



Factors important for the shelf-life of minimally processed lettuce

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Factors important for the shelf-life of minimally processed lettuce

Ph.D. Thesis
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Preface

This thesis is part of the requirement for obtaining a PhD degree at University of Copenhagen. The PhD was founded by the University of Copenhagen through a scholarship. The work was carried out in the facilities of the Quality and Technology group, Department of Food Science at the Faculty of Science. This project also involved GASA Odense, Rijk Zwaan, Bladgrønt and Bemis as discussion partners and suppliers of raw materials and MAP products. The lettuce used in the experiments were kindly donated by Rijk Zwaan and sowed in the commercial facilities of Bladgrønt. I would like to thank Mogen and Ulla Mogense for providing me part of their lettuce field to grow the lettuce used in this project and Gasa Odense for processing and packaging the plant material. Additionally, to Bemis for supplying the film packages.

I would like to thank especially to Mikael Agerlin Petersen for his guidance as supervisor and for sharing with me his knowledge about aroma, and to Abdel and to the late Mehdi D. Farahani for their technical assistance during my research. I would to thank to all the people of quality and technology group, especially to Margaret Owusu my friend and former officemate and to Marta and Camilla, for the nice working atmosphere and friendship.

Finally, to my husband, thanks for your patience and support and to my son Erik, who always draw a smile in my face and for reminding me what is important in life. Above all, I thank God for giving me the opportunity to work in this PhD project.

Summary

The minimally processed vegetable industry has been increasing rapidly due to change in lifestyle. Both women and men work outside home and have less time to cook and need more convenience and time saving products, which also present fresh and healthy characteristics. Iceberg lettuce (*Lactuca sativa* L.) is one of the most popular fresh-cut vegetables. Although an increase in the number of mixed salads in retail food chains is evident, their short shelf-life due to rapid browning and off-odour is a problem that need research. Therefore, the aim of this PhD project was to investigate factors important for the shelf-life of minimally processed iceberg lettuce and to propose a new methodology to measure browning in cut lettuce.

Browning has been pointed out as the main factor limiting shelf-life in cut lettuce. The problem becomes complex because browning of cut lettuce is difficult to measure. A novel method using image analysis for the measurement of browning in minimally processed lettuce was developed and presented in **paper I**. The method used a flatbed scanner for image acquisition, colour dye patches for colour correction, and colour thresholding to quantify the browning, that was expressed as brown area fraction. Cut lettuce was stored at 5°C for 6 days and plus 1 day at room temperature (day 7). Changes in browning were assessed at 2, 6 and 7 days of storage using image analysis. The result showed an increase in browning as time and temperature of storage increased. It was concluded that this technique can be used for measuring the browning in cut lettuce.

Few studies are done on the formation of volatiles in cut lettuce. Temperature of storage and methods of preparation that minimized quality loss are highly desirable. As such, cutting direction and storage temperature were investigated to elucidate their influence on aroma formation and respiration rate in minimally processed lettuce, are presented in **paper II**. Lettuce was cut longitudinal and transverse to the mid-rib and stored at 6 and 10°C for 4 and 5 days. Changes in respiration rate were analyzed through the storage time, and aroma analysis was carried out after 4 and 5 days of storage in January and

March, respectively. Respiration rate increased with increasing storage temperatures. Aroma formation was also influenced by storage temperature. Higher storage temperature allowed the increase of α -longipinene, 2-methylbutanal and 3-methylbutanal. Transversal cut to the rib was strongly related with volatiles of lipoxygenase (LOX) pathway i.e. cis-3-hexenal, cis-3-hexenol and trans-2-hexenol, meanwhile longitudinal cut enhanced the formation of volatiles from other metabolic routes. Therefore, it was concluded that transversal cut cause a more severe damage to the tissue than longitudinal cut based on aroma production of LOX volatiles.

It has been indicated that cultivar, season, packaging and storage time influence the type and concentration of volatile compounds, browning, chemical constituents and texture in vegetables. As part of this project, a more integrated study was undertaken for first time in lettuce to our knowledge. The study took into account the influence of cultivar, season, packaging and storage time. In order to achieve this, iceberg lettuce cultivars Platinas, Diamantinas and Morinas were harvested from June to September 2009. Lettuces were minimally processed and stored under three different treatments: two passive modified atmosphere packaging (MAP) built up by films of different permeabilities, F1 (OPALEN 65 AF) and F2 (OPP/PE-L 2040 AF), and storage in air. All packages were stored at 5°C. Gas composition, volatile compounds, physicochemical constituents, PPO activity and browning were assessed at 1, 5, 8 and 11 days of storage in packaged lettuce, whereas in air stored samples only at 1 and 5 days of storage. Additionally, respiration rate was only assessed in air stored samples and GC-O analysis was undertaken after 1 and 11 days of storage for cultivar Morinas packaged in passive MAP F2 in September. The study was divided into three papers, **paper III**, **IV** and **paper V**. The study in **paper III** was limited to analyze the volatiles compounds as a function of packaging and storage time and was used as basis for a more comprehensive analyses as presented in **paper IV**.

Paper III revealed that packaging and storage time had an influence on the volatiles of cut iceberg lettuce allowing the formation of desirable aroma but also on the development of off-odors. This result indicates that extremely low O₂ and high CO₂ conditions found

in the passive MAP F1 and F2 after 11 days of storage enhanced the formation of volatiles of anaerobic metabolism such as ethyl acetate and 2,3-butanedione.

In paper IV 52 volatile compounds were identified and of these 21 potent odorants were shown to contribute to the aroma of cut lettuce. Among them elemene, caryophyllene, β -selinene and 2,3-butanedione, enhanced under anaerobic conditions and likely to be off-odours. In August high production of these odorants probably compromised the quality in terms of odour. The findings suggest that most of the potent odorants enhanced their relative area under anaerobic conditions built up in the passive MAPs during the storage time, and are likely to produce off-odour. Levels of odorants such as 2,3-butanedione, elemene, caryophyllene and β -selinene were significantly enhanced under anaerobic conditions after 11 days of storage, being significantly higher in passive MAP F1. Regarding the cultivars, Morinas and Diamantinas was the less tolerant to high CO₂ resulting in significantly higher amount of 2,3-butanedione.

Paper V was related to browning and other physicochemical characteristics of minimally processed lettuce such as soluble sugars, organic acids, chlorogenic acid, pH, polyphenol oxidase activity and firmness. Gas chromatography mass spectrometry (GC-MS) method was developed for the analysis of soluble sugars, organic acids and chlorogenic acid in cut lettuce. Our results showed that season and storage time mainly influence over physicochemical characteristics of packaged and air stored cut lettuce, and in less degree the cultivar. In June, fructose, glucose, sucrose, malic acid and firmness were kept high under anaerobic conditions. Differences in the content of O₂ and CO₂ between the passive MAPs and air stored samples demonstrated to influence the formation of browning and other physicochemical characteristics as storage time increased. It was concluded that browning was remarkable controlled in passive MAPs samples, irrespective of season and cultivar due to extremely low O₂ and high CO₂ conditions, however, after 11 days of storage, this condition favored tissue softening, decreased of sugars and malolactic fermentation, mainly in passive MAP F1.

List of publications

Paper I: Deza-Durand, K.M., Petersen, M.A., Poll, L., Larsen, M. 2009. Image analysis for measuring enzymatic browning in minimally processed lettuce, in: Sørensen, H., Sørensen, S., Sørensen, A.D., Sørensen, J.C., Andersen, K.E., Bjerregaard, C., Møller, P. (Eds.), Euro Food Chem XV-FOOD FOR THE FUTURE-the contribution of chemistry to improvement of food quality. Book I of Proceedings. Faculty of Life Science, University of Copenhagen, Denmark, pp.111-114.

Paper II: Deza-Durand, K.M., Petersen, M.A. 2011. The effect of cutting direction on aroma compounds and respiration rate of fresh-cut iceberg lettuce (*Lactuca sativa* L.) Postharvest Biology and Technology, volume 61, issue 1, pp. 83-90.

Paper III: Deza-Durand, K.M., Petersen, M.A., Roepstorff, M., Poll, L. 2011. Influence of packaging and storage time on aroma compounds of minimally processed lettuce, in: Hofmann, T., Meyerhof, W., Schieberle, P. (Eds.), Advances and challenges in flavor chemistry and biology. Proceedings of the 9th Wartburg Symposium. DFA, Germany, pp. 305-309.

Paper IV: Deza-Durand, K.M., Petersen, M.A. 2013. Effect of season, cultivar, packaging and storage time on volatile formation of cut iceberg lettuce. Postharvest Biology and Technology (submitted).

Paper V: Deza-Durand, K.M., Petersen, M.A. 2013. Changes in physicochemical characteristics of packaged and air stored cut iceberg lettuce upon storage and season (manuscript under preparation).

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Papers I-V

Chapter 1. Introduction

Cut Iceberg lettuce (*Lactuca sativa* L.) is considered one of the most popular of minimally processed vegetables, and a growing demand for this convenient and time saving product has occurred in developed countries over the last few years (Ragaert et al., 2004). This vegetable is very popular due to its crispy texture, attractive green leaves, neutral taste and green aroma (Rico et al., 2007).

During cutting the disruption of the cell induces numerous physiological changes such as an increase in the respiration and mixing of phenolic acids and polyphenol oxidase, which causes browning (Couture et al., 1993; Saltveit, 2003). Tissue disruption can also initiate the formation of volatiles, like C6 aldehydes from LOX pathway. These volatiles are important odorants of lettuce, but in higher concentration can become off-odours. Important factors that influence the browning and volatiles in fruits and vegetables are season, cultivar, method of preparation, packaging, storage temperature and storage time, among others (Matheis et al., 1983; Heimdal et al., 1995; Smyth et al., 1998; Fukumoto et al., 2004; Hodges and Toivonen, 2008; Cantos et al., 2001).

Volatile products of cut lettuce have received little attention, even though they are important parameters in assessing its quality (Smyth et al., 1998; Nielsen and Poll, 2006; Lonchamp et al., 2009). Methods of preparation of cut lettuce that minimize cutting damage are highly desirable. There are some volatiles that are more specific to mechanical injury such as C6 aldehydes from LOX pathway, which can be used as indicators of severe cutting damage. Furthermore, the influence of different storage temperatures related to the development of volatiles in cut lettuce is unknown, but in packaged broccoli stored at 10 °C volatile compounds increased their concentration in comparison with broccolis stored at 4 °C (Jacobsson et al., 2004).

Modified atmosphere packaging has showed to be effective to extend the shelf-life of fresh-cut lettuce by reducing browning (Bolin and Huxsoll, 1991; Heimdal et al., 1995). However, off-odours can be developed due to extremely low O₂ and high CO₂ levels build up in the package. Therefore, off-odour can be a serious limiting factor for the quality and marketability of cut lettuce.

Fresh cut lettuce industry in Denmark uses different cultivars of lettuce depending on availability during season. Thus the problem becomes complex because large cultivar variation could implicate changes in the rate of enzymatic browning and in both quantitative and qualitative formation of off-odours (Forney et al., 2000). Likewise, season could affect the volatile formation in lettuce. For example in *Brassica* specie, the changes of sulfur volatiles within a season are caused by variations in the amount of aroma precursors, i.e. glucosinolates, as a result of changes in environmental conditions (Mattheis and Fellman, 1999; Vallejo et al., 2003).

Furthermore, objective measurements of browning in cut lettuce have been made using colorimeters (Heimdal et al., 1995), however, it has been claimed to be less successful to obtain an accurate colour representation due to point by point measurements (O'Sullivan et al., 2003). Under this context, other methodology like image analysis is a prominent choice, which has been reported to be representative, consistent and cost effective (O'Sullivan et al., 2003).

Aim of the thesis

The overall objective of this PhD thesis was to generate more knowledge about the formation of volatiles, browning and other physicochemical characteristics such as glucose, fructose, sucrose, malic acid, tartaric acid, ascorbic acid, chlorogenic acid, pH, polyphenol oxidase activity and firmness as a function of season, package, method of preparation, temperature of storage and storage time. The specific objectives of this research were:

- To investigate the aroma compounds and respiration rate of cut lettuce as a function of cutting direction and storage temperature (**paper II**).
- To investigate the changes in aroma compounds of cut lettuce as a function of season, cultivar, package and storage time (**paper III and IV**).
- To develop a new technique to assess the browning of cut lettuce (**paper I**)
- To investigate the changes of browning and other physicochemical characteristics of cut lettuce as a function of season, cultivar, package and storage time (**paper V**).

The results of this PhD thesis have been published in peer reviewed journals (paper II) and in conference proceedings (paper I and III) and submitted to peer reviewed journals (paper IV). Paper V is still under preparation.

Thesis outline

The thesis is divided into seven chapters. A brief description is given below.

Chapter 1. *Introduction*, gives an introduction to the problems associated to minimally processed iceberg lettuce. It also presents the scope of the thesis and lists the specific objectives.

Chapters 2. *The lettuce*, provides an overview of iceberg lettuce, e.g. taxonomy, cultivation and respiration rate.

Chapter 3. *Minimally processed lettuce*, describes each step of the processing and its influence on browning and aroma of lettuce.

Chapter 4. *Aroma of minimally processed lettuce*, provides a definition of aroma and describes the possible metabolic routes for the formation of volatiles in lettuce. It also includes an introduction to the analytical methods used for the analysis of volatiles e.g. dynamic headspace sampling, GC-MS and GC-O. Effect of important factors on the formation of volatiles in cut lettuce is provided through of paper II, III and IV.

Chapter 5. *Enzymatic browning and other physicochemical characteristic of minimally processed lettuce*, gives an overview of the process of browning and methodology for its measurement e.g. image analysis (paper I). It also provides theoretical background for measurement of texture in lettuce and introduction to the analysis of sugar, acids,

ascorbic acid and chlorogenic acids using GC-MS. Paper V is used to explain the effect of important factors on browning and physicochemical properties of lettuce. Chapter 6 and provides *conclusion* and *perspectives* of the PhD research.

Chapter 2. The lettuce

2.1. Taxonomy

The specie *Lactuca sativa* comprises seven main groups of cultivars: Butterhead lettuce, Iceberg or crisphead lettuce, Romaine/Cos lettuce, Cutting lettuce, Stalk lettuce, Latin lettuce and Oilseed lettuce. Among them, iceberg lettuce is the most popular minimally processed leafy vegetable. Its consumption has increased dramatically in recent years due to consumers needing more convenience and time saving products, which also present fresh and healthy characteristics (Ragaert et al., 2004; Rico et al., 2007).

Table 1. Classification of Iceberg lettuce

<i>Taxonomic hierarchy</i>	<i>Latin name</i>
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Asterales
Family	Asteraceae
Genus	<i>Lactuca</i> L.
Specie	<i>Lactuca sativa</i> L.

(<http://plants.usda.gov/java/ClassificationServlet?source=profile&symbol=LACTU&display=63>)

2.2. Anatomy

Lettuce leave is made of vascular and photosynthetic tissue (Toole et al., 2000), where a thick white mid-rib (vascular tissue) constitutes the majority of the leaf (photosynthetic tissue). The leaves are tightly wrapped and interlocked providing a crispy texture to lettuce (Fig.1) (de Vries, 1997; Toole et al; 2000; Křístková et al. 2008). Previous works indicate that photosynthetic and vascular tissues possess different phenolic metabolism as well as textural characteristics (Toole et al; 2000). Vascular tissue has lower polyphenol oxidase, peroxidase and phenylamonium lyase activity than photosynthetic tissue

(Heimdal et al., 1995; Fukumoto et al., 2002). However the potential for browning development is higher in vascular tissue, mainly in outer than inner leaves probably due to vascular tissue having a higher total volume and cut area (Fukumoto et al., 2002). On the other hand, there is no information, to our knowledge, regarding differences in the formation of volatiles between both tissues. In **paper II** it is hypothesized that the differences in the formation of volatiles between transversal and longitudinal cutting could be related to increase cutting surface area and damage to more cells of the mid-rib (vascular tissue) which enhanced the formation of LOX volatiles. Further research is needed in this area to understand volatile formation in fresh-cut lettuce.



a) Whole head (outside)



b) Cut in half (inside view)

Figure 1. Iceberg lettuce (*Lactuca sativa* L.)

2.3. Cultivation

Iceberg lettuce is produced commercially in North and Central America, Asia and Europe (Mou, 2008). In Denmark, the season of growing lettuce begins in April and ends in September. The rest of the year lettuce is imported mainly from Spain and Holland to supply the market (Gasa, oral communication). Field production of iceberg lettuce is carried out using transplanting seedlings (Fig.2) (Kristensen et al., 1987). Seedlings are planted from April to July. In June and September lettuce takes approximately 8 weeks to

grow to commercial maturity and in July and August it takes 6 to 7 weeks. Lettuces are harvested manually at commercial maturity when the head is well formed, compact and has a weight in the range from 400 to 500g (personal communication with the growers). Commercial maturity is defined as the stage of development when a plant or plant part has characteristics for an economical utilization for a particular purpose by the consumer (Shewfelt, 1986). Head firmness under hand pressure is used for classification of maturity (Kader et al., 1973). It is important to mention that in this manuscript and in the **papers II to V** the term “maturity” refers to “commercial maturity”.

Several cultivars of iceberg lettuce are available for cultivation in Denmark (Rijk Zwaan catalog 2007). Growers choose a cultivar based mainly on its resistance against tipburn and other diseases, well formed head and good speed for early production. However a cultivar that performs well in the field is not necessarily advisable for industry, as shown in **paper III**.



a) seedling of lettuce



b) commercially mature lettuce

Figure 2. (a) Seedling of lettuce and (b) commercially mature lettuce for harvest

Iceberg lettuce is very popular due to its crispy texture, attractive green leaves, neutral taste and green aroma (Rico et al., 2007). It is consumed fresh in salads, and it is a good dietary source of micronutrients such as vitamin A, C and E, and minerals such as calcium and iron (Hedges and Lister, 2005).

2.4. Respiratory metabolism of vegetables: an overview

While lettuce is growing it obtains all the energy it needs from the balance between utilization of carbon compounds (respiration) and acquisition (photosynthesis). However, once lettuce is harvested this balance is changed and the source of organic compounds is the built up reserves (Kays, 1991; Saltveit and Kader, 2003; Maguire et al., 2004). Respiration is the central process in living cells that release energy through the utilization of organic compounds, which is used to drive energy to catabolic and anabolic reactions inside the cell (Wills et al., 1982; Kays, 1991). Respiration can be aerobic (in the presence of oxygen) or anaerobic (in the absence of oxygen) (Wills et al., 1982). Under aerobic conditions, the complete oxidation of glucose involves three main reactions: glycolysis or Embeden-Meyerhof- Parnas (EMP) pathway, tricarboxylic acid cycle (TCA) or Krebs cycle and the electron transport system (Wills et al., 1982; Kays, 1999; Kader and Saltveit, 2003). A brief description of both types of respiration is given in the forthcoming subsections.

2.4.1. Glycolysis

Glycolysis occurs in the cytoplasm and produces two molecules of pyruvate from one molecule of glucose (Fig.3). The glycolysis involves a series of 10 enzymatic reactions, where the key enzyme of the process is the enzyme phosphofructokinase, which initiates the process (Kader and Saltveit, 2003). During the reaction two ATP (adenosine triphosphate) molecules and two NADH (reduced nicotinamide adenine dinucleotide) molecules are formed (Wills et al., 1982; Kays, 1999; Kader and Saltveit, 2003).

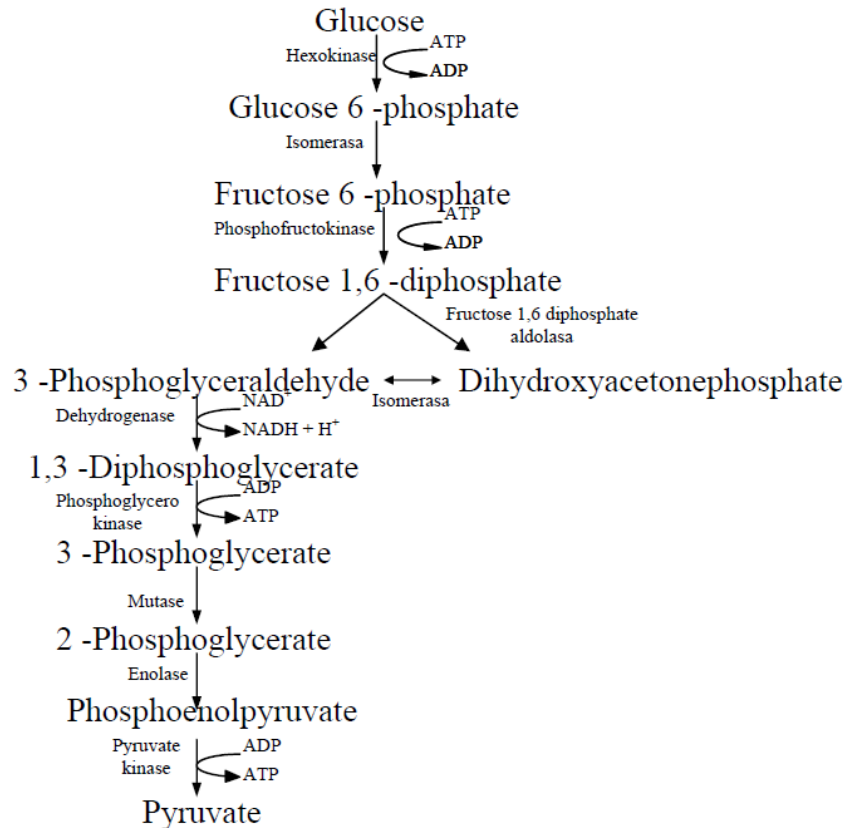


Figure 3. The glycolysis (Deza-Durand, 2006)

2.4.2. Tricarboxylic acid cycle (TCA)

The TCA cycle occurs in the mitochondrial matrix (Fig. 4). First, pyruvate moves by diffusion to the mitochondria, where it is decarboxylated to form acetate which condenses with a co enzyme A to form acetyl CoA. Further, acetyl CoA is condensed with oxaloacetate and enters to the cycle, in which, through seven sequential enzymatic reaction citric acid is formed (Wills et al., 1982). Citric acid is subsequently converted to oxaloacetate which readily reacts with another acetyl CoA molecule. Each molecule of pyruvate metabolized by TCA produces organic acids, three molecule of CO₂, one molecule of FADH₂ (reduced flavin adenine dinucleotide) and four molecules of NADH process (Kader and Saltveit, 2003).

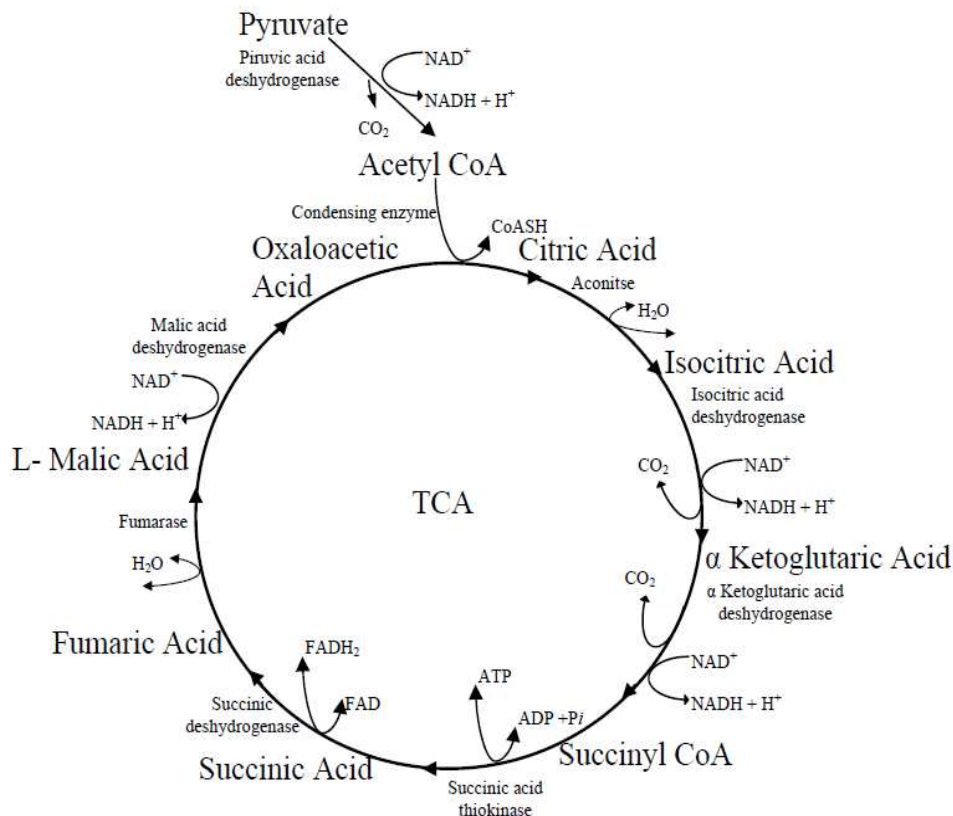


Figure 4. The tricarboxylic acid cycle (TCA) (Deza-Durand, 2006)

2.4.3. The electron transport system

The third pathway, the electron transport system occurs in the cristae of the mitochondria and involves the oxidation of FADH₂ and NADH obtained in the TCA and glycolysis to produce energy in form of ATP. In the process one NADH molecule produces three ATP molecules, while one FADH₂ molecule produces two ATP molecules (Wills et al., 1982; Kader and Saltveit, 2003).

2.4.4. Anaerobic respiration

In the absence of oxygen, glycolysis replaces the TCA cycle as the main source of energy for the plant tissue (Kader and Saltveit, 2003). The pyruvate is accumulated and further decarboxylated to acetaldehyde with a release of one molecule of CO₂. Subsequently, acetaldehyde is reduced to ethanol by the enzyme alcohol dehydrogenase with the

regeneration of NAD⁺. Two molecules of ATP are produced under anaerobic conditions against 37 produced under aerobic conditions (Wills et al., 1982; Kader and Saltveit, 2003).

2.5. Respiration rate of lettuce

Lettuce head has been classified as a commodity with moderate respiration rate (Kader, 2002). However, cutting lettuce for minimally processing speeds up the respiration rate (**paper II**, Kader and Saltveit, 2003). Respiration rate has been associated with the perishability of the product. Therefore it is assumed that higher respiration rate reduces the shelf-life of lettuce. Cutting direction has been indicated to affect the respiration rate of commodities such as tomatoes (Brecht, 1995) and green bananas (Abe et al., 1998). For lettuce it was demonstrated that the increase in respiration rate was higher in transverse than longitudinal cut sections, but temporary (**paper II**) as observed in Fig. 5. The increase of respiration rate in lettuce after cutting was due to enhanced aerobic mitochondrial respiration by enzymes such as phosphofructokinase and cytochrome oxidase (Asahi, 1978). However, this increase was observed until normal aerobic respiration is reestablished which depends on the commodity (Toivonen and DeEll, 2002). In addition, other methods of preparation such as shredding lettuce increases the respiration rate in comparison to cutting lettuce with a sharp knife or tearing by hand due to less damage to the tissue (Kader and Saltveit; 2003; Toivonen and DeEll, 2007)

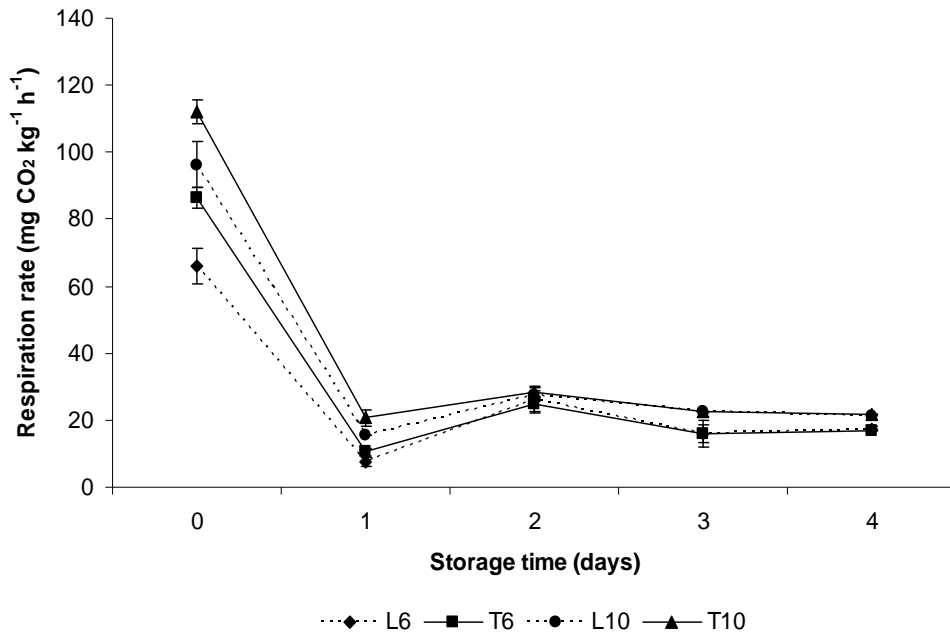


Figure.5. Respiration rate of iceberg lettuce cut transverse and longitudinal to the mid-rib stored in air at 6 and 10 °C for 5 days in March 2008. Abbreviations: L6, lettuce cut longitudinal stored at 6 °C; T6, lettuce cut transverse stored at 6 °C; L10, lettuce cut longitudinal stored at 10 °C; T10, lettuce cut transverse stored at 10 °C.

In **paper II** and **IV** was found that respiration rate was mainly affected by storage temperature, season and maturity at harvest (Table 2 and 3) (Wills et al., 1982; Toivonen and DeEll, 2007). Analysis of variance showed significantly higher respiration rates of cut lettuce at 10 °C than at 6 °C of storage (**paper II**). Higher storage temperatures are expected to increase the respiration rate due to increased reaction rates of many pathways in cell respiration (Wills et al., 1982; Watada and Qi, 1999). In **paper II**, data from January exhibited significantly higher mean values of respiration rates than that from March, lettuce heads bought in January were mature, whereas those from March were over-mature, which could explain the trend to higher respiration rates in January due to young cells being more active than old cells (Wills et al., 1982; Kays, 1991).

Moreover in **paper IV**, mean value of respiration rate was around 100% higher in lettuces harvested in August in comparison with June, July and September ($p \leq 0.05$). Regarding the cultivars, respiration rate of cultivar Morinas and Platinas were

significantly higher than Diamantinas ($p \leq 0.05$), but these differences seemed to be minor in comparison with season as differences were mainly around 8%.

Table 2. Respiration rate of fresh-cut lettuce under different cutting directions stored at 6 and 10°C in January and March 2008.

Factors	Respiration rate (mgCO ₂ kg ⁻¹ h ⁻¹)
<i>Temperature of storage</i>	
10°C	43.0 ± 30.8 (46) b
6°C	31.7 ± 22.4 (48) a
<i>Replicates of the experiments</i>	
January 2008	41.9 ± 21.1 (36) b
March 2008	34.3 ± 30.3 (58) a

Data expressed as mean ± standard deviation. Values on parenthesis represent the number of samples used for the calculation of the mean. Different letters indicate significant differences at $p \leq 0.05$.

Table 3. Respiration rate of cultivars Platinas, Morinas and Diamantinas stored in air at 5 °C after 1 day of storage in June, July, August and September 2009.

Factors	Respiration rate (mgCO ₂ kg ⁻¹ h ⁻¹)
<i>Season</i>	
June	59.3 ± 7.8 (9) ^a
July	60.2 ± 6.7 (9) ^a
August	112.5 ± 13.2 (9) ^b
September	60.3 ± 9.7 (9) ^a
<i>Cultivars</i>	
Platinas	76.7 ± 28.3 (12) ^b
Morinas	74.2 ± 28.1 (12) ^b
Diamantinas	68.3 ± 18.5 (12) ^a

Data expressed as mean ± standard deviation. Values on parenthesis represent the number of samples used for the calculation of the mean. Different letters indicate significant differences at $p \leq 0.05$.

Anaerobic respiration is triggered when O₂ is depleted. This condition was observed in **paper IV and V**, passive MAP F1 and F2 allowed rapid development of anaerobic conditions after 5 days (0.02% O₂) with high accumulation of CO₂. Under this extreme atmosphere browning was not observed, but, odorants likely to be off-odors increased after 11 days of storage, loss of firmness and soluble sugars and malolactic fermentation was observed as well. Smyth et al (1998) also reported that at O₂ content between 0.3-0.5 % color retention is excellent and off-flavors were limited in packaged cut lettuce. The influence of extreme atmosphere on the formation of odorants and browning will be discussed in Chapter 4 and 5.

Chapter 3. Minimally processed lettuce

Minimally processed or fresh-cut vegetables are vegetables that have been cut in small pieces and are ready-to-eat (RTE) (Saltveit, 2003). Among them, iceberg lettuce is one of the most popular RTE vegetables. Preparing fresh-cut lettuce includes unit operations such as reception of raw material, coring, cutting, washing, centrifuging, packaging and storage (see Fig. 6). Processing of lettuce allows physical, biochemical and physiological changes to enhance the loss of quality in the product (Saltveit, 2003; Rico et al., 2007). Quality of a fresh-cut lettuce can be defined as “the combination of physical, chemical and sensory attributes of the produce, those are of importance to determine the degree of acceptability by the consumer” (Watada 1986; Rico et al., 2007).

The main limitation of fresh-cut lettuce’s shelf-life is the development of browning (Heimdal et al., 1995, **paper I, V**), but off-odour is also a serious limiting factor (**paper III, IV**). The term “shelf-life” can be defined as the time period before the product attributes drop below the quality limit under specified storage conditions (Shewfelt, 1986; Rico et al., 2007). Browning and off-odour are still problems for the minimally processed industry in Denmark (GASA, oral communication). Therefore, this chapter will describe each step of minimally processing lettuce and its influence on browning and aroma of lettuce.

3.1. Raw material

The initial quality of a commodity to be minimally processed has high relevance in the final product (Shewfelt, 1986). In general, a high quality iceberg lettuce should be bright green, free of defects, crisp and turgid (Saltveit, 2000). Likewise, a compact head and adequate break-strength of iceberg lettuce’s leaves are characteristics highly desirable for the processor (GASA, oral communication). Maturity is also important for the processing of lettuce. Immature lettuce has soft and not fairly compact head which is difficult to cut by machine (Kader et al., 1973). On the contrary, overmature lettuce is ideal for cutting due to its hard and very compact state, but it is also more susceptible to postharvest

disorders during storage (Saltveit, 2000). Therefore, mature lettuce with a firm head is preferred because it provides a final product with better quality characteristics.

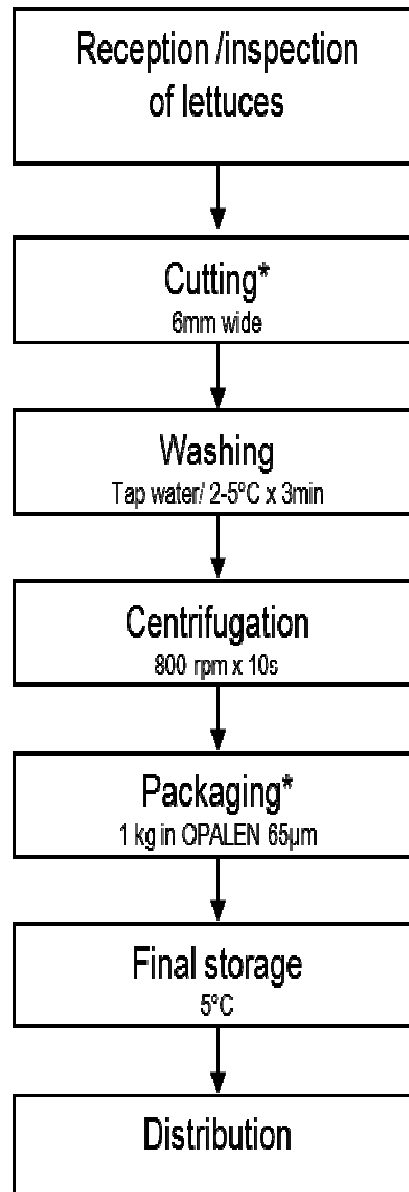


Figure 6. Minimally processed of iceberg lettuce

*: steps in the process examined in this thesis.

3.2. Cutting

The objective of cutting is to reduce the size of the vegetable, which improves product handling and provides a more convenient and time saving product (Sanguansri, 1997; Ragaert et al., 2004). At the factory external leaves and core of lettuce are removed manually before cutting (GASA, oral communication). Cutting of lettuce leads to the disruption of cells, promoting numerous physiological changes such as an increase in the respiration rate and enzymatic browning by mixing of polyphenol oxidase enzyme and phenolic acids, which reduce the shelf-life of the product (Couture et al., 1993; Saltveit, 2003). Likewise, tissue disruption releases enzymes which allow the formation of volatiles such as alcohols, aldehydes, terpenes, esters and acids (Belitz et al., 2004).

Methods of preparation of fresh-cut lettuce which minimize cutting damage are highly desirable. Bolin and Huxsoll (1991) found that tearing lettuce and cutting with a sharp knife led to a lower respiration rate and deterioration than shredding. Shredding is a term used to describe leafy vegetables cut in thin slices. Likewise, Martinez et al. (2008) found that a cutting grade less than 5 mm increase significantly the respiration rate of lettuce, which may lead to a higher speed of deterioration. Likewise, cutting direction influences the formation of volatiles in lettuce. In **paper II**, lettuce leaves were cut longitudinally and transversely to the mid rib. Transverse cutting was found to be a more severe method of preparation than longitudinal cutting, based on increased levels of volatiles produced through the LOX pathway.

3.3. Washing

The objective of washing is to remove exudates from cut tissue as well as soil and other possible contaminate, and to reduce the temperature of the produce (Kader and Saltveit, 2003). It is noteworthy that sanitizing agents such as chlorine are used in the wash water, mainly to reduce the microbial load in fresh-cut vegetables (Soliva-Fortuny and Martin-Belloso, 2003). Special attention has been appointed in the reduction of browning by the use of chlorine and warm water in the washing step (Baur et al., 2004; Delaquis et al., 2000). However the aroma of cut lettuce was affected negatively (Delaquis et al., 2000).

In Denmark the minimally processed vegetable industry doesn't use sanitizing agents (personal communication, Gasa) due to concern about the formation of harmful disinfection by the presence of products such as chloroamines and trihalometanes (Simons and Sanguansri, 1994). Instead, cold tap water is widely used to clean and sanitize the product and there has not been any indication of sanitation problems yet (personal communication, Gasa).

3.4. Centrifugation

The aim of centrifugation is to remove the excess of water retained by the product during washing (Moretti et al., 2007). Centrifugation is widely used in fresh-cut industry, but other methods such as vibration screen and forced air tunnel can be used for water removal (Bolin and Huxsoll, 1990; Moretti et al, 2007). For lettuce, it has been reported that slight desiccation of the produce improved the shelf-life of lettuce (Bolin et al., 1977; Bolin and Huxsoll 1991). However, an over-centrifugated product can increase the mechanical damage in the tissue i.e. cracks and crush, speeding up the loss of quality (Toole et al., 2000; Saltveit, 2003; Moretti et al., 2007).

3.5. Packaging

Modified atmosphere packaging (MAP) has been successfully used in cut lettuce to reduce browning by creating an atmosphere with low O₂ and high CO₂ (relative to air) and by storing at specific cold temperature (Zagory, 1998; Soliva-Fortuny and Martin-Belloso, 2003). Modified atmosphere can be created actively or passively. Active MAP is build up by flushing out air with a gas mixture (O₂, CO₂ and N₂) into the bag prior to sealing (Zagory, 2000). Passive MAP is developed by the interaction of packaging film gas permeability and respiration of the product (Talasila and Cameron, 1997). Passive MAP is used in the fresh-cut industry in Denmark. Since passive MAP depends on respiration rate of the commodity and film, it is important to choose a film that allows O₂ to enter at a rate that is consumed by the commodity and leave out the CO₂ at a rate that avoids extreme accumulation (Rakotonirainy et al., 2001; Saltveit et al., 2003). Low

density polyethylene, polyvinyl chloride and polypropylene are the main films used for packaging fruits and vegetables (Lee et al., 1996; Kader and Saltveit, 2003).

MAP has been successfully used for the reduction of enzymatic browning in cut lettuce (Heimdal et al., 1995; Smyth et al., 1998). However, extremely low O₂ and high CO₂ levels in the package can produce undesirable odours, alongside with a product with good appearance (Heimdal et al., 1995).

In **paper IV** and **paper V** lettuces were minimally processed and stored under three different treatments: two passive modified atmosphere packaging (MAP) built up by films of different permeabilities, F1 (OPALEN 65 AF) and F2 (OPP/PE-L 2040 AF), and storage in air. In table 4 is shown the different characteristics of film F1 and film F2. It was found that extremely low O₂ (<0.05%) and high CO₂ (>20%) built up by passive MAP F1 did not allow the formation of browning, however odorants likely to be off-odours increased after 11 days of storage. Moreover, the tolerance to extreme atmosphere for the formation of off-odours can change with the season and cultivar (Smyth et al., 1998). That was observed in **paper II** which is further discussed in Chapter 4.

Table 4. Characteristics of the films used in paper IV and V

Treatment	Film material	Transmission CO ₂	Transmission O ₂	Type of atmosphere
Passive MAP F1	OPALEN 65 AF (65 µm)	158	35	Passive MAP
Passive MAP F2	OPP/PE-L 2040 AF (60 µm)	Unknown	68	Passive MAP

The permeance data of the two films was provided by the manufacturer Bemis, Denmark. Transmission rate CO₂ and O₂ (cm³ m⁻² 24 h⁻¹ atm⁻¹), at 23 °C and 50% RH for film F1 and at 23 °C and 85% RH for film F2.

OPALEN: oriented polyamide/polyethylene.

OPP/PE-L: oriented polypropylene/linear low-density polyethylene.

MAP: Modified atmosphere packaging.

Chapter 4. Aroma of minimally processed lettuce

4.1. Volatile compounds and odorants identified in minimally processed lettuce

Most plant volatiles are lipophilic liquids with high vapor pressure (Pichersky et al., 2006). A total of 77 volatiles have been identified in this commodity (Table 5). Aldehydes, alcohols and terpenes constituted the main groups of volatiles. As observed in table 2, **paper II, III and IV** provide a major contribution of the number of volatiles identified in lettuce to our knowledge. Differences to other studies could be laid in differences of the analytical method as well as differences in the setup of the experiment, such as cultivar, growing conditions and packaging. Among the volatiles found, 20 were identified by GC-O as potent odorants in lettuce (Nielsen and Poll, 2006; Lonchamp et al., 2009; **paper IV**). Potent odorants are volatiles that contribute to the perceived aroma of food (Forney et al., 2000; Belitz et al., 2004). The contribution of a volatile to the aroma depends on its odor threshold and concentration in the food (Forney et al., 2000; Belitz et al., 2004). Cis-3-hexenal, cis-3-hexenol, trans-2-hexenal, 2-ethyl-1-hexanol, elemene, caryophyllene, copaene and (+) cyclosativene and 2-methoxy-3-isopropylpyrazine contributed to the green notes in lettuce (Nielsen and Poll, 2006; Lonchamp et al., 2009 **paper IV**). **Paper IV** showed that the number of odorants increased during storage time in packed cut lettuce. 2,3-Butanedione, 2-methylpropanal, hexanal, benzothiazole, β -selinene and five unknowns were detected only after 11 days of storage (Table 6). These compounds were most probably contributors to off-odour due to their sweet, rancid, unpleasant and spoiled vegetables aroma notes. Likewise, off-odour can arise from changes in the concentration of desirable compounds (Belitz et al., 2004; Poll et al., 2006). For example, in **paper II** was found that caryophyllene and/or elemene contributed to the leafy aroma of lettuce after 1 day of storage, but were after 11 days perceived as strong chemical due to higher concentration. The use of and/or indicates an uncertainty of which of both compounds was responsible for the odour due to problem

with identification between GC-MS and GC-O data. Off-odour can be described as an unpleasant odor that is not characteristic of lettuce aroma.

Table 5. Some volatiles compounds identified in iceberg lettuce

<i>VOLATILE COMPOUNDS</i>	<i>IDENTIFICATION</i>	<i>LITERATURE</i>
<i>Aldehydes</i>		
2-methylpropanal	GC-MS, GC-O	p(II); p(IV)
2/3-methylbutanal	GC-MS, GC-O	p(II); p(IV)
hexanal	GC-MS, GC-O	p(II); p(IV)
cis-3-hexenal	GC-MS, GC-O	a; b; p(II); p(IV)
trans-2-hexenal	GC-MS, GC-O	p(II); p(IV)
propanal	GC-MS	p(II); p(IV)
2-propenal	GC-MS	p(II); p(IV)
pentanal	GC-MS	p(II); p(IV)
Heptanal	GC-MS	p(II)
octanal	GC-MS	p(II); p(IV)
nonanal	GC-MS	p(II); p(IV)
2,4-hexadienal	GC-MS	p(II); p(IV)
decanal	GC-MS	p(II); p(IV)
acetaldehyde	GC-MS	e
benzaldehyde	GC-MS	p(II); p(IV)
<i>Alcohols</i>		
Ethanol	GC-MS	e
1-butanol	GC-MS	p(II); p(IV)
1-penten-3-ol	GC-MS	p(II); p(IV)
cis-3-hexenol	GC-MS, GC-O	a; b; p(II); p(IV)
1-propanol	GC-MS	p(II); p(IV)
2-methyl-1-propanol	GC-MS	c; p(II); p(IV)
2-methoxypropoxy-2-propanol	GC-MS	p(II)
1-pentanol	GC-MS	p(II); p(IV)
1-hexanol	GC-MS	p(II); p(IV)
trans-2-hexen-1-ol	GC-MS	p(II); p(IV)
2-ethyl-1-hexanol	GC-MS, GC-O	e; p(II), p(IV)
octanol	GC-MS	p(II); p(IV)
nonanol	GC-MS	p(II)
phenol	GC-MS	p(II); p(IV)
2-phenoxyethanol	GC-MS	e
3-methylbutanol	GC-MS	c
2-methylbutanol	GC-MS	c

3-methyl-1-pentanol	GC-MS	c
Terpenes		
caryophyllene and or elemene	GC-MS, GC-O	e, p(II); p(IV)
α -pinene	GC-MS	p(II); p(IV)
limonene	GC-MS	p(II); p(IV)
p-cymene	GC-MS	p(II); p(IV)
terpinolene	GC-MS, GC-O	d; p(II); p(IV)
α -humulene	GC-MS	p(II); p(IV)
α -selinene	GC-MS	p(II); p(IV)
β -selinene	GC-MS, GC-O	p(II); p(IV)
α -longipinene	GC-MS, GC-O	e; p(II)
α -muurolene		p(II)
copaene	GC-MS, GC-O	e
(+) cyclosativene	GC-MS, GC-O	e
(E)- α -bisabolene	GC-MS, GC-O	d
germacrene	GC-MS, GC-O	d
valencene	GC-MS, GC-O	d
Ketones		
2,3-butanedione	GC-MS, GC-O	p(IV)
3-hydroxy-2-butanone	GC-MS	p(IV)
2-butanone	GC-MS	p(II); p(IV)
6-methyl-5-hepten-2-one	GC-MS	p(II); p(IV)
acetophenone	GC-MS	p(II); p(IV)
Geranylacetone	GC-MS	p(II)
Acids		
acetic acid		
propanoic acid	GC-MS	p(II); p(IV)
butanoic acid	GC-MS	p(II); p(IV)
2-methyl butyric acid	GC-MS	p(II); p(IV)
hexanoic acid	GC-MS	p(II); p(IV)
Sulfur compounds		
dimethyl sulfide	GC-MS, GC-O	p(II); p(IV)
dimethyl sulfoxide	GC-MS	p(II); p(IV)
benzothiazole	GC-MS, GC-O	p(II); p(IV)
Esters		
ethyl formate	GC-MS	p(IV)
ethyl acetate	GC-MS	c; p(II); p(IV)
cis-3-hexenyl acetate	GC-MS	a; b; p(II)
propanoic acid ethyl ester	GC-MS	c
2-methylpropanoic acid ethyl ester	GC-MS	c
butanoic acid ethyl ester	GC-MS	c
3-methylbutanoic acid ethyl ester	GC-MS	c

3-methylbutylacetate	GC-MS	c
hexanoic acid ethyl ester	GC-MS	c
Pyrazine		
2-methoxy-3-isopropyl pyrazine	GC-MS, GC-O	p(II); p(IV)
Furans		
2-ethylfuran	GC-MS	p(II); p(IV)
2-pentylfuran	GC-MS	c; p(II); p(IV)
Miscellaneous		
ethyl tiglate	GC-MS	p(IV)
styrene	GC-MS	p(IV)

a: Arey et al., (1991); b: Charron et al., (1996); c: Smyth et al., (1998); d: Nielsen and Poll (2006); e: Lonchamp et al., (2009), p(IV): paper II, p(IV): paper IV.

Table 6. Some potent odorants found in packaged cut lettuce stored at 5 °C at 1 and 11 days of storage in September 2009 (**paper IV**).

Volatile compounds	Odor descriptor	
	1 day of storage	11 days of storage
2/3-methylbutanal	sweet, cacao	strong sweet, cacao
hexanal	n.d.	lettuce, fruity
trans-2-hexenal	vegetables	unpleasant, fatty
cis-3-hexenol	alcohol, chili, soil	soil, weak grass, tea
elemene and/or caryophyllene	lettuce, grass, flower, soil	strong chemical, grass, chili
dimethyl sulfide	boiled broccoli, shellfish	off-odour, broccoli, shellfish
2,3-butanedione	n.d.	sweet, caramel

n.d. = not detected

4.2. Plant biosynthetic pathways of volatile compounds

Volatiles in lettuce as well as in vegetables and fruits originate from fatty acids, amino acids and carbohydrate groups (Salunkhe and Do, 1976; Schwab et al., 2008). There is a lack of information on biosynthesis of volatiles in lettuce. It is assumed that volatiles in lettuce are formed through metabolic routes similar to those previously identified in tomatoes, carrots, broccoli, onion and other crops (Salunkhe and Do, 1976, Chin and Lindsay, 1993; Toivonen, 1997; Baldwin et al., 2000; Belitz et al., 2004; Reineccius, 2005). Differences in the volatile profile between lettuce and other vegetable crops, suggest that differences in the availability of substrate and or enzyme that determine the type and amount of formed volatiles exist, indicating that each vegetable specie is

capable of synthesizing their own characteristic volatile pattern (Salunkhe and Do, 1976, Pichersky et al., 2006). Likewise, in **paper II** and **IV** were found that mature lettuce produces a higher level of volatiles than over-mature lettuce. Moreover, differences in metabolic behavior between inner leaves (old leaves) and outer leaves (young leaves) and photosynthetic and vascular tissue could also influence in the formation of volatiles, as suggested in **paper II**. A better understanding of mechanisms of formation of volatiles in lettuce will provide a tool to control the aroma of lettuce.

The following section describes the possible routes for the formation of the main volatiles identified in this study.

4.2.1. Aldehydes and alcohols

Aldehydes can be formed from fatty acids that are oxidised via the lipoxygenase (LOX) pathway (Baldwin et al., 2000; Belitz et al., 2004). The pathway starts with the oxidation of linoleic or linolenic acid by LOX enzyme in presence of oxygen (Galliard et al., 1977). The mechanism of action is summarized in three steps: first, the activation of the native enzyme by the oxidation of Fe^{2+} to Fe^{3+} , subsequently, the removal of an H^+ from the substrate molecule complexed with the enzyme and finally the insertion of O_2 in the linoleic or linolenic acid at the position of carbon 9 or 13, resulting in 9-hydroperoxide or 13-hydroperoxide (Gardner, 1988; Robinson et al., 1995). As observed in Fig.7, the breakdown of hydroperoxides is further catalyzed by hydroperoxide lyase (HPL) to hexanal and cis-3-hexenal (Galliard et al., 1977; Baldwin et al., 2000). Likewise, cis-3-hexenal can be isomerized to trans-2-hexenal, either enzymatically or nonenzymatically (Galliard et al., 1977; Feussner and Wasternack, 1998; Baldwin et al., 2000). cis-3-Hexenal and trans-2-hexenal contributed to the green leafy aroma in lettuce (**paper IV**).

Aldehydes can also derive from branch-chain amino acids (Fig.8) (Belitz et al., 2004; Schwab et al., 2008). The reaction is initiated by aminotransferase forming 2-keto acid, which is further decarboxylated to produce branched chain aldehydes (Ardö, 1996; Marielly and Casey, 2004). Amino acids leucine, isoleucine and valine are converted to 3-methylbutanal, 2-methylbutanal and 2-methylpropanal, respectively (Ardö, 1996). The

sweet odor notes of these volatiles probably contributed to the off-odour in packaged cut lettuce after 11 days of storage (**paper IV**). Aldehydes can be reduced to their respective alcohols by the action of the enzyme alcohol dehydrogenase (Baldwin et al., 2000, Belitz et al., 2004). 1-Penten-3-ol and cis-3-hexenol were found to be potent odorants in fresh-cut lettuce (**paper IV**).

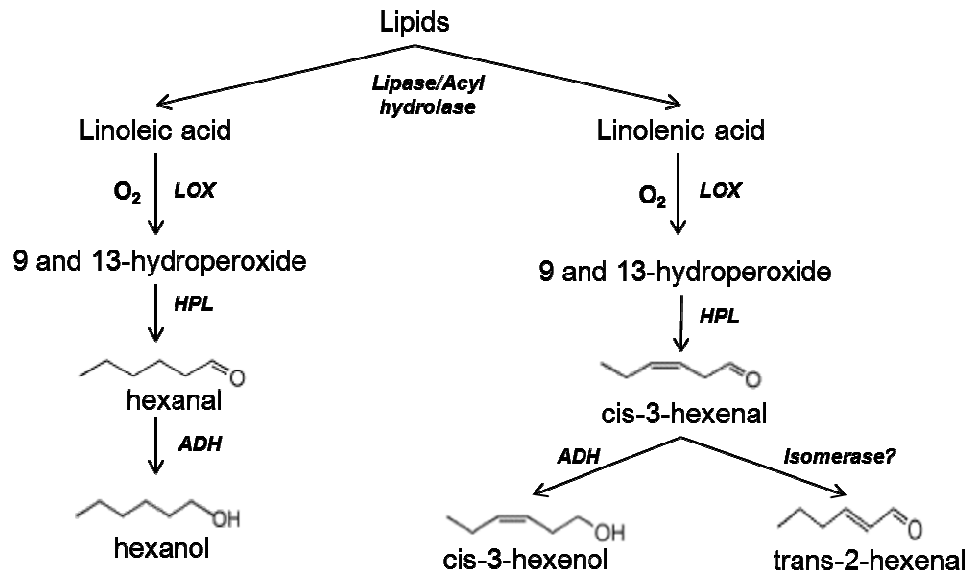


Figure 7. LOX pathway for the formation of C6 aldehydes and alcohols from degradation of lipids in plants (Gallaird et al., 1977; Gardner, 1995; Riley et al., 1996; Baldwin et al., 2000). LOX= lipoxygenase; HPL= hydroperoxide lyase; ADH= alcohol dehydrogenase.

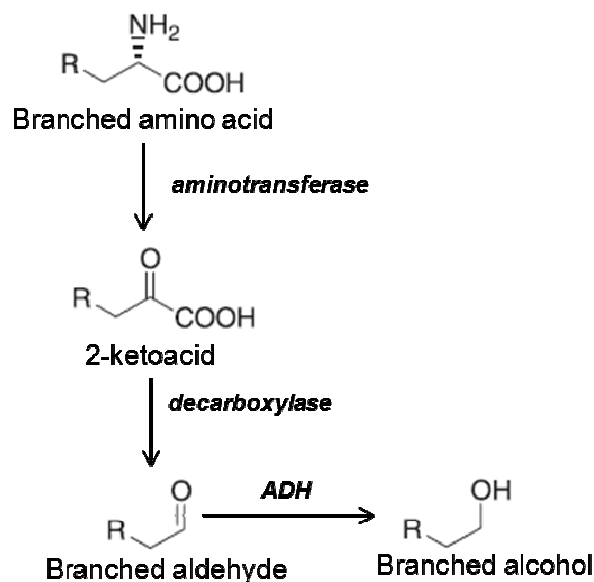


Figure 8. Pathway for the formation of branched aldehydes and alcohols from branched amino acids (Schwab et al., 2008). ADH= alcohol dehydrogenase.

4.2.2. Terpenes

Terpenes in the form of sesquiterpenes, such as caryophyllene, elemene and β -selinene contributed to the leafy aroma of cut lettuce (**paper IV**, Lonchamp et al., 2009). Sesquiterpenes are formed via the mevalonate (MVA) pathway in the cytosol (Pichersky et al., 2006; Tholl, 2006). The condensation of two molecules of isopentenyl pyrophosphate (IPP) and one molecule of its isomer dimethylallyl pyrophosphate (DMAPP) forms farnesyl pyrophosphate (FPP), which is subsequently catalyzed to sesquiterpenes by a terpene synthetase (Fig.9). In the plastids the methylerythritol phosphate (MPE) route also produces IPP and DMAPP molecules and is responsible for the formation of monoterpenes. Under certain conditions, such as stress, IPP and DMAPP molecules from this route can be transported from plastids to cytosol for the formation of sesquiterpenes (Piel et al., 1998; Hampel et al., 2004). It is believed that similar behavior might occur in packaged cut lettuce and cause the increase of caryophyllene and/or elemene under anaerobic conditions (**paper IV**).

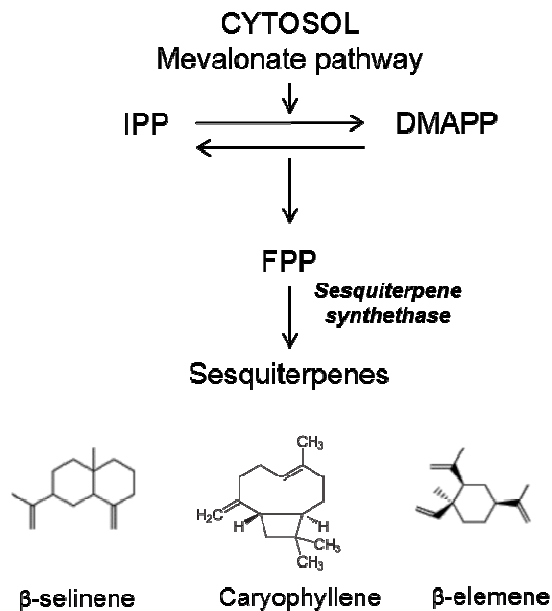


Figure 9. Pathway for the formation of terpenes in plants (Pichersky et al., 2006; Tholl, 2006)

4.2.3. Volatiles from anaerobic metabolism

MAP with extremely low O_2 (<1%) and high CO_2 (>20%) may lead to anaerobic respiration with the formation of off-odours (Kader, 1997). In our study 2,3-butanedione, 3-hydroxy-2-butanone, ethyl acetate and ethyl formate were identified as volatiles from anaerobic metabolism (**paper III** and **IV**). Among them only 2,3-butanedione was identified by GC-O (**paper IV**), which likely contributes to the off-odour in cut lettuce due to its low odor threshold (5ppb) (Burdock, 2005). Previous work by Smyth et al. (1998) identified ethanol, acetaldehyde and short-chain methyl-branched alcohols as volatiles from extremely low O_2 in packaged cut lettuce.

The formation of these volatiles under anaerobic conditions is the result of accumulation of pyruvate by the Emden-Meyerhof pathway in the cytoplasm. Pyruvate is then converted to acetaldehyde by pyruvate decarboxylase and further to ethanol by the action of alcohol dehydrogenase. The high level of CO_2 reduces the pH of the cytoplasm, which enhances the activity of the enzymes previous mentioned (Siriphanich and Kader, 1986; Ke et al., 1994). As a result an increasing level of ethanol may also stimulate the

formation of ethyl esters such as ethyl acetate and formate (Larsen, 1994; Forney et al., 2000).

4.3. Analytical methods for volatile analysis in lettuce

In **paper II, III** and **IV** volatiles were isolated from homogenized lettuce using dynamic headspace sampling. For separation and identification of volatiles in cut lettuce a gas chromatography (GC) coupled with a mass spectrometry (MS) was used. However, this technique does not provide information of the contribution of an individual compound to the aroma of lettuce. To this end, gas chromatography olfactometry (GC-O) was used. Table 7 shows the principle and the aim behind the analysis of volatiles. Further description of the operation parameters of the GC-MS and GC-O (FID) equipment can be found in **paper II** and **IV**.

Table 7. Principles of the analytical techniques for volatile analysis in lettuce (**paper II, IV**).

Technique	Aim	Principle
Dynamic headspace sampling	Isolation of volatiles (purge and trap)	Sample volatiles were constantly purged from an aqueous matrix to a trap (Tenax), which allowed the enrichment of more volatiles from the sample, increasing sensitivity of the analysis. Volatiles trapped in an adsorbent were thermally desorbed direct to the GC.
Gas chromatography-mass spectrometry (GC-MS)	GC Separation	Volatiles were swept by a carrier gas (mobile phase) through the column. Stronger interaction between the volatile and the column inner surface (stationary phase) led to longer retention time. This interaction is temperature dependent. For an adequate separation of the volatiles the GC was operated in a temperature range between 40 and 240 °C.
	MS Identification	Each volatile eluted direct to the detector. There, the volatile was ionized and fragmented and the ions formed were separated according to their mass/charge ratio. The output from the analysis was a mass spectrum which was used for identification.

Gas chromatography-Olfactometry (GC-O)	To determine the potent odorants in a sample	GC-O was performed in a conventional GC equipped with a sniffing port. The flow from the column was split between the detector (flame ionizant detector) and the sniffing port, where the human nose was used as a detector, providing simultaneous detection of the potent odorants. From each individual volatile that elute from the port, the judge was instructed to describe the odour and retention time.
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In GC-O analysis, the human nose is used as a detector (Pollien et al., 1997). It is known that the olfactory system is more sensitive than any physical detector e.g. FID and MS to some compounds (Delahunty et al., 2006).

For the identification of the potent odorants in **paper IV**, the samples were run in duplicates, with the GC-O and GC-MS under the same conditions. Likewise, a standard mixture of references was run in the GC-MS and GC-O equipment. The GC-O retention time of the sample and GC-O and GC-MS retention time of the reference mixture was interpolated to found the potent odorant in the GC-MS chromatogram of the sample. Once the potent odorants were identified in the GC-MS chromatogram, their retention time was plotted against the retention time of the GC-O for the same sample, see Fig.10. As can be seen in Figure 10 all points fall within the tendency, soft s-shape, which confirms that the identification of potent odorants was as expected. The use of and/or in elemene and/or caryophyllene indicates an uncertainty of which of both compounds was responsible for the odour.

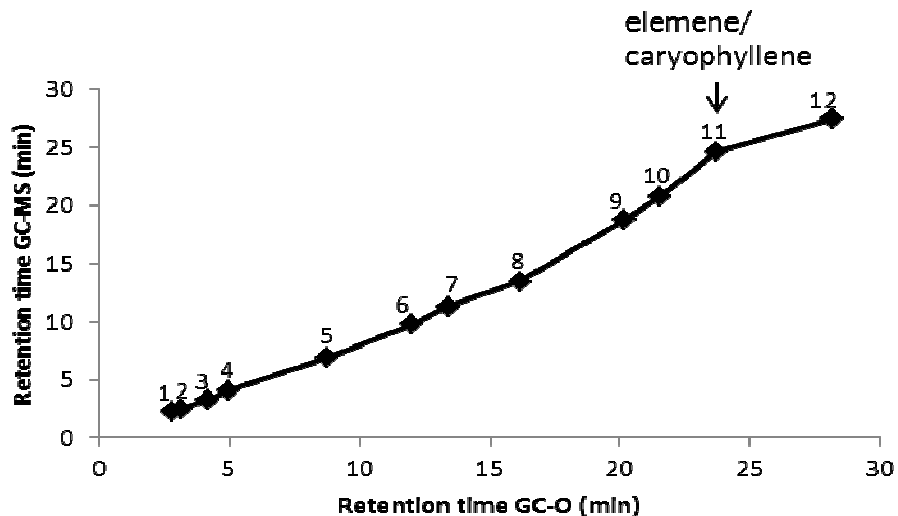


Figure 10. Plot of GC-MS retention time vs. GC-O retention time for the identification of potent odorants. Abbreviations: 1: dimethyl sulfide, 2: 2-methylpropanal, 3: 2/3-methylbutanal, 4: 2,3-butanedione, 5: hexanal, 6: cis-3-hexenal, 7: 1-penten-3-ol, 8: trans-2-hexenal, 9: cis-3-hexenol, 10: 2-methoxy-3-isopropylpyrazine, 11: elemene/caryophyllene, 12: β-selinene.

Therefore, identification of the potent odorants was also examined by using retention index (RI). Figure 11 shows the plot of retention index against the GC-MS retention time of some identified potent odorants. Retention index was taken from literature, (Sumitami et al., 1994; Cha and Cadwallader, 1998; Le Guen et al., 2000; Ruther, 2000; Pennarum et al., 2003; Varming et al., 2004; Gancel et al., 2005; Chen et al., 2009; Mesa-Arango, 2009) where DBWAX column was used. From Figure 11, it can be seen that elemene and caryophyllene elute too close as such correctly identified. Elemene and caryophyllene are terpenes with similar aroma descriptions in literature (www.flavournet.org) that correspond to the description provided by the judges. Therefore, due to uncertainty to discriminate which of them produce the odor it was decided to use and/or for these compounds.

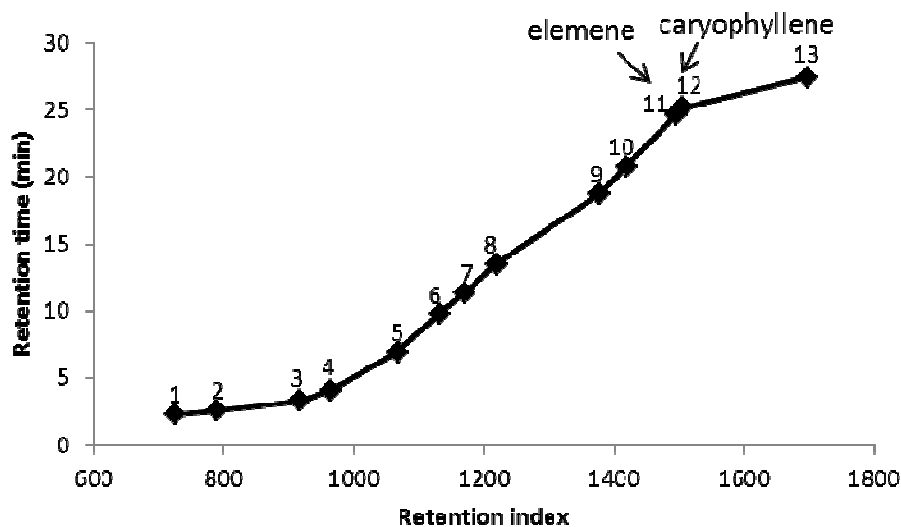


Figure 11. Plot of GC-MS retention time and RI of potent odorants. *Abbreviations: 1: dimethyl sulfide, 2: 2-methylpropanal, 3: 2/3-methylbutanal, 4: 2,3-butanedione, 5: hexanal, 6: cis-3-hexenal, 7: 1-penten-3-ol, 8: trans-2-hexenal, 9: cis-3-hexenol, 10: 2-methoxy-3-isopropylpyrazine, 11: elemene, 12: caryophyllene, 13: β-selinene.*

4.4. Effect of important factors in the formation of volatiles in cut lettuce

Cultivar, season, method of preparation, packaging, temperature and storage time influence the type and concentration of volatiles in vegetables (Smyth et al., 1998; Hodges and Toivonen, 2008, **paper II, III, IV**).

Few studies are done on the formation of volatiles in fresh-cut lettuce. Among them, the investigations were focused on the effect of packaging, storage temperature and time (Smyth et al., 1998; Lonchamp 2009). In **paper IV** a more integrated study was undertaken for first time in lettuce to our knowledge. The study took into account the influence of cultivar, season as well as packaging, storage temperature and time. Likewise, the potential of producing volatile compounds due to the direction of the cut (method of preparation) in combination with storage temperature was discussed for first time in **paper II**.

In this chapter, **paper II, III** and **IV** will be outlined for the discussion regarding factors that affect the volatile compounds in lettuce.

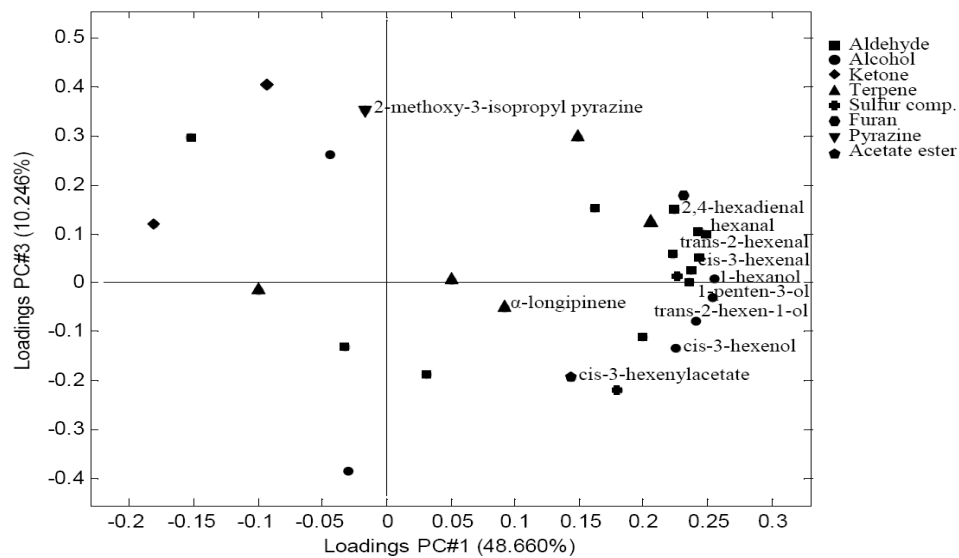
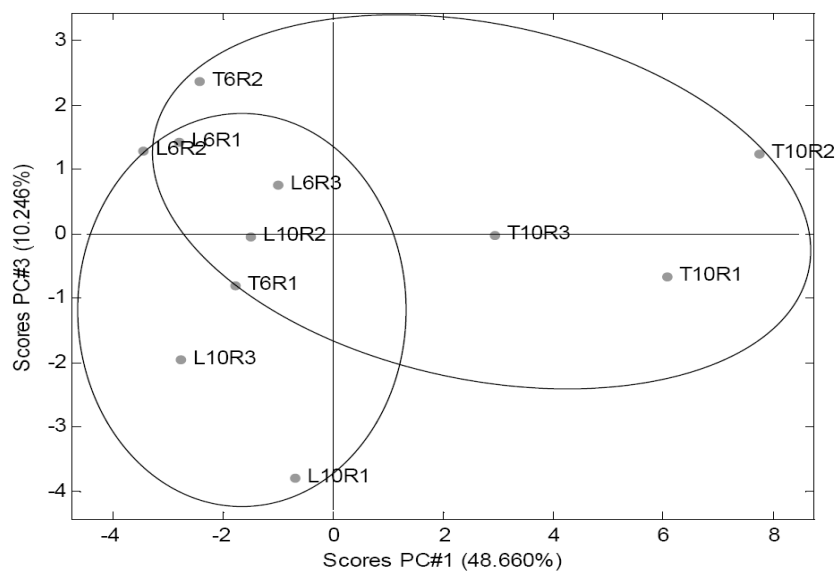
4.4.1. The effect of the method of preparation and temperature of storage

Paper II: The effect of cutting direction on the aroma compounds of fresh-cut iceberg lettuce

The cutting direction in combination with higher storage temperatures appears to play a significant role in the formation of volatiles in lettuce. In **paper II** iceberg lettuce was cut longitudinal and transverse to the mid-rib and stored in air at 6 and 10°C for 4 days in January 2008 and 5 days in March 2008. Volatiles were isolated at the end of the storage using dynamic headspace sampling and identified by GC-MS.

A PCA plot (Fig. 12a) of data from January 2008 shows that volatiles from the LOX pathway were higher in the transverse cut samples stored at high temperature. Particularly, 1-penten-3-ol, hexanal, hexanol, 2,4-hexadienal and trans-2-hexenal, were found to be significantly higher when lettuce was cut transversely and stored at 10°C ($p \leq 0,05$). Among the compounds, trans-2-hexenal was the most affected. It increased up to 10 times more than other LOX volatiles. Furthermore, in the March 2008 data (Fig. 12b), cis-3-hexenal and cis-3-hexenol were also strongly associated with transverse cutting but at 6 °C, while trans-2-hexenal, trans-2-hexen-1-ol, 2-ethyl-1-hexanol, 2,4-hexadienal, hexanal, 1-hexenol and 1-penten-3-ol seemed to be related to higher storage temperatures. LOX has been shown to be a stress-related enzyme (Hildebrand, 1989), and an increase in these compounds by cutting the lettuce in the transverse direction might indicate a greater disruption of membranes, which in combination with high storage temperature probably increased activity of enzymes related to senescence and lipid degradation such as acyl hydrolase and LOX.

a) January 2008



b) March 2008

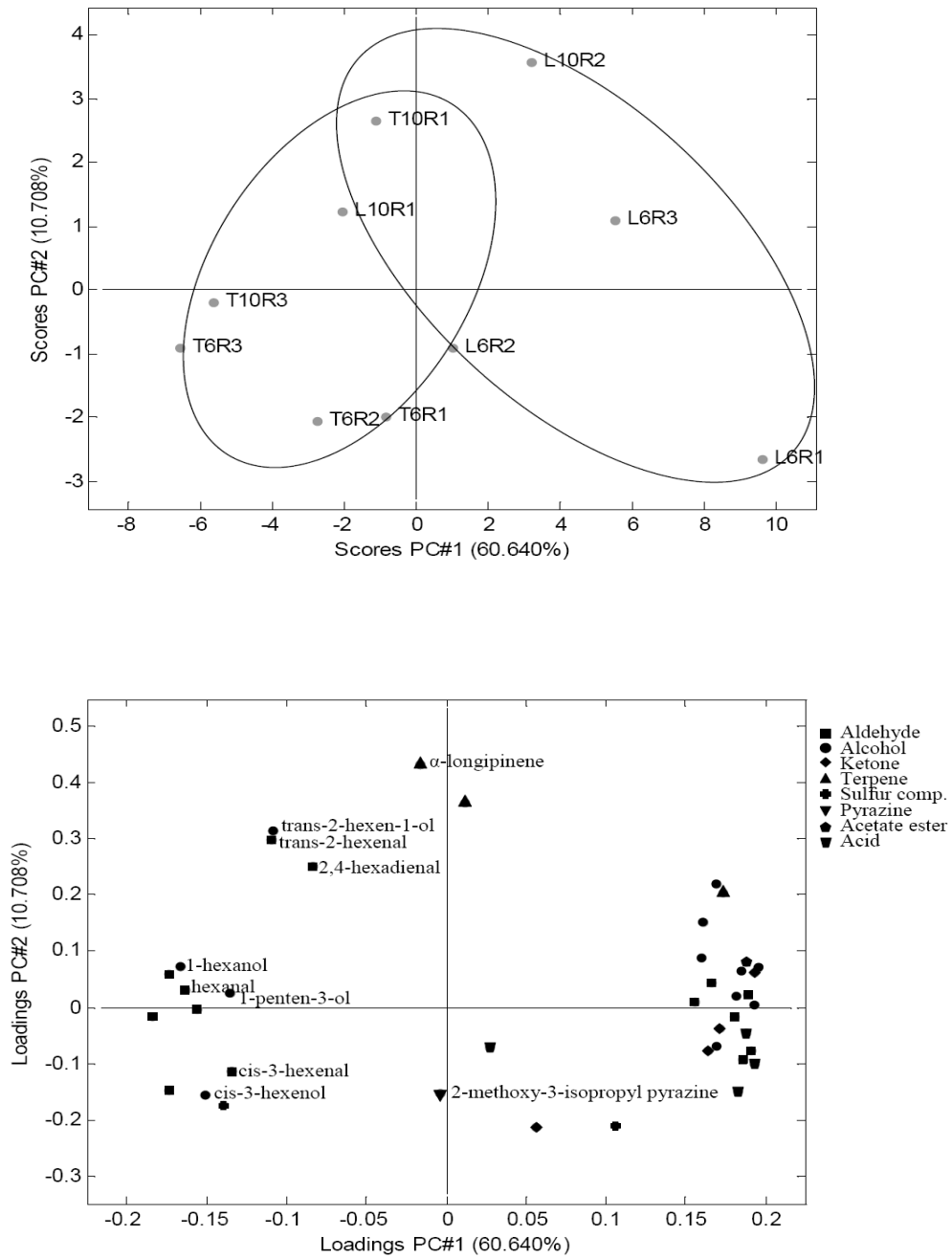


Figure 12. PCA analysis of fresh-cut lettuce cut transverse and longitudinal stored in air at 6 and 10°C after 4 days in January 2008 (a), and after 5 days in March 2008 (b). Abbreviations: T6= Lettuce cut transverse stored at 6°C, L6= Lettuce cut longitudinal stored at 6°C, T10= Lettuce cut transverse stored at 10°C, L10= Lettuce cut longitudinal stored at 10°C, Replicates= R1, R2, R3.

The state of maturity of lettuce also had an effect on the production of LOX volatiles between experiments. For example, in January, lettuce heads were mature, which means that LOX activity might be high in comparison with overmature lettuce found in March. Matsui et al. (1997) showed that LOX activity is reduced with an increase in maturity. Moreover, the diversity of volatiles from other enzymatic reactions observed in the March samples from the longitudinal cutting (Table 8) could also be influenced by the stage of maturity. Further research is needed on the metabolic routes for volatile formation in lettuce and their relation to severity of tissue damage and/or to different parts of lettuce that could have different metabolic behavior i.e. inner and outer leaves, photosynthetic and vascular tissue.

Table 8. Interaction between experiment and type of cutting on the relative area of aroma compounds of lettuce stored in air for 4 and 5 days in January and March.

<i>Experiment</i>	January 2008		March 2008	
<i>Cutting</i>	Transverse	Longitudinal	Transverse	Longitudinal
<i>Aroma compounds</i>				
2-butanone	0 ^a	0 ^a	0.03 ^a	0.06 ^b
1-butanol	0 ^a	0 ^a	0.01 ^a	0.03 ^b
1-pentanol	0 ^a	0 ^a	0.002 ^a	0.01 ^b
1,2-methoxypropoxy -2-propanol	0 ^a	0 ^a	0.02 ^a	0.05 ^b
octanol	0 ^a	0 ^a	0.0026 ^a	0.01 ^b
phenol	0 ^a	0 ^a	0.02 ^a	0.07 ^b
propanoic acid	0 ^a	0 ^a	0.0035 ^b	0.01 ^c
butanoic acid	0 ^a	0 ^a	0.01 ^a	0.03 ^b
ethyl acetate	0 ^a	0 ^a	0.01 ^a	0.02 ^b
nonanal	0.01 ^{ab}	0.01 ^a	0.03 ^b	0.05 ^c
decanal	0.01 ^a	0.01 ^a	0.01 ^b	0.03 ^c
benzaldehyde	0.01 ^a	0.01 ^a	0.02 ^a	0.03 ^b
limonene	0.02 ^{ab}	0.01 ^a	0.02 ^a	0.05 ^b

Values with different letters across a row are significantly different ($p \leq 0.05$).

4.4.2. The effect of season, cultivar, packaging and storage time

Paper IV: Effect of season, cultivar, packaging and storage time on volatile formation of iceberg cut lettuce

In **paper IV** changes in volatile compounds of minimally processed iceberg lettuce was measured as a function of season, cultivar, packaging and storage time. In order to achieve this, iceberg lettuce cultivars Platinas, Diamantinas and Morinas were harvested from June to September 2009. Lettuces were minimally processed and stored under three different treatments: two passive modified atmosphere packaging (MAP) built up by films of different permeabilities, F1 (OPALEN 65 AF) and F2 (OPP/PE-L 2040 AF), and storage in air. All packages were stored at 5°C. Volatile compounds were assessed at 1, 5, 8 and 11 days of storage in packaged lettuce, whereas in air stored samples volatiles were analyzed only at 1 and 5 days of storage. Additionally, GC-O analysis was undertaken after 1 and 11 days of storage for cultivar Morinas packaged in passive MAP F2 in September.

Fifty two volatiles were identified in this study; of these 21 including 8 unknowns were identified by GC-O as potent odorants. A PLS-DA was done to discriminate the odorants within season. To this end a model was developed using the 13 potent odorants identified by GC-O and gas composition of passive MAP and air stored samples of cultivars Platinas, Morinas and Diamantinas stored for up to 11 days in June, July, August and September 2009 (Fig. 13). From the score plot can be seen that PC1 and PC2 discriminate within season. Changes in potent odorants within season could be attributed to climatic conditions, which could explain their position along PC1. In the other hand, lettuces harvested in June and August were mature in comparison with over-mature lettuces harvested in July and September, which might have attributed their position along PC2. In order to understand the formation of odorants within season, a comparison between months with lettuces with same maturity was undertaken, as shown in the loading plot of Fig. 13. For examples, in June cooler temperatures and long days may have promoted the synthesis of amino acid such as valine and s-methylmethionine for the formation of 2-methoxy-3-isopropyl pyrazine and DMS. In contrast, August was mainly

characterized by having high air temperature. This condition stressed the lettuce by the fact of a high metabolism was found in August. This could reduce the tolerance of cut lettuce to anaerobic conditions and accelerate the deterioration, resulted in an enhancement of 2, 3 butanedione, caryophyllene, β -selinene and elemene, which are likely to be off-odours. This indicates that probably it would be more difficult to maintain low production of potent odorants likely to be off-odours in August.

Figure 14 shows the score and loading plot of a PLSDA for discrimination between storage time. From the score plot, it can be seen that all the samples were displaced clockwise from day 1 to day 11 of storage. The displacement of samples evidenced changes in the amount of potent odorants as storage time increased. After 1 day of storage, aerobic conditions predominated in the passive MAPs and air stored samples, which seemed to increase the level of cis-3-hexenol. As storage time increased the level of O₂ decreased and the CO₂ content increased in the passive MAPs. Therefore, most of the odorants seemed to increase after 8 days of storage, after being exposed to extremely low O₂ and high CO₂. Further exposition, up to 11 days, enhanced the formation of odorants that were described as unpleasent such as 2,3-butanedione, β -selinene, caryophyllene and elemene. Changes in the formation of odorants as storage time increased could be the result of membrane deterioration by prolonged exposition to extremely low O₂ and high content, mainly developed in passive MAP F1 which enhanced the formation of odorants likely to be off-odours (Chin and Lindsay, 1993).

In general, the results indicated that mainly season and storage time and in less degree cultivar influence the formation of potent odorants packaged and air stored cut lettuce. Among cultivars, differences in the formation of individual potent odorants were not significant, with exception of 2,3-butanedione which was significantly higher in the cultivars Morinas and Diamantinas after 11 days of storage in passive MAP F1 in August ($p \leq 0.05$).

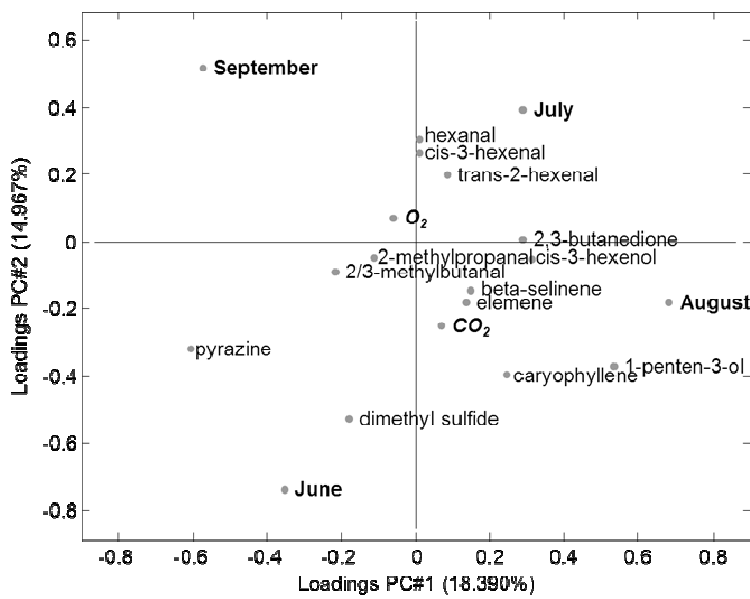
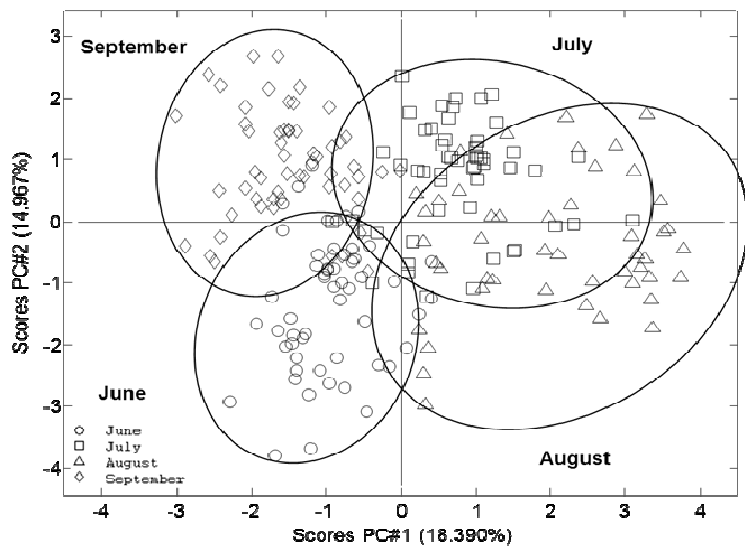


Figure 13. A PLS-DA score and loading plots of potent odorants and gas composition from passive MAP and air stored samples of cultivars Platinas, Morinas and Diamantinas stored for up to 11 days at 5 °C in June, July, August and September 2009.

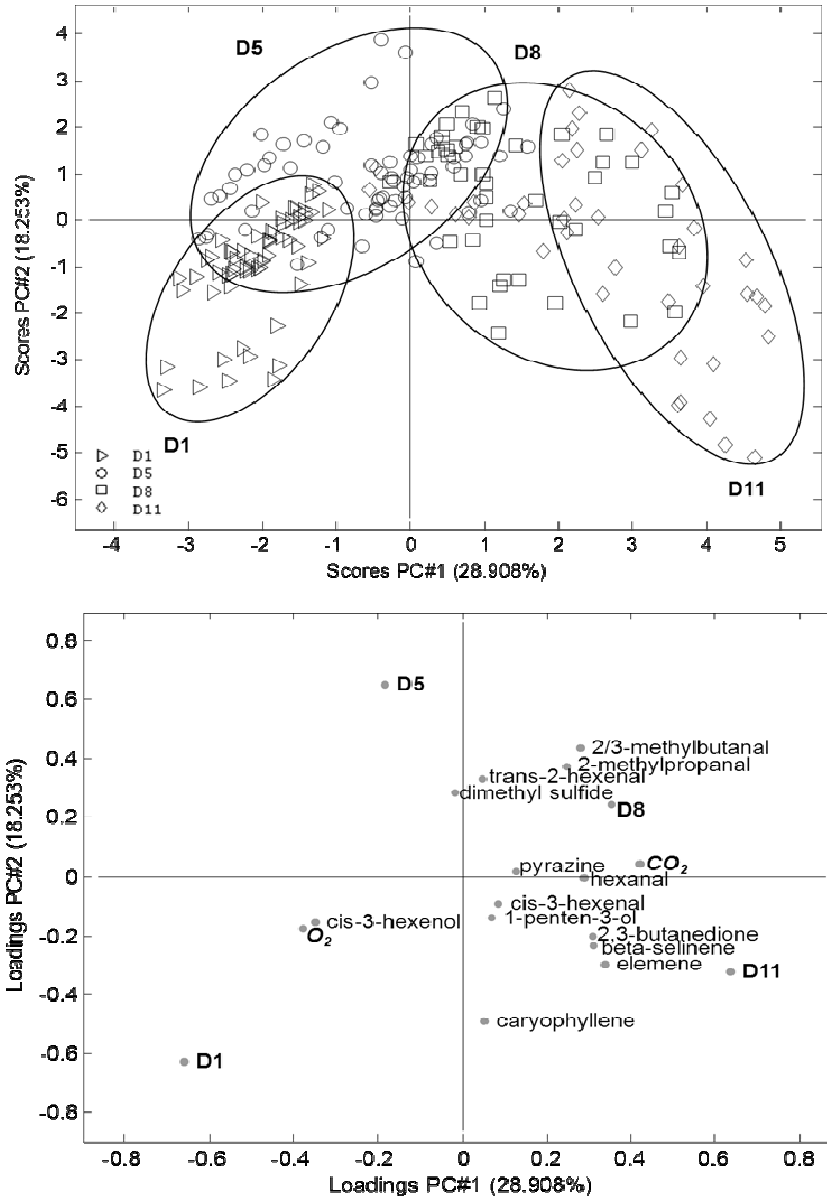


Figure 14. PLS-DA score and loading plots of odorants and gas composition of data from passive MAPs and air stored samples of cultivars Platinas, Morinas and Diamantinas harvested in June, July, August and September 2009 stored for up to 11 days at 5°C. Abbreviations: D1, first day of storage; D5, fifth day of storage; D8, eighth day of storage; D11, eleventh day of storage.

Chapter 5. Enzymatic browning and other physicochemical characteristics of minimally processed lettuce

5.1. Formation of browning in minimally processed lettuce

Browning has been reported as the main limitation of the shelf-life of minimally processed lettuce (Heimdal et al., 1995). The mechanism of enzymatic browning in lettuce is initiated by cell damage by cutting, which allows the interaction of polyphenol oxidase (PPO) and phenolic compounds (Toivonen and Brummell, 2008). As a result, quinones are formed which react non-enzymatically with other quinones, amino acids or proteins to produce melanin pigments, responsible for the brown color in the cutting edge of lettuce (Ramirez et al., 2003; Doğan and Salman, 2007). It is noteworthy that there are other enzymes involved in this process, such as phenylalanine ammonia-lyase, which leads with the biosynthesis of phenolic acids, and peroxidase, that can also form melanines, but its role depends on the presence of H₂O₂ in the cell, which is generally very low (Lopez-Galvez, 1996; Richard-Forget and Gauillard, 1997; Fujita et al., 2006). PPO has been indicated as the key enzyme for the development of enzymatic browning (Martinez and Whitaker, 1995). In this study, special attention has been on polyphenol oxidase due to its influence in enzymatic browning.

5.2. The PPO: An overview

PPO has been isolated from different sources such as bacteria, fungi, arthropods, mammals and plants. In plants PPO has been found in the plastids in soluble form and membrane-bound (Martinez and Whitaker, 1995).

PPO is an oxidoreductase that catalyses the oxidation of phenolic acids in presence of O₂ (Ramirez et al., 2003). PPO is able to catalyze two different reactions: 1) hydroxylation of monohydroxyphenols and 2) the oxidation of *o*-dihydroxyphenols to *o*-quinone (Ramirez et al., 2003; Doğan et al., 2007). In lettuce, PPO from vascular and photosynthetic tissue is specific in their cleavage for *o*-dihydroxyphenol and can be

classified as a catechol oxidase (E.C.1.10.3.1) (Heimdal et al., 1994; Ramirez et al., 2003; Doğan and Salman, 2007).

The *o*-dihydroxyphenol oxidase activity involves the oxidation of 2 molecules of substrate to obtain 2 molecules of *o*-quinone (Ramirez et al., 2003). The proposed mechanism of oxidation of *o*-dihydroxyphenols is shown in Fig. 15. The active site of PPO has two copper atoms that show different functional states during the catalytic activity: *met*, *deoxy* and *oxy* (Ramirez et al., 2003). The mechanism of action can be summarized in 5 steps: 1) the addition of a substrate (*o*-dihydroxyphenol) binds to the *met* form [Cu(II)], 2) producing the *deoxy* form of the enzyme [Cu (I)] and a molecule of *o*-benzoquinone, subsequently, 3) the *deoxy* form bind the O₂ to give the *oxy* form [Cu(II)], 4) which then bind a molecule of catechol to give the ternary complex enzyme Cu(II).O₂. *o*-dihydroxyphenol, 5) finally two hydrogens are removed to obtain the *o*-quinone and the *met* form of the enzyme completing the cycle (Lerch, 1983; Solomon et al., 1992; Ramirez et al., 2003).

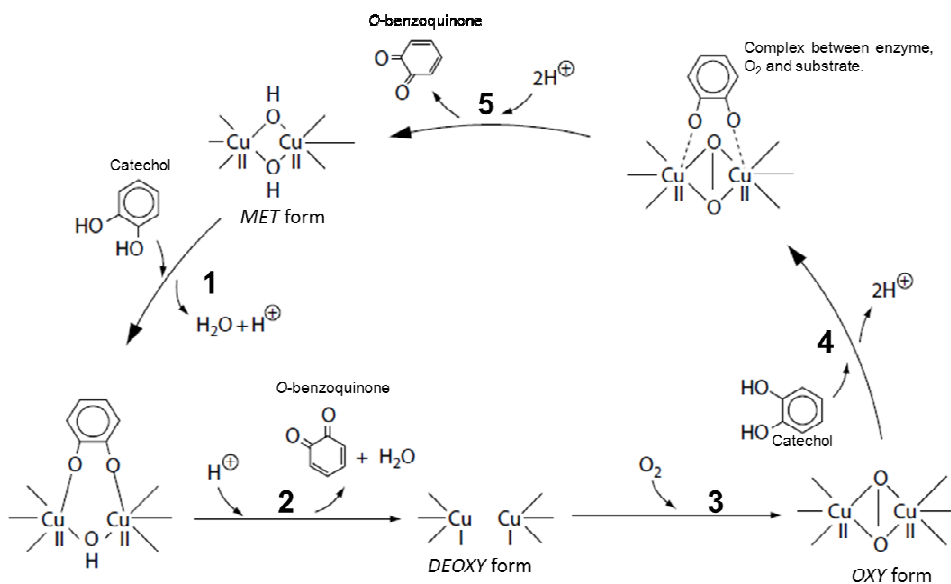


Figure 15. Proposed mechanism of action of PPO for oxidation of *o*-dihydroxyphenol using catechol as substrate (Lerch, 1983; Solomon et al., 1992; Ramirez et al., 2003).

The *o*-quinones are very reactive non-enzymatically with O₂, other quinones, sulfhydryl compounds, amines, amino acids and proteins to produce melanin pigments responsible for the brown color (Ramirez et al., 2003; Doğan and Salman, 2007).

5.3. The PPO substrates in lettuce

In lettuce potential *o*-dihydroxy substrate for PPO are chlorogenic acid (5-*O*-caffeoylquinic acid), chicoric acid (dicaffeoyl tartaric acid), isochlorogenic (dicaffeoylquinic acid) acid and caftaric acid (caffeoyl tartaric acid (Cantos et al., 2001; Baur et al., 2004; Sobolev et al., 2005). The main storage organelle of these compounds is the vacuole (Queiroz et al., 2008), but chlorogenic acid and chicoric acid have also been found located in chloroplast, epidermis and vascular bundles in lettuce (Heimdal, 1995). Once lettuce is cut, chlorogenic acid is accumulated in lettuce midrib caused by a *de novo* formation by enzyme phenylalanine ammonia lyase (PAL) (Ke and Saltveit, 1989; Cantos et al., 2001). For instance, in **paper V** was found a high content of chlorogenic acid with 87.21 mg/100g of fresh weight (FW) in cut lettuce stored in air after 5 days of storage at 5°C, whereas in packaged cut lettuce, passive MAP F2 and F1, the level was 48.98 and 35.99 mg/100g FW, respectively, probably due to differences in *de novo* biosynthesis of phenolic acids by PAL enzyme under high CO₂ (Mateos et al., 1993).

5.4. Analysis of enzymatic browning in lettuce

5.4.1. Polyphenol oxidase activity

In **paper V** a spectrophotometric method was used for the measurement of PPO activity in cut lettuce. Spectrometry methods are based on changes in absorbance of the product or substrate as a function of time (Copeland, 1996). Polarographic methods have also been used for PPO activity in lettuce (Heimdal et al., 1995). The latter method measures the oxygen depletion during the enzyme reaction (Copeland, 1996).

Irrespective of the method, the assay of bisubstrate reactions is performed as for pseudo-monosubstrate reaction (Sørensen et al., 1999). Initial velocity of PPO can be determined by maintaining one substrate, e.g. O₂, at high concentration relative to the other substrate (e.g. chlorogenic acid), where O₂ concentration can be considered constant during the reaction (Sørensen et al., 1999). During the assay, PPO is irreversibly inactivated. Inactivation is due to a free radical-catalyzed fragmentation of one or more of the six histidine residues of the enzyme that bind the two coppers at the active site (Ramirez et al., 2003).

Some characteristics of PPO of iceberg lettuce have been described by Heimdal et al. (1994) and Gawlik-Dziki et al. (2008). Heimdal et al. (1994) reported for both vascular and photosynthetic tissue, a pH optimum ranged from 5 to 8 with an optimum temperature between 25 to 35 °C, using chlorogenic acid as substrate.

5.4.2. Image analysis

Objective measurements of browning in cut lettuce have been made using colorimeters (Heimdal et al., 1995). Measurements with colorimeters usually takes several points to compensate the un-uniformly surfaces of cut lettuce and then provide an average colour, which might not represent the colour of the sample (O'Sullivan et al., 2003). Therefore, to overcome this problem, an image analysis of cut lettuce was developed in **paper I** and used for evaluations of browning in **paper V**.

For image acquisition a flatbed scanner was used, which provided a better representation of colour by the fact that the flatbed scanner captures a bigger area of the sample, around 600 times of a Minolta colorimeter. When images are captured changes in lighting conditions of the scanners could affect the colours in the image obtaining unreliable information for comparison. In order to avoid this problem, a colour correction was performed.

For colour correction a transformation was sought to each image to bring the colours of the reference patches to match a reference image. The mean RGB pixel values of each

colour patch in the image to be corrected were used to create a 20x3 matrix P_I of pixel values with one row per patch. A least square linear transformation into the corresponding matrix P_R for the reference image was obtained as follows:

$$L = [P_R^T P_R]^{-1} P_R^T P_I$$

To obtain the corrected pixel value p_c for each pixel in the image we computed:

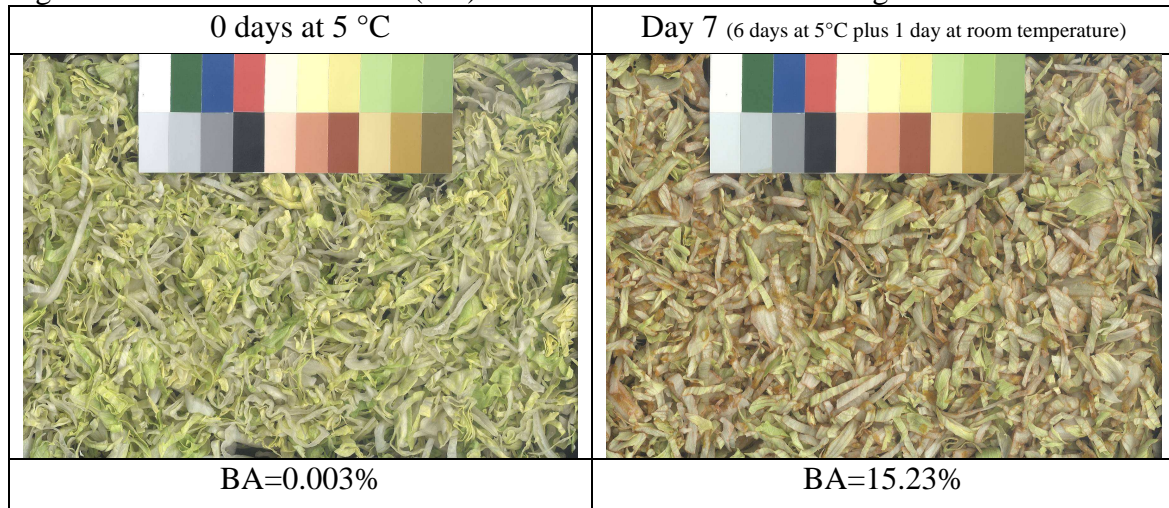
$$p_c = L * p$$

where p is the uncorrected RGB pixel value.

Once images were colour corrected they were used for further analysis. A thresholding to extract pixels of brown colour was applied to each corrected image and the brown area fraction (BA) was calculated as explained in **paper I**. Thresholding is a selection of a range of brightness, hue and saturation that allowed the extraction and quantification of the browning in the images, which was perceived as a pinking colour adjacent to the wound area.

In **paper I** it was demonstrated that image analysis is a useful technique for measuring browning in cut lettuce. This method provides a better representation of colour by using a big area of sampling and accurate quantification of browning by thresholding the colour corrected images (Fig. 16).

Figure 16. Brown area fraction (BA) of cut lettuce at different storage conditions.



5.5. Important physicochemical characteristic of minimally processed lettuce: texture, soluble sugars and organic acids

5.5.1. Firmness

Texture is defined as the sensory expression (touch, vision, and hearing) of the structural elements of a food and how this structure reacts under an applied force (Bourne, 1978). In **paper V** the texturometer with a Kramer shear cell probe was used to evaluate the texture of cut lettuce, which was expressed as firmness. Firmness was defined as the maximum force to break the sample (Prakash et al., 2000; Han et al., 2004; Baur et al., 2005; **paper V**). Firmness is influenced by the structure of the vegetable, particularly cell shape and size, cell wall thickness and strength and the extent of cell to cell adhesion, alongside with the turgor (Toivonen and Brummel, 2008). These characteristics are highly related to the type of tissue (Toole et al., 2000). For instance, lettuce has two different types of tissue, photosynthetic and vascular (veins) (Martin-Diana et al., 2006). The vascular tissue has reinforcing fibers within the parenchyma which give a major contribution to the tissue strength (Tool et al., 2000).

In cut lettuce, loss of water and degradative process of cell wall by senescence are the main causes of loss of firmness (Prakash et al., 2000; Martin-Diana et al., 2006; Martinez

et al., 2007). Loss of water could be due to the lack of cuticle and sub-epidermal layers in the cut area (Wills et al., 1982; Toivonen and Brummell, 2008). The decrease in water in the tissue leads to a loss of turgor, reducing the firmness of cut lettuce (Prakash et al., 2000; Martin-Diana et al., 2006). Water loss can be reduced by packaging (Toivonen and Brummell, 2008). However, excessive accumulation of CO₂ in the packages has shown to enhance tissue softening in packaged cut lettuce in **paper V**. Firmness decreased through the storage time in packaged cut lettuce, being significantly lower in passive MAP F1 after 11 days of storage ($p \leq 0.05$). Probably, in packaged cut lettuce the extensive exposition to high CO₂ after 11 days of storage might have promoted loss of membrane compartmentalization. Loss of membrane compartmentalization might imply loss of turgor due to damage to cellular membrane (Faust et al., 1967). Hamza et al. (1996) also found that excessive accumulation of CO₂ in the packages enhance tissue softening in packaged romaine lettuce.

5.5.2. Soluble sugars and organic acids

Soluble sugars and organic acids are substrate for respiration in plant cells. Fructose, glucose and sucrose are the major soluble sugars in lettuce (Bolin and Huxsoll, 1991, Poulsen et al., 1991; Heimdal et al., 1995). Among the organic acids, malic acid is the major organic acid in lettuce followed by tartaric acid (Souci et al., 2000; Chandra et al., 2009; Flores et al., 2012, **paper V**). During storage of lettuce cut or intact, the content of soluble sugars and organic acids decrease as a consequence of respiration of the plant cell (Heimdal et al., 1995; Chandra et al., 2009, **paper V**). For instance, in cut lettuce stored in air was found a sharp drop in sucrose and malic acid probably in order to meet a higher respiratory demand than under passive MAPs (**paper V**). To reduce the losses of sugars and organic acids in lettuce and other vegetables, MAP in combination with low storage temperatures is used, but extremely low O₂ and high CO₂ can trigger anaerobic reactions, as observed in **paper V**. Longer the storage under anaerobic conditions higher the loss of soluble sugars in packaged cut lettuce and presence of malolactic fermentation (**paper V**).

In addition, ascorbic acid has the capability to reduce *o*-quinones to *o*-diphenols, reducing the severity of browning (Cantos et al., 2001; Degl'Innocenti et al., 2005). There are a few studies regarding the action of ascorbic acid on browning in cut lettuce (Heimdal et al., 1995; Cantos et al., 2001). For instance, Heimdal et al. (1995) found that an inhibition of browning in cut lettuce packaged in moderate vacuum after 10 days of storage at 5 °C might be caused by reduction of ascorbic acid to dehydroascorbic.

5.6. Analytical technique for the determination of soluble sugars, organic acids and chlorogenic acid using GC-MS

In **paper V** a simultaneous analysis of malic acid, tartaric acid, chlorogenic acid, ascorbic acid and soluble sugars such as glucose, fructose and sucrose was made using GC-MS. To be detected by GC-MS, these metabolites have to be converted to a volatile non polar and stable derivative form (Roessner et al., 2000). The most common used derivatization method for GC-MS involves the conversion of the original metabolite into their trimethylsilyl (TMS) or methoxime derivatives. In **paper V** trimethylsilyl derivatization was used (Roessner et al., 2000; Kanani and Klapa, 2007). To this end sugars and acids were exposed to silylation. The derivatization procedures imply to convert the OH group of the acid and carbohydrate molecule in an ether or ester group (Sparkman et al., 2011). Silylation reduces polarity, enhances volatility and thermal stability (Fluka chemie, 2012).

Liquid chromatography, capillary electrophoresis-MS and nuclear magnetic resonance are other analytical techniques used for simultaneous analysis of compounds under study in **paper V** (Kanani and Klapa, 2007). The advantage of GC-MS over these methods rely on a better separation of compounds in the gas phase than in liquid phase, high sensitivity that decrease the amount of the biological material needed for accurate measurements, and better identification power by MS due to extensive compound databases (Kanani et al., 2008).

5.7. Analysis of TMS derivatives

When GC-MS is used the peak area of the derivative is proportional to the concentration of the original metabolite. However, some biases can distort the proportionality relationship of the original metabolite concentration and the peak area of the metabolite derivative (Kanani and Klapa, 2007). The reasons are that a) some metabolites form more than one derivative and b) derivatization reaction has not been completed (Gehrke and Leimer, 1971; Kanani and Klapa, 2007). Therefore, experiments regarding the time of derivatization for all the metabolites under study in **paper V** were done.

In a solution glucose and fructose exist as a mixture of anomeric and acyclic forms. Derivatizations lead to four isomers for fructose and two for glucose. Each isomer has a peak in the GC-MS profile, as observed in Fig. 17 (Bradbury, 1990). Whereas, malic acid, tartaric acid, ascorbic acid, chlorogenic acid and sucrose formed only one peak. To evaluate this data a similar approach as presented by Kanani and Klapa (2007) and Kanani et al. (2008) was undertaken in **paper V**. In the case of malic acid, tartaric acid, ascorbic acid and chlorogenic acid the only peak is proportional to the concentration of the metabolite in the sample. However, for glucose, both TMS isomer peaks are proportional to the concentration of the metabolite in the sample. Thus, the largest one of these was chosen. For fructose, which formed four isomers, the largest one is no longer proportional to the concentration of the metabolite, as such all isomers peaks was summed up to maintain the proportionality relationship between the derivative and the original metabolite concentration.

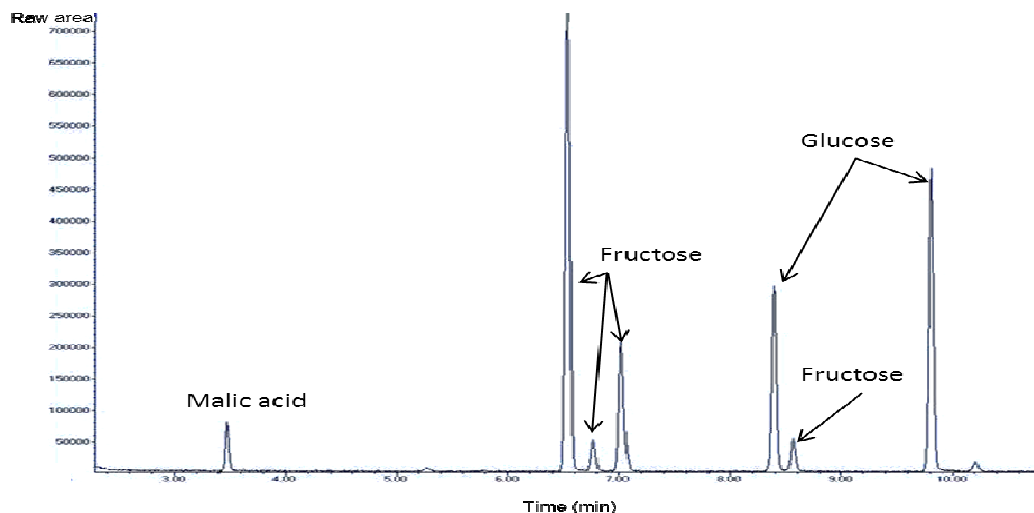


Figure 17. Total ion chromatogram (GC-MS) of glucose, fructose and malic acid as their TMS derivate of cut lettuce.

5.8. Influence of important factors in the formation of browning and other physicochemical characteristics in cut lettuce

Paper V: Changes in physicochemical characteristics of packaged and air stored cut iceberg lettuce upon storage and season

In **paper V** iceberg lettuce of cultivars Platinas and Morinas was harvested in June, July, August and September 2009. Once lettuces were harvested, they were minimally processed and packaged in two passive modified atmospheres (MAP) built up by films of different permeabilities, F1 (OPALEN 65 AF) and F2 (OPP/PE-L 2040 AF), and storage in air. All packages were stored at 5°C. GC-MS was used for the analysis of glucose, fructose, sucrose, malic acid, tartaric acid, chlorogenic acid and ascorbic acid. Browning was evaluated using images taken with a scanner and subjected to color correction and thresholding. PPO activity and texture were also evaluated. The analyses were taken at 1, 5, 8 and 11 days of storage for packaged lettuce and at 1 and 5 days of storage for air stored samples

From the score plot of Fig. 18, it can be seen that June and August was clearly discriminated within season. From the loading plot it can be seen that in June most physicochemical characteristics had high values under anaerobic conditions. Differences in these values might be attributed to differences in climatic conditions within season. For instance, June 2009 was characterized with an average temperature of 13.5 °C and more hours of sunshine (271 hours). It has been indicated that long period of photosynthetic activity in plants, results in increased production of photo assimilates such as carbohydrates (Taiz and Zeiger, 1998) and malic acid in grape berries (Hawker, 1969). Long days in June might explain the increase of soluble sugars and malic acid observed in packaged cut lettuce. The accumulation of carbohydrates could also contribute to changes in cell wall components, which are major contributors to firmness in lettuce (Martin-Diana et al., 2006; Toivonen and Brummell, 2008).

Among physicochemical characteristics, concentrations of sucrose and malic acid decreased as storage time increased (Fig.19). The drop in sucrose and malic acid in air storage samples was probably in order to meet a higher respiratory demanded than under passive MAPs. Respiration rate of cut fruits and vegetables is reduced under low O₂ and high CO₂ (Kader, 1986), but extremely low O₂ and high CO₂ can trigger anaerobic conditions, as observed in passive MAPs. Longer the storage under anaerobic conditions higher the loss of sucrose and reduction of malic acid, probably as a consequence of malolactic fermentation by lactic acid bacteria mainly found in passive MAP F1 after 11 days of storage ($p \leq 0.05$) (Cabrita et al., 2008). The extensive exposition to high CO₂ in passive MAP F1 also promoted loss of firmness (Fig.19) and decrease in pH. High CO₂ cause loss of membrane compartmentalization, which might have implied loss of turgor due to damage to cellular membrane (Faust, 1967).

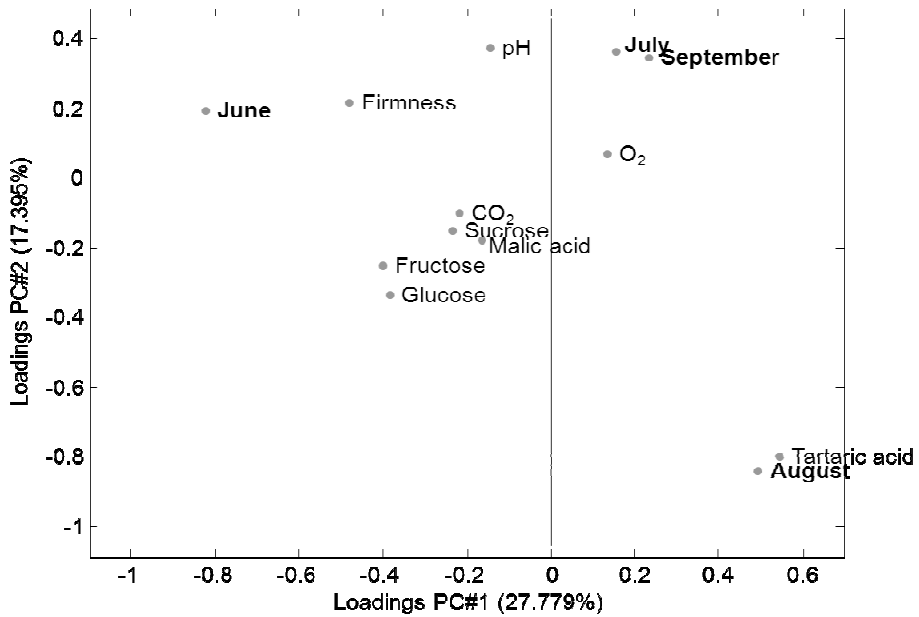
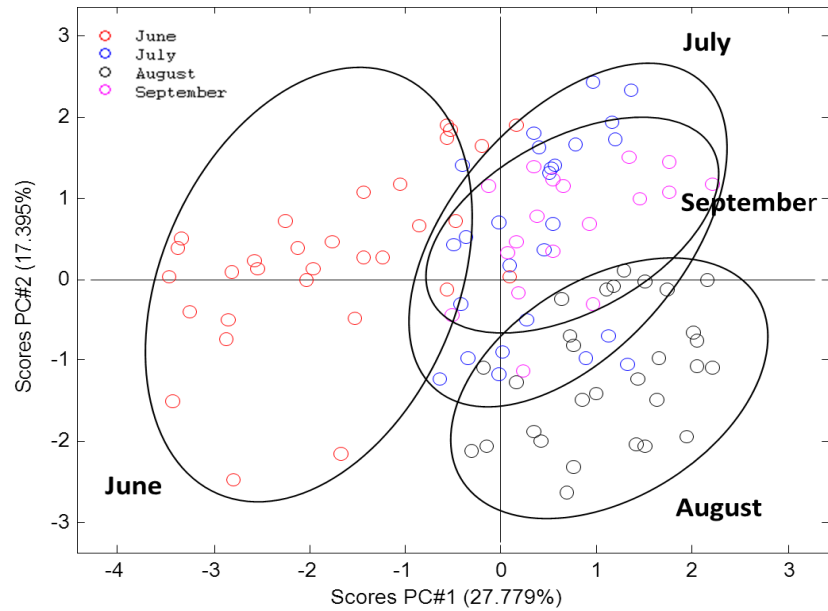
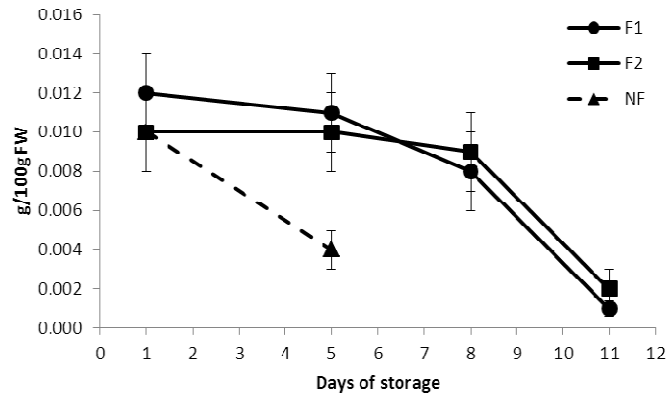
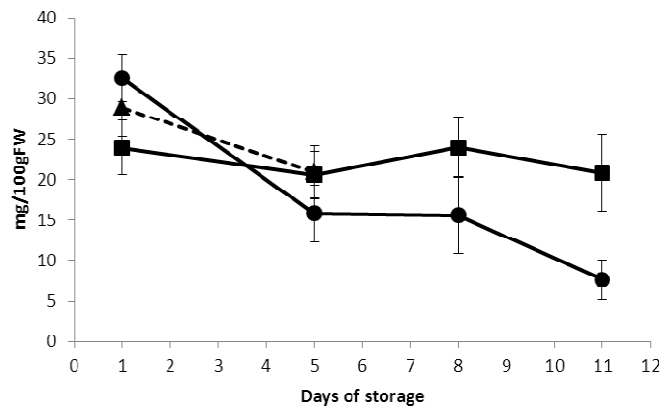


Figure 18. PLS-DA PLS-DA model for gas composition and physicochemical characteristics of cut lettuce cultivar Platinas and Morinas packaged in passive MAPs for up to 11 days and stored in air for 5 days at 5 °C in June, July, August and September 2009.

a) Sucrose



b) Malic acid



c) Firmness

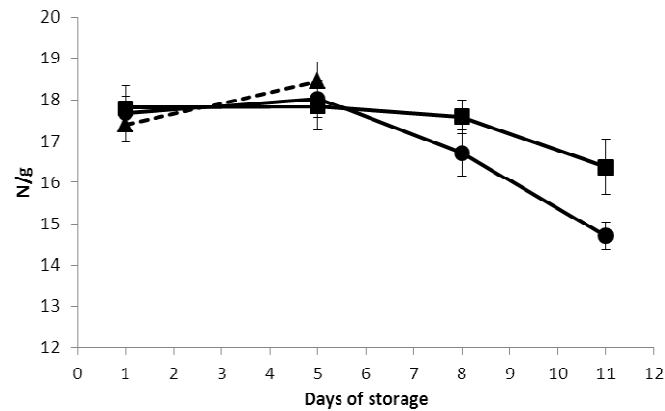


Figure 19. Changes in the concentration of selected soluble sugars, organic acids and firmness of cut lettuce stored for up to 11 days in passive MAP F1 and F2 and for 5 days in air at 5 °C. Vertical lines represent the standard error of the mean (n=15).

Browning has been indicated to be the main limitation of the shelf-life of cut lettuce (Heimdal et al., 1995). As expected, the browning area (BA) increased in air stored samples as the time of storage increased. Browning was not found at 1 day of storage, but cut lettuce gradually turned brown during 5 days of storage. Cut lettuce stored in air presented favorable conditions for the development of browning. In air stored samples the concentration of chlorogenic acid was significantly higher than in packaged samples ($p \leq 0.05$) and PPO activity remained constant after 5 days of storage ($p \geq 0.05$).

On the contrary, browning was not observed in packaged samples during the storage time. Probably the inhibition of browning was a consequence of extremely low O_2 content and high CO_2 (Heimdal et al., 1995; Smyth et al., 1998). The results indicated that chlorogenic acid decreased as storage time increased in passive MAPs samples ($p \leq 0.05$), and PPO activity of cut lettuce packaged in passive MAP F2 sharply decrease after 1 day of storage ($p \leq 0.05$) (Table 9).

Table 9. Changes in ascorbic acid and chlorogenic acid of cut lettuce packaged in passive MAPs and PPO activity of passive MAP F2 as storage time increase.

Storage time (Days)	Ascorbic acid (mg/gFW)	Chlorogenic acid (mg/gFW)	PPO activity (U ml ⁻¹)
1	25.28 ± 23.84 (17) ^b	53.57 ± 38.17 (17) ^a	0.28 ± 0.10 (11) ^b
5	7.61 ± 7.26 (20) ^a	30.14 ± 45.34 (20) ^{ab}	0.15 ± 0.08 (5) ^a
8	10.37 ± 10.16 (25) ^a	63.49 ± 65.88 (25) ^b	0.16 ± 0.06 (5) ^a
11	2.43 ± 3.09 (16) ^a	15.33 ± 17.18 (16) ^a	

Data expressed as mean ± standard deviation. Values in parentheses represent the number of samples used for the calculation of the mean. Different letters indicate significant differences at $p \leq 0.05$. Abbreviations: FW= fresh weight.

Season and cultivar are important factors for the development of browning (Matheis, 1983). Analysis of variance showed that there was more potential of browning in September and August than the rest of months ($p \leq 0.05$) and more for cultivar Platinas than Morinas ($p \leq 0.05$) (Table 10). Therefore a packaging to build up low O_2 and high CO_2 is of importance to control browning independent of the season and cultivar, as we proved in this study by the fact of browning was not observed in packaged samples.

However, there is a risk for the formation of off-odors (**paper IV**), tissue softening, decreased of sugars and malolactic fermentation, mainly in passive MAP F1 after 11 days of storage.

Table 10. Brown area fraction (BA) of two cultivars of cut lettuce stored in air at 5 °C during season 2009.

Factors	BA(%)
<i>Cultivars</i>	
Morinas	9.0 ± 4.5 (8) ^a
Platinas	13.0 ± 4.8 (6) ^b
<i>Season 2009</i>	
June	7.0 ± 1.3 (2) ^{ab}
July	6.0 ± 3.2 (4) ^a
August	12.0 ± 0.7 (4) ^{bc}
September	15.0 ± 5.3 (4) ^c

Data expressed as mean ± standard deviation. Values in parentheses represent the number of samples used for the calculation of the mean. Different letters indicate significant differences at $p \leq 0.05$.

Chapter 6. Conclusions and Perspectives

Conclusions

- This PhD thesis provides a better understanding of changes of volatile compounds, fructose, glucose, sucrose, malic acid, chlorogenic acid, tartaric acid, ascorbic acid, firmness, browning and respiration rate, as a part of quality changes induced by season, cultivar, packaging, storage time, storage temperature and method of preparation.
- Respiration rate was not a good an indicator of stress by cutting direction
- Transversal cutting was a more severe method of preparation than longitudinal cutting based on the increase in the levels of volatiles produced through the LOX pathway.
- Respiration rate was mainly affected by temperature of storage and season.
- A total of 52 volatile compounds were identified in this PhD project and of these 21 were shown to potent odorants of cut lettuce.
- Among the potent odorants, elemene, caryophyllene, β -selinene and 2,3-butanedione, enhanced under extremely low O₂ and high CO₂ built up in passive MAP F1 and likely to be off-odours.
- In August high production of this odorants was found and probably compromised the quality in terms of odour.
- Regarding the cultivars, Morinas and Diamantinas produce a significant amount of the undesirable odorant 2,3-butanedione.
- Browning was higher in August and September in samples stored under air.
- Browning was higher in cultivar Platinas in air stored samples.
- In June soluble sugars, malic acid and firmness were kept high under anaerobic conditions
- Browning was remarkably controlled in both passive MAPs due to extremely low O₂ and high CO₂ conditions; as a result, a product with good appearance was obtained.

However the increase of potent odorants likely to be off-odours were a limiting factor for shelf-life of packaged cut lettuce.

- Cut lettuce packed in passive MAP F1 after 11 days of storage showed a worse overall quality than MAP F2 due to tissue softening, decrease of sugars, malolactic fermentation and enhanced the production of odorants likely to be off-odours.
- The passive MAP built with film F2 seemed to be the most promising packaging.
- Storage of packaged cut lettuce for up to 11 days should be avoided.
- In this thesis was demonstrated that image analysis is a technique that allows an accurate quantification of browning by thresholding the colour corrected images.
- GC-MS was demonstrated to be a powerful tool for the identification and quantification of soluble sugars, organic acids, chlorogenic acid and ascorbic acid in cut lettuce.

Perspectives- Recommendations for future research

- To characterize the relationship between cutting, washing and drying with physiological and quality attributes of lettuce or other vegetables.
- To develop a sensory analysis for the determination of the shelf-life of cut lettuce in passive MAP F2.
- Industrial implementation of image analysis technique developed in this study as an automatic tool to assess browning and other color related characteristics of vegetables and fruits, i.e as a quality control procedure.
- In future works is suggested to investigate the influence of microbial growth in the formation of volatiles in cut lettuce.
- A more comprehensive study in lettuce volatiles and physicochemical constituents of cut lettuce covering at least two seasons is needed.
- Extend the application of GC-MS for the analysis of non-volatiles in other vegetables.

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