves. The nitrite determination was carried out in order to test whether there is an accumulation of this compound in the tissue after VI, as it is known that nitrite is harmful to human health (Hamirani et al. 2008). The nitrite content was practically constant at a very low level in all VI-treated spinach leaves, and no accumulation of nitrite was observed even 72 h after VI.

**Fig. 3** Change of glucose (**a**) and fructose (**b**) contents in baby spinach leaves during storage at 8 °C up to 72 h. Leaves were either non-impregnated (*filled circle*) or vacuum impregnated with sucrose solution (*empty circles*)
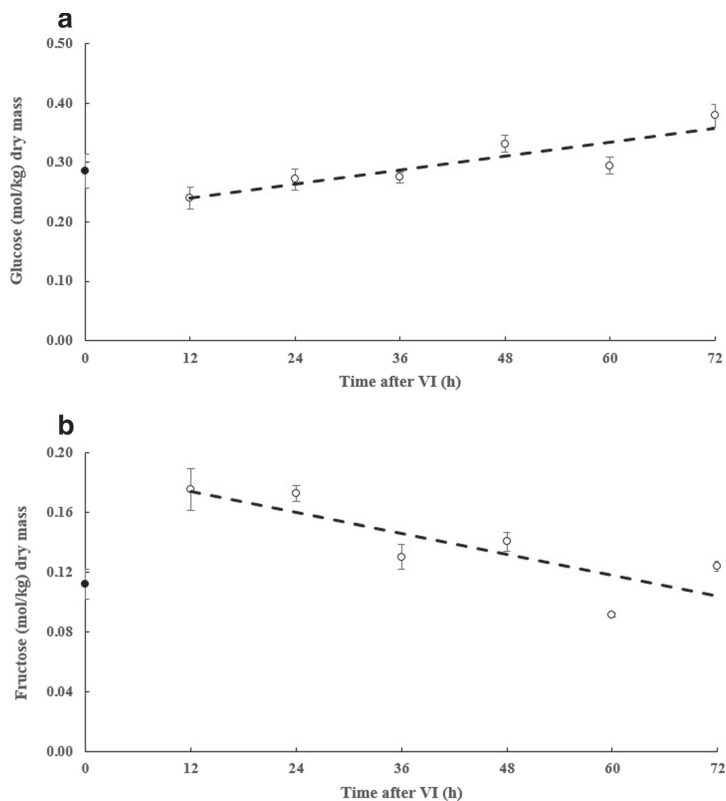


**Fig. 4** Nitrite content in baby spinach leaves during storage at 8 °C up to 72 h. Leaves were either non-impregnated (*filled circle*) or vacuum impregnated with sucrose solution (*empty circles*)
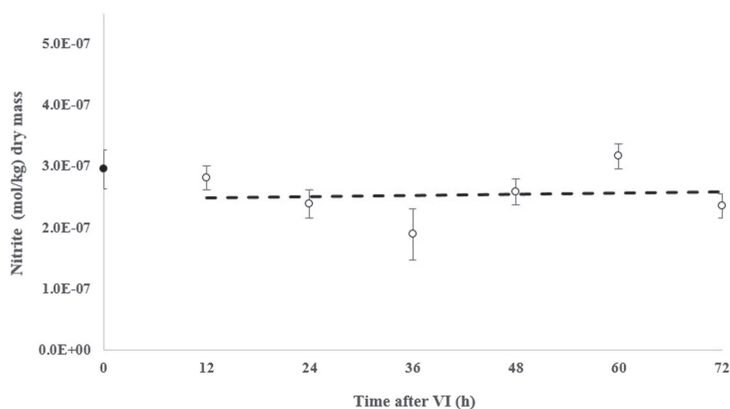
Figure 5 shows that the exogenously fed sucrose did not result in a clear accumulation or decrease of the soluble protein concentration with time after VI.

## Discussion

### Sucrose Feeding by VI Is Used as a Substrate to Metabolize Nitrate

Our results clearly show that feeding sucrose by VI into baby spinach tissue was associated with a marked reduction of their nitrate content (Fig. 2a). Sucrose is known to induce NR gene expression, mimicking the induction of light in the transcription of NR involved in the nitrate assimilation pathway. Plants will only reduce nitrate when sufficient sugar is present in the cells (Cheng et al. 1992; Morcuende et al. 1998; Kaiser and Brendle-Behnisch 1991). Interestingly, our results show that nitrate has been reduced even when the leaves were placed in darkness, suggesting that the marked nitrate reduction that occurred in the cell was solely due to sucrose feeding by VI.

The reduction of the fed sucrose throughout the storage time (Fig. 2b) might be the result of the use of sucrose as a substrate for both the metabolization of stored nitrate and for respiration. This suggestion is supported by Panarese et al. (2014), who showed that feeding sugars such as sucrose and trehalose provokes a drastic increase in the metabolic activity of baby spinach, mainly involving mitochondrial oxygen-consuming pathways. Our results show that the initial decrease of nitrate was observed 12 h after VI, indicating that the tissue starts to induce the NR enzyme and metabolize the stored nitrate at an appreciable rate.

Whilst a reduction of sucrose and fructose was observed, the increase of glucose (Fig. 3a) might be due to the effect of unused glucose. Either sucrose or glucose can be used as a substrate to metabolize the nitrate, and in our case, the sucrose might have been favored. Morcuende et al. (1998) reported

that either the sucrose or glucose can replace the light in stimulating the increase of NR activity in plant cells; however, this stimulation was higher when the sucrose was supplied to detached tobacco leaves rather than the glucose. Invertase activity, converting the added sucrose to glucose and fructose, as well as starch degradation, could also have contributed to the accumulation of glucose (Farrar et al. 2000).

### Sucrose Regulation of NR Expression

The activation of the NR enzyme may provoke the conversion of nitrate to nitrite. This study has shown that nitrite was not accumulated when spinach leaves were impregnated with sucrose solution (Fig. 4). This is consistent with previous reports, where it has been proven that only a part of the oxidized nitrate remains in the form of nitrite while the majority is subjected to rapid transformation of nitrite to ammonium (Beevers and Hageman 1969; Walters and Smith 1981). However, soluble proteins have not yet accumulated in the tissue after VI, as sucrose has been used as a substrate for cellular respiration and induces the enzymatic process for nitrate metabolization (Fig. 5). Nonetheless, conversion of all metabolized nitrate (4.6 mmol/kg dry mass over 72 h) to protein would correspond to an accumulation of approximately 500 mg of protein over the same time span. This is 20 % of the initial protein value and within the variation observed over time. Yet, it can also be speculated that the nitrogen is no longer available for protein synthesis as it might be released to the surroundings by the denitrification process, a process where nitrate is converted to nitrogen gas (Weier et al. 1993).

### Concluding Remarks

Experimental evidence showed that sucrose feeding by vacuum impregnation has significantly reduced the nitrate content in baby spinach leaves. Sucrose is used as



Fig. 5 Soluble protein content in baby spinach leaves during storage at 8 °C up to 72 h. Leaves were either non-impregnated (*filled circle*) or vacuum impregnated with sucrose solution (*empty circles*)
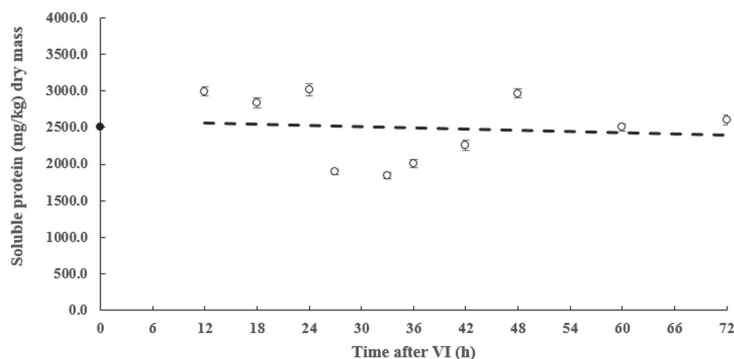
a substrate to metabolize the stored nitrate even in dark conditions. This method could potentially be useful in the industry to reduce the high nitrate content in spinach, which is harmful to human health, and subsequently improve their quality characteristics.

This study has set the basis for further investigations on

1. Changes of other important nutritional factors such as ascorbic acid. These changes are not possible to predict, but investigation with redox markers may give information on the change of ascorbate and/or ascorbate to dehydroascorbate ratio.
2. The effect of modified atmosphere packaging after impregnation on nitrogen metabolism.
3. Continuous atmosphere composition measurements of the impregnated, packed spinach would provide additional information on the leaf metabolism.

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Paper IV

# Short communication:

# Effect of vacuum impregnation of baby spinach leaves on package gas composition

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# Abstract

Baby spinach leaves were vacuum impregnated with solutions of sucrose, calcium lactate or ascorbic acid. Their gross metabolic activity measured with isothermal calorimetry as well as the atmospheric composition created inside the packed leaves at two different storage temperatures, 5 ºC and 21 ºC, were measured. Results show that, at both measured temperatures, the gross metabolic activity of baby spinach leaves increased after impregnation and the size of this increase depends on the impregnated substance and the temperature. The impregnating sucrose plays a major role for the atmosphere created inside the packed spinach during storage at both studied temperatures.

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# 1. Introduction

Minimally processed fruits and vegetables (MPFV) are products that maintain attributes and quality similar to those of fresh products (Alzamora, López-Malo, & Tapia, 2000). Minimal processing involves any procedure, short of complete conservation procedures (e.g. canning, drying, freezing, etc.) that adds value to the agricultural product. Peeling, washing, slicing or shredding are common minimal processing procedures making the product ready to use (Barry-Ryan & O'Beirne, 1997). A combination of modified atmosphere packaging (MAP) and refrigeration is often used in order to ensure long storage life and high quality of fruits and vegetables (Gómez Galindo, Rocculi, Wadsö, & Sjöholm, 2005).

Processing operations used for preparing a ready-to-eat product create practical problems regarding shelf-life, safety and packaging. This has presented a continuous challenge to food scientists, as consumers are demanding for convenience and quality foods, owing to the lifestyle changes that has influenced their eating habits (Gómez Galindo, Rocculi, Wadsö, & Sjöholm, 2005). MPFV should have storage life of at least 4-7 days, but preferably longer depending on the market (Ahvenainen, 1996; Barry-Ryan & O'Beirne, 1997). Microbial spoilage, physiological ageing, biochemical changes and loss of nutritional quality are common factors that contribute to food deterioration.

MAP provides a means of modifying the composition of the internal atmosphere of the packed product. The aim is to create an optimal gas balance inside the package, by lowering the metabolic activity of a product. In general, the level of oxygen ($O_2$) inside the package is reduced, lowering the rate of respiration and thus deterioration (Jacobsson, 2004). Active or passive modification can be achieved by changing the atmosphere inside the package (Zagory & Kader, 1988). Active MAP involves removing some of the air from inside the package and replacing it with a mixture of inert gases in order to obtain the desired atmosphere. In passive MAP, the respiration of the plant tissue and the gas diffusion characteristics of the film packaging material modify the atmosphere inside the package. The decrease of $O_2$ concentration and the increase of carbon dioxide ($CO_2$) concentration inside the package reduces the respiration rate of the product (Kader & Saltveit, 2003).

Several technologies have been developed through the years aiming at keeping the fresh-like characteristics of packed vegetables for longer time in the shelves. These technologies either target the growth of microorganisms that would potentially cause deterioration of the product or target the metabolic activity of the product in order to influence their respiration rate

(for a review see (Ma, Zhang, Bhandari, & Gao, 2017)). In this paper, the application of vacuum impregnation of different substances into the tissue of baby spinach is studied as means of affecting the metabolic activity of the leaves during MAP.

In a previous investigation by our group, it was demonstrated that introducing different substances into baby spinach leaves by vacuum impregnation provoked different effects on the short term gross metabolic activity measured with isothermal calorimetry as well as changes in the composition of sugars (sucrose, glucose and fructose) and starch (Yusof, Wadsö, Rasmusson, & Gómez Galindo, 2017). We here aim at exploring the effect of impregnation on the modified atmosphere of packed spinach leaves.

As suggested by Gómez Galindo et al. (2005), isothermal calorimetry may provide a versatile tool to conduct fundamental metabolic studies of the effect of different processing steps on MAP. We here show a preliminary study where isothermal calorimetry measurements of short term metabolic responses as well as measurements of the changes in the atmosphere created inside packed, impregnated leaves during storage at two different temperatures are reported and discussed.

# 2. Materials and methods

## 2.1 Plant materials

Baby spinach leaves (*Spinacia oleracea* cv. Misano F1) were grown in a greenhouse with 16 h of light at 20 °C during day and night times. The greenhouse lamps were 400 W metal halide lamps with a photosynthethic photon flux of 100 µmol/m²/s. Two seeds were sowed 1.5 cm below the soil surface and 4.0 cm apart from each other. The dimension of plants growing tray was 54 cm × 32 cm and there were 42 spinach plants grown in each tray. The spinach trays were watered every second day. Leaves from five weeks old spinach were harvested. At the time of harvesting, the length of each leaf blade was $7.0 \pm 0.1$ cm with $2.0 \pm 0.1$ cm petiole and the width at the center of the leaf was $3.0 \pm 0.3$ cm. Leaves were harvested at 10 in the morning, which was 4 h after the start of the light period and only non-shaded leaves were used. Leaves were harvested randomly from plants located in 5 trays, placed into sealed plastic bags and transported to the laboratory within 10 min. For each experimental replication, the harvesting of the leaves was done from 5 new trays where the plants were 5 weeks old.

## 2.2 Solutions

An isotonic sucrose solution 0.6 mol/l (pH 5.8) in equilibrium with the spinach leaves were designed with respect to the cell sap. The isotonic solution concentration was determined by immersing three spinach leaves in a series of solutions with different concentrations. The variation of tissue weight was recorded every hour until equilibrium. Ascorbic acid and calcium lactate solutions were prepared at 5.7 mmol/l (pH 3.2) and 50 mmol/l (pH 7.1) respectively. These concentrations were chosen based on the most commonly used concentrations for vacuum impregnation of fruits and vegetables (Bieganska-Marecik & Czapski, 2007; Radziejewska-Kubzdela, Biegańska-Marecik, & Kidoń, 2014; Yurttas, Moreira, & Castell-Perez, 2014). The chosen concentrations did not noticeably change the taste of the leaves immediately after VI. The impregnating solutions were kept at $5.0 \pm 0.1$ ºC before the VI treatment took place.

## 2.3 Vacuum Impregnation

Leaves were submerged in the solutions and immediately subjected to VI. VI was carried out in a chamber connected to a vacuum controller (SIA, Bologna, Italy) and a vacuum pump, as described by Panarese, Dejmek, Rocculi, and Galindo (2013). The chamber was covered with aluminum foil to keep dark condition during VI. The setup was placed in a temperature controlled room to carry out the impregnation at $21.0 \pm 0.7$ ºC and $5.0 \pm 0.1$ ºC.

Based on preliminary experiments, to establish maximum weight gain and to avoid tissue damage, a protocol with a minimum absolute pressure of 150 mbar was chosen. The chosen pressure profile ensured that the cell viability was maintained (cell viability after VI was verified by vital staining with fluorescein diacetate (FDA) as described by Phoon, Gómez Galindo, Vicente, and Dejmek (2008). During the first phase of VI, the pressure was gradually decreased from 1000 mbar to 150 mbar in 11 min and was kept at 150 mbar for 1 min. During the second phase, vacuum was released and the pressure progressively increased to atmospheric pressure during 7 min and was kept at atmospheric pressure for 13 min. The total treatment time was 32 min and this cycle was repeated twice. After VI, the excess impregnation solution on the surface of the spinach leaves was removed with tissue and the weight gain of each leaf was recorded. Immediately after impregnation, samples were transferred to calorimetry ampoules.

## 2.4 Packing and storage

The spinach leaves ($35 \pm 0.3$ g), either untreated or VI treated were packed in a micro-perforated polypropylene bags (18 cm $\times$ 18 cm) with an oxygen transmission rate (OTR) of 1300 cc/m$^2$/d/atm (Flextrus Group AB, Lund, Sweden). The film thickness was 30 µm and each bag had 22 perforations of 80 µm each. For each impregnated substance, time point and temperature, 3 bags were prepared. All bags were exposed to light during storage at two different temperatures; $5.0 \pm 0.1$ ºC and $21.0 \pm 0.7$ ºC, for 4 days.

## 2.5 Isothermal calorimetry measurements of heat production

The calorimetric measurements were performed with a two-channel isothermal calorimeter (Biocal 2000, Calmetrix Inc., USA), where each calorimeter is equipped with its own reference

cell. The reference was 91 g aluminum. The primary output from the heat flow sensors in the calorimeter (voltage) was recorded every minute by a computer. The corresponding thermal powers (heat production rates) were calculated according to Eq. 1.

$$P = \varepsilon \frac{(Vs - Vbl)}{m} \qquad\qquad \text{(Eq. 1)}$$

Where $P$ is the specific thermal power of the spinach sample ($\mu$W/g), $\varepsilon$ is the calibration coefficient of the calorimeter ($\mu$W/$\mu$V), $V_s$ the voltage signal from the calorimeter ($\mu$V), $V_{bl}$ the voltage recorded for the baseline ($\mu$W), and $m$ is the mass of the sample (g). Baselines were recorded before every new measurement.

Isothermal calorimetry measurements were performed at 21 ºC and 5 ºC. No condensation or temperature fluctuations were registered in the equipment at the lower temperature as it was placed in a cold room at 5.0 ± 0.1 ºC. Eight leaves (7.8 ± 0.1 g) were placed in a 1.1 L closed plastic container with wet tissue on the bottom for 3 h at 21 ºC in the darkness. After this incubation period, the leaves were subjected to two treatments, described below. Four replications were made for each of the impregnated solutions and the control.

i) The leaves were placed into sealed 125 ml plastic ampoules which were placed in the calorimeter. After the initial disturbance, the signal was recorded for two hours. They were then removed from the ampoule and VI was applied. The leaves were subsequently placed back in the ampoule and the calorimeter. After the initial disturbance, the signal was recorded for two more hours.

ii) Untreated leaves (control) were placed in the ampoule and, after the initial disturbance, the signal was recorded for two hours. They were removed from the ampoule for 64 min but VI was not applied; instead, the leaves were placed in a container with saturated atmosphere. The leaves were subsequently placed back in the ampoule and the calorimeter. After the initial disturbance, the signal was recorded for two more hours.

In a separate experiment, the signal from untreated leaves was recorded for six consecutive hours.

## 2.6 Atmosphere analysis

During the storage period, the concentration of oxygen and carbon dioxide inside the packages was measured using an $O_2$ and $CO_2$ analyzer (Check Mate 9900 PBI-DAN sensor

Co., Denmark) after 15 min, 24 h, 48 h, and 96 h of storage at 5 ºC and 21 ºC. The instrument was calibrated with air. A syringe was inserted into the package for measuring the gas composition. Only one measurement was taken on each package at each temperature and time point. After the measurement, the package was discarded.  For each substance, temperature and time point, three packages were measured. The weight of each package was taken before and after storage at each time point in order to determine the moisture loss at both temperatures.

## 2.7 Statistical Analysis

The statistical significance ($p < 0.05$) of the treatments was tested by means of one-way analysis of variance (ANOVA) using Excel (Microsoft Office, Redmond, WA USA). The Tukey-Kramer multiple comparison test was used to evaluate true differences in treatment means.

# 3. Results

## 3.1 Weight gain after impregnation with different substances

Table 1. Weight gain (%) of spinach leaves after VI treatment with sucrose, calcium lactate or ascorbic acid solutions. Reported are average and standard deviation of six measurements. Different letters indicate statistical significance ($p < 0.05$).

| Impregnated substances | Weight gain (%) |
|---|---|
| Sucrose | $24.0 \pm 2.3^a$ |
| Calcium lactate | $48.3 \pm 1.9^b$ |
| Ascorbic acid | $64.3 \pm 2.9^c$ |

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 showed the highest weight gain.

## 3.2 Effect of different impregnating solutes on metabolic heat production at 21 ºC and at 5 ºC

The results from the calorimetric measurements on spinach leaves when impregnated with sucrose, calcium lactate and ascorbic acid solutions are reported in Figure 1. In this figure, to facilitate the comparison between the treatments, the signals during the 1 h stabilization periods are not shown, and the continuous measurement of the control sample was added (dashed line). A significant increase of thermal power was observed when the spinach leaves were impregnated with sucrose and calcium lactate solutions at both temperatures. At 21 ºC (Figure 1A), the metabolic response to the impregnation of sucrose is higher than that of lactate, whereas at 5 ºC (Figure 1B) the metabolic response to the impregnation of lactate is higher than that of sucrose. No change on the metabolic heat production could be measured at 5 ºC after the leaves were impregnated with ascorbic acid solution; however, a statistically significant increase ($p < 0.05$) was detected when the metabolic heat production was measured at 21 ºC.
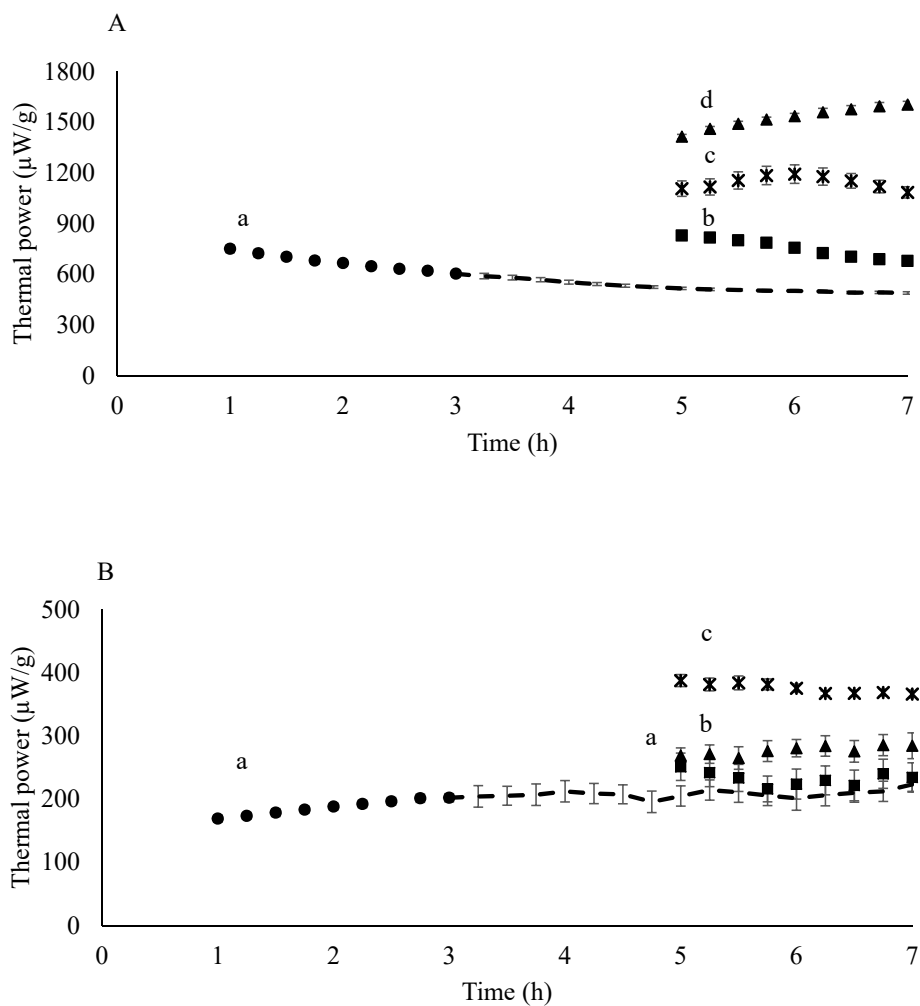
Figure 1. Calorimetric measurements of metabolic thermal power of untreated (●) and VI treated spinach leaves with 0.6 mol/l sucrose (pH 5.8) (▲), 50 mmol/l calcium lactate (pH 7.1) **(x)** and 5.7 mmol/l ascorbic acid (pH 3.2) (■) at (A) 21 ℃ and (B) 5 ℃. In a separate experiment, the signal from the untreated leaves (dashed line) was recorded for six consecutive hours. Statistically significant difference of the different curves (p < 0.05) is represented by different letters above the recorded thermal power curves. Bars represent ± standard error of the mean of the thermal power.

## 3.3 Changes in $O_2$ and $CO_2$ composition during storage of packed leaves

Figure 2 shows that all impregnated substances have different effects on the modification of the atmosphere around the spinach leaves at both studied temperatures. Among all substances used, sucrose impregnated leaves shows the highest $O_2$ consumption and $CO_2$ production throughout the storage period at both temperatures followed by the calcium lactate impregnated leaves (Table 2). At both temperatures, the gas composition of the impregnated leaves changed during the first 1 day of storage and did not significantly change for the next 3 days. An exception is the ascorbic acid impregnated leaves stored at 20 ºC which showed an increase on $O_2$ consumption with the concomitant increase of $CO_2$ production after 4 days of storage.
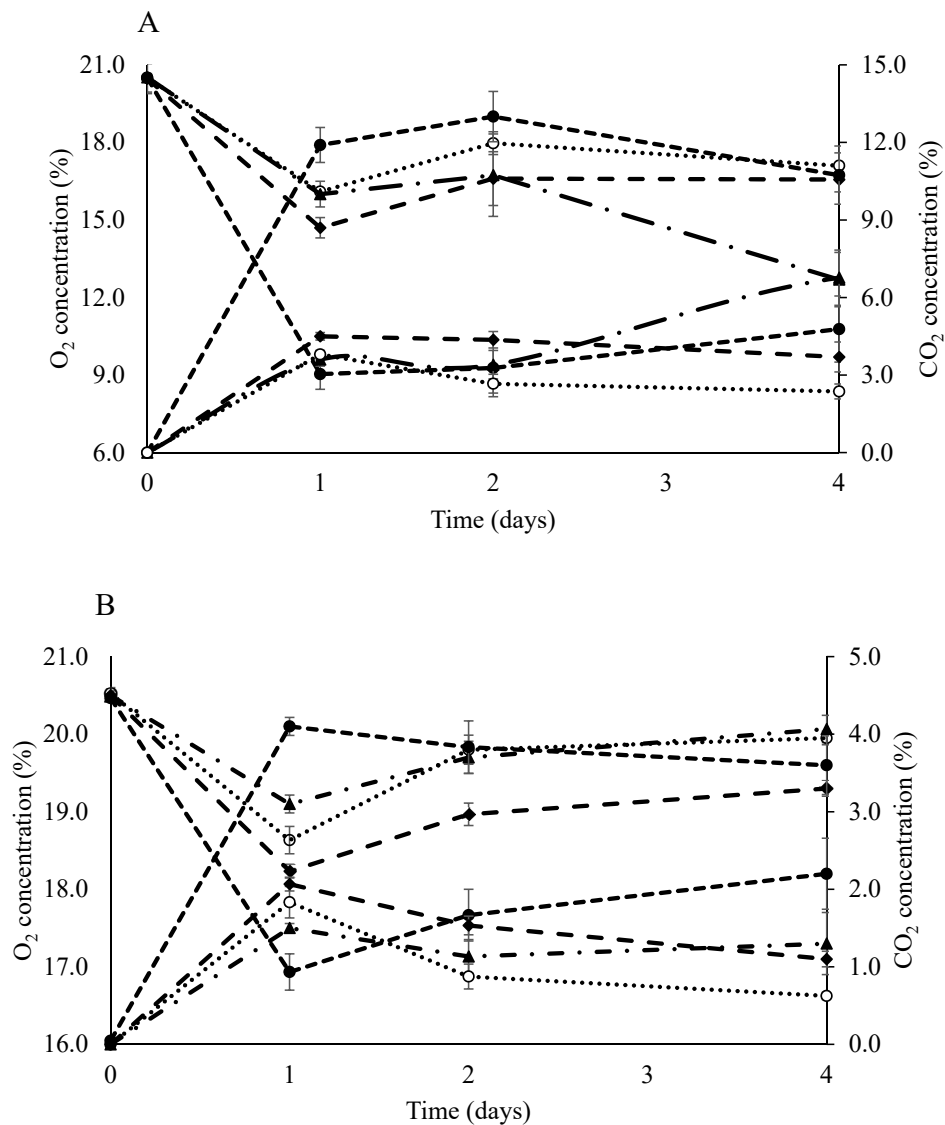
Figure 2. Changes in the composition of oxygen (primary axis) and carbon dioxide (secondary axis) of untreated baby spinach leaves (o) and VI treated spinach leaves with 0.6 mol/l sucrose solution (pH 5.8) (●), 50 mmol/l calcium lactate (pH 7.1) (♦) and 5.7 mmol/l ascorbic acid (pH 3.2) (▲) when stored at (A) 21 ºC and (B) 5 ºC for 4 days. Curves starting at 21 % and 0 % are for $O_2$ and $CO_2$, respectively. Bars represent ± standard error of the mean of the gas composition.

Table 2. $O_2$ and $CO_2$ concentrations inside the packages during 4 days of storage and weight loss after the end of the storage period at two different storage temperatures. Values in a column followed by a different letter were significantly different at $p < 0.05$ according to Tukey-Kramer's test.

| Impregnating substances | Storage temp. (°C) | $O_2$ | | | | $CO_2$ | | | | Weight loss (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0d | 1d | 2d | 4d | 0d | 1d | 2d | 4d | |
| Untreated | | 20.5a | 16.1a | 18.0a | 17.1a | 0.0a | 3.8a | 2.7a | 2.4a | 1.1a |
| Sucrose | | 20.5a | 9.0b | 9.3b | 10.8b | 0.0a | 11.9b | 13.0b | 10.7b | 0.5a |
| Calcium lactate | 21 | 20.5a | 14.7a | 16.6a | 16.6ac | 0.0a | 4.5a | 4.4a | 3.7ac | 0.6a |
| Ascorbic acid | | 20.5a | 16.0a | 16.7a | 12.7bc | 0.0a | 3.6a | 3.4a | 6.8bc | 0.7a |
| Untreated | | 20.5a | 18.6ab | 19.7a | 20.1a | 0.0a | 1.8ab | 0.9a | 0.6a | 0.1a |
| Sucrose | | 20.5a | 16.9c | 17.7b | 18.2bc | 0.0a | 4.1c | 3.8b | 3.6b | 0.2a |
| Calcium lactate | 5 | 20.5a | 18.2a | 19.0a | 19.3ac | 0.0a | 2.1a | 1.5a | 1.1a | 0.1a |
| Ascorbic acid | | 20.5a | 19.1b | 19.7a | 20.1a | 0.0a | 1.5b | 1.1a | 1.3a | 0.3a |

The weight loss at the end of the storage period showed to be dependent on the storage temperature (Table 2) but it was not influenced by the impregnated substance. The weight losses found here are minor for the packed spinach, and are not expected to have significant effects on the quality of the product. No significant differences in weight loss was observed between untreated and impregnated leaves, packed and stored at different temperatures.

# 4. Discussion

The results presented in this paper showed that, at both measured temperatures, the metabolic activity of baby spinach leaves increased after impregnation with different substances (Figure 1). These results are consistent with previous reports from our group (Panarese et al., 2014; Yusof et al., 2017), where metabolization of impregnated substances by the cells in the tissue proved to be the cause of this increase. We cannot, however, rule out the possibility that an osmotic effect of the impregnated solutions, reflected on the weight gain results (Table 1), influenced the measured metabolic activity.

Results also show that impregnation with sucrose plays a major role for the atmosphere created inside the packed spinach during storage at both studied temperatures. Comparing Figures 2 and 3, there can seem to be a disagreement between the calorimetry and the atmosphere composition results. However, the calorimetric measurements were only made for a few hours after impregnation and the first measurement of atmospheric composition was made after 24 h. It would be expected that the changes in atmospheric compositions and the produced heat would be approximately proportional, but as this is not seen when comparing the short term calorimetric data with the longer term atmospheric composition data, it is probable that, e.g., the metabolic activity after sucrose impregnation increased significantly after the calorimetric measurement was ended. It would have been interesting to continue the calorimetric measurement for longer times, but as it is made in closed vials the atmospheric conditions would have changed significantly – and in different ways compared to the packages – if longer measurements had been made. In the present calorimetric measurements the oxygen concentration never decreased more than 1 %, from 21 to 20 %.  Extended measurements might be possible with a setup that would allow a continuous flow of air inside the ampoule.

For all impregnated substances, the $O_2$ concentration inside the package was above the critical concentration (0.8 %) for developing anaerobic respiration (Ko, Watada, Schlimme, & Bouwkamp, 1996) and the leaves had good appearance after the 4 days of storage at both temperatures. However, it was observed that the ascorbic acid impregnated leaves deteriorated faster than the control and the leaves impregnated with other substances during storage at 21 ºC. 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.

# 5. Conclusions

This study explores the effect of vacuum impregnation with different substances on the gross metabolic heat production and the composition of the atmosphere created inside packed baby spinach leaves. The following are the main results:

- Vacuum impregnation of baby spinach leaves with sucrose and calcium lactate solutions increased their gross metabolic activity at 5 ºC and 21 ºC. Impregnation with ascorbic acid solution provoked an increase on the gross metabolic activity of the leaves only at 21 ºC.
- Impregnation with sucrose plays a major role for the atmosphere created inside the packed spinach during storage at both studied temperatures. Among all substances used, sucrose impregnated leaves shows the highest $O_2$ consumption and $CO_2$ production throughout the storage period at both temperatures.
- Short term drastic increases of metabolic activity of the treated spinach measured by calorimetry differed from the measurements of $O_2$ consumption and $CO_2$ production by the impregnated, packed product as both measurements may reflect metabolic changes taking place in different time scales.

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