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Manipulating postharvest quality and nutritional content of spinach

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Declaration

The work reported in this thesis is an original research carried out by myself, with the guidance from my director of studies and supervisory team. No material reported in this thesis has been submitted for any other degree or diploma in another institution. All relevant references used in this thesis are included in the text and written in the references section following Harper Adams University Guide to referencing.

This thesis is dedicated to a few Very Special Women

that appealed to my heart and imagination,

and to my Mother and Brother for their unending support.

I appreciate them more than words can say...

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Abstract

The high nutritional value of leafy salads and convenience to the consumer has resulted in continuing growth of the leafy vegetable market. The shelf-life of leafy vegetables, including spinach, is relatively short (7-14 days) and is influenced by storage conditions.

This project investigated the effect of storage temperature and light exposure on quality maintenance during the storage of baby leaf spinach. A series of experiments (Experiments 1-5) were conducted to conclude that quality loss of spinach leaves is accelerated with increasing temperature and light intensity during storage, temperature having a greater effect (Experiment 5). Low intensity light, however, improved leaf texture maintenance when compared with samples stored in the dark. In addition to observed responses, results from Experiments 1 and 2 (reported in Chapter 3) helped to identify leaf textural and visual quality as the best indicators of shelf-life.

The fresh produce industry is keen on developing new methods, *e.g*. pre-storage treatments that will enhance or maintain nutritional value of the product, retain its colour and texture. There is enough evidence in the literature to suggest that pre-storage hot water treatment might be a good option. Most of the studies, however, have been conducted on lettuce. Thus, the decision was made to investigate whether hot water treatment is also a good solution for improving the quality or extending the shelf-life of baby leaf spinach. Based on the results from Experiments 6 and 7, it was concluded that hot water treatments have limited commercial potential for quality improvement of spinach leaves.

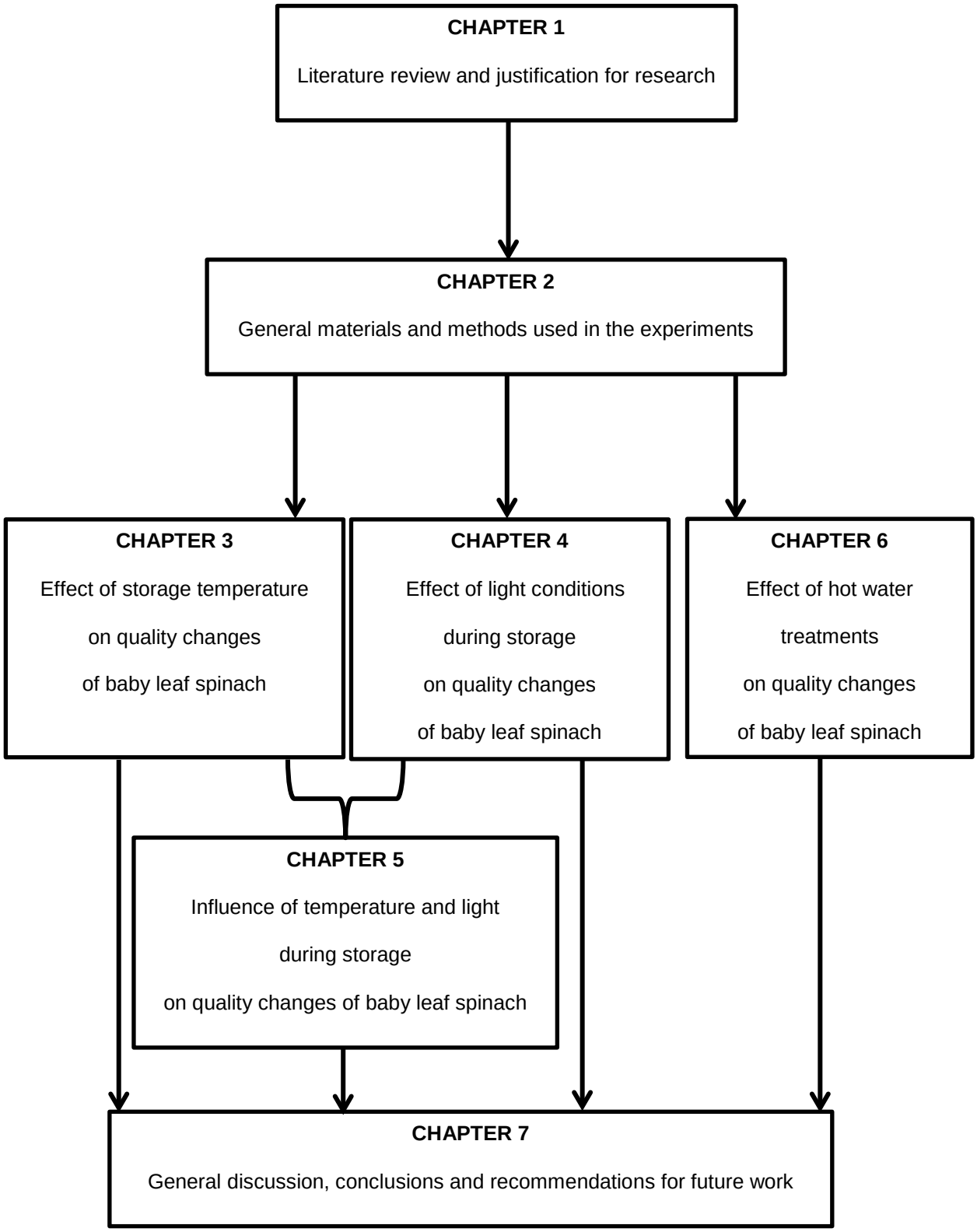
Outline of the thesis

In Chapter one, a brief introduction to the problems associated with the short shelf-life of leafy vegetables is given. The literature review covers the aspect of heat treatment prior to storage on quality changes during subsequent storage. Furthermore, the literature relevant to quality changes and the effects of temperature and light exposure during the storage of leafy vegetables has been reviewed. Quality loss is associated with changes in texture, visual and nutritional quality, and microbial contamination.

Chapter two describes general methodology used in the experiments detailed in the thesis. In instances where it is necessary, additions to the methodologies are given in the appropriate Chapters.

Chapter three and four report the results from experiments that investigated the effects of temperature and light exposure during storage on quality changes of baby leaf spinach. Only one stressor (either temperature or light) was used at a time. In Chapter five, the effects of temperature and light exposure, both stressors applied simultaneously, on quality changes of baby leaf spinach are reported.

Chapter six presents the findings from experiments that investigated whether pre-storage hot water treatments can potentially be used by industry to enhance or maintain postharvest quality of spinach leaves. Finally, Chapter seven contains discussion of the findings from all experiments detailed in the thesis, final conclusions and recommendations for future work.



Schematic outline of the thesis

Statement of advanced studies

In addition to the experiments detailed in this thesis (during the period of this study), the author:

- Obtained a Postgraduate Certificate in Research Skills awarded by Harper Adams University College;
- Published the following paper in Postharvest Biology and Technology:

Glowacz, M., Mogren, L. M., Reade, J. P. H., Cobb, A. H. and Monaghan, J. M. 2013. Can hot water treatments enhance or maintain postharvest quality of spinach leaves? *Postharvest Biology and Technology,* 81, pp. 23-28.

- Gave presentations at the following conferences and meetings:

SHE 2012, 2nd Symposium on Horticulture in Europe, July 1-5, 2012, Angers, France
"Influence of temperature and light exposure during storage on quality changes of spinach leaves"

ISHS, II International Conference on Quality Management of Fresh Cut Produce Convenience Food for a Tasteful Life, 17-21 July 2011, Torino, Italy
"Hot water treatment after harvest preserves nutritional quality of spinach during storage"

Seminar on 29th of February 2012, Harper Adams University College, Edgmond, Newport, Shropshire, UK
"Manipulating postharvest quality of spinach"

- Attended a number of research seminars and talks organised at Harper Adams University, Edgmond, Newport, Shropshire, UK

List of abbreviations

*a**	leaf greenness value
AsA	ascorbic acid
AOS	active oxygen species
APX	ascorbate peroxidase
*b**	leaf yellowness value
CAT	catalase
cfu	colony forming unit
CV	coefficient of variance
d	days
DHA	dehydroascorbic acid
DHAR	dehydroascorbic acid reductase
DMAPP	dimethylallyl diphosphate
DW	dry weight
FSA	Food Standards Agency
FW	fresh weight
GDP-mannose	guanosine 5′-diphospho-mannose
GGPP	geranylgeranyl diphosphate
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
h	hours
IPP	isopentenyl diphosphate
*L**	leaf lightness value
LYC	lycopene cyclase
MDHA	monodehydroascorbic acid
MDHAR	monodehydroascorbate reductase
min	minutes
NAD(P)H	nicotinamide adenine dinucleotide phosphate

NSY	neoxanthin synthase
OTR	oxygen transmission rate
PAL	phenylalanine ammonia-lyase
PME	pectin methylesterase
POD	peroxidase
ppm	parts per million
PPO	polyphenol oxidase
PPPP	pre-phytoene pyrophosphate
PSY	phytoene synthase
s	seconds
SOD	superoxide dismutase
sp.	species
UV	ultraviolet
VDE	violaxanthin deepoxidase
wk	weeks
ZEP	zeaxanthin epoxidase

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Chapter 1 Literature review

1.1 Spinach – growth, harvest and storage

Plate 1.1 Spinach as grown in the field (view from the top)

Spinach is quick-maturing (3 weeks), cool-season vegetable crop. Spinach produces a rosette of leaves (Plate 1.1) that may be wrinkled (savoy or semi-savoy types) or smooth (flat leaf types). Leaves are typically oval, rounded, or triangular and are born on a short stem. In general, baby leaf spinach is planted on 80-inch-wide (203 cm) beds (Plate 1.2).

Plate 1.2 Spinach beds in the field (overview)

Spinach seeds are planted 1-2 cm deep, depending on the method of planting and soil conditions. It can be grown successfully on a variety of soils, but a fertile sandy loam high in organic matter is preferred. Spinach has relatively shallow root system and relies on frequent, short irrigations to maintain uniformly moist soil for maximum leaf production. Spinach that is sold as bagged salad mixes is usually mechanically harvested (Plate 1.3 A). A machine with a front cutter bar is run on the top of the plant beds. The cutter bar clips the leaves and attached petioles off the plant. The height of the cutter bar can be adjusted to control the amount of petiole that is included. The leaves are lifted by conveyor belt into bins on trailers (Plate 1.3 B) and transported to the processing plant for sorting, washing, air drying, and packaging into a variety of different bagged spinach products.

Plate 1.3 Process of mechanical harvesting of spinach leaves. (A) A machine with a front cutter bar runs on the top of the plant beds. (B) The leaves are lifted by conveyor belt into bins on trailers.

There is a need for keeping loads from overheating. If the processing stage is delayed, the spinach is typically vacuum cooled and stored for a short period of time at low (close to 1 °C) temperature; spinach is quite perishable and will yellow when stored at higher than recommended temperatures. The main cause of postharvest losses in spinach, however, is not leaf yellowing but rather decay associated with mechanical damage during harvest and postharvest operations.

Spinach is widely grown across the UK by a number of companies (*e.g.* Anglia Salads, Essex; Emmett Ltd, Lincolnshire; G's Fresh, Cambridgeshire and West Midlands; PDM Produce Ltd, Shropshire; Intercrop Ltd and Southern Salads, Kent; Kemp Herbs, Norfolk; Vitacress Salads Ltd, Hampshire; Scotherbs Ltd, Scotland) that are members of British Leafy Salads Association.

The high nutritional value of leafy salads and convenience to the consumer has resulted in continuing growth of the leafy vegetable market, *i.e.* in 2010, this market accounted for over £700 million of UK business. Consumers care more and more about what they eat and fresh produce has been recognised as a healthy food, rich in antioxidants (Pandrangi and LaBorde, 2004; Bergquist *et al*., 2006; Llorach *et al*., 2008). The key issue for retailers regarding leafy vegetables is their short shelf-life (7-14 days), which is influenced by initial quality at harvest (Clarkson *et al.*, 2003, Newman *et al.*, 2005, Zhang *et al.*, 2007, Wagstaff *et al.*, 2010) and subsequent storage conditions (Garcia-Gimeno and Zurera-Cosano, 1997, Piagentini *et al.*, 2005, Martinez-Sanchez *et al.*, 2006). Due to the fact, that shelf-life of leafy vegetables is relatively short, new technique for maintaining textural, visual and nutritional quality of fresh produce is required. Care must also be taken regarding microbial contamination of leafy vegetables either via reducing microbial counts (using different pre-storage treatments listed in Table 1.1) or reducing microbial growth during storage (selecting appropriate storage conditions). The end of shelf-life means that the product is no longer suitable for sale and consumption. Furthermore, it needs to be disposed and this may cause a financial loss to the retailer and producer.

1.2 Opportunities for manipulating postharvest quality of leafy vegetables

There is a large volume of literature reporting quality loss during the storage of leafy vegetables (Allende *et al.*, 2004b, Murata *et al.*, 2004, Pandrangi and LaBorde, 2004, Hagen *et al.*, 2009). This is often a result of a combination of physical and/or biochemical changes, and microbial spoilage, the main attributes used to assess the quality of leafy vegetables are texture, sensory and nutritional quality, and microbial contamination. Thus, it is of interest to the fresh produce industry, to develop a method (pre-storage treatment) that will enhance or maintain nutritional value of the product, as well as retain its colour and texture.

There are number of pre-storage treatments that could be used by the industry (Table 1.1). Most of them are used mainly as disinfectants, however, it is worth mentioning that some of the washing treatments, including pure water (Murata *et al*., 2004; Gomez *et al*., 2008; Koukounaras *et al*., 2009), chlorinated water (Delaquis *et al*., 1999, 2004; Li *et al*., 2001b; Garcia *et al*., 2003) or aqueous ozone (Alexopoulos *et al*., 2013; Baur *et al*., 2004; Martinez-Sanchez *et al*., 2008a) can preserve the quality of fresh produce during subsequent storage. In addition, some other treatments like gaseous ozone (Klockow and Keener, 2009; Bermudez-Aguirre and Barbosa-Canovas, 2013), UV-light treatments (Artes-Hernandez *et al*., 2009; Selma *et al*., 2008; Escalona *et al*., 2010) and irradiation (Rajkowski and Fan, 2008; Lester *et al*., 2010a) have also been used, as an alternative to chlorine, to reduce microbial counts on leafy vegetables prior to storage as these treatments do not leave any residues on the product.

The question that needs to be answered is whether it is possible to find optimal conditions for the storage of spinach leaves? Another question that needs to be addressed is whether short, pre-storage treatments can enhance the quality of spinach leaves during subsequent storage or at least reduce or delay spinach deterioration, thus extending its shelf-life?

Table 1.1 Possible ways (pre-storage treatments) for postharvest quality manipulation of leafy vegetables

Pre-storage treatment	Crop	Effect on Quality	Reference
Hot water treatment	spinach	↑ chlorophyll retention (+), AsA: DHA ratio (+)	Gomez *et al*., 2008
		↓ solute leakage (+)	Gomez *et al*., 2008
	lettuce	↑ colour (+)	Li *et al*., 2001b
		↑ visual quality (+)	Baur *et al*., 2005
		↑ general appearance (+)	Li *et al*., 2001b; Murata *et al*., 2004
		↑ organoleptic quality (+)	Murata *et al*., 2004
		↓ browning (+)	Li *et al*., 2001b; Fukumoto *et al*., 2002; Kang and Saltveit, 2003;
			Delaquis *et al*., 2004 ; Murata *et al*., 2004; Baur *et al*., 2005; Moreira *et al*., 2006
		↓ PAL (+)	Fukumoto *et al*., 2002; Kang and Saltveit, 2003; Murata *et al*., 2004; Baur *et al*., 2005
		↓ POD (+)	Fukumoto *et al*., 2002
		↓ initial microbial population (+)	Li *et al*., 2001b; Baur *et al*., 2005; Moreira *et al*., 2006
		↓ total microbial population (+)	Delaquis *et al*., 2004; Baur *et al*., 2005
		↓ reduced pathogens (+)	Rajkowski and Fan, 2008
		↓ phenolics (±)	Fukumoto *et al*., 2002; Kang and Saltveit, 2003; Murata *et al*., 2004
		↑ microbial growth (-)	Li *et al*., 2001b; Murata *et al*., 2004; Moreira *et al*., 2006
		↑ respiration (-)	Baur *et al*., 2005
		↓ AsA (-), colour (-), texture (-)	Moreira *et al*., 2006
	rocket	↑ chlorophyll retention (+), colour (+), soluble solids (+)	Koukounaras *et al*., 2009
		↓ yellowing (+)	Koukounaras *et al*., 2009
		↑ respiration (-)	Martinez-Sanchez *et al*., 2008a
Chlorinated water	spinach	↓ microbial population (+)	Rahman *et al*., 2010
	lettuce	↑ visual quality (+)	Delaquis *et al*., 1999; Baur *et al*., 2005
		↓ browning (+),	Delaquis *et al*., 1999; Odumeru et al., 2003; Delaquis *et al*., 2004; Baur *et al*., 2005
		↓ initial microbial population (+)	Delaquis *et al*., 1999; Li *et al*., 2001b; Garcia *et al*., 2003; Odumeru *et al*., 2003;
			Baur *et al*., 2005; Olmez and Akbas, 2009; Alexopoulos *et al*., 2013; Bermudez-Aguirre and Barbosa-Canovas, 2013
		↓ respiration (+),	Odumeru *et al*., 2003
		↓ visual quality (-)	Bermudez-Aguirre and Barbosa-Canovas, 2013
		↑ microbial growth (-)	Baur *et al*., 2004; Baur *et al*., 2005
		↑ respiration (-)	Li *et al*., 2001b; Vandekinderen *et al*., 2008
		↓ AsA (-), carotenoids (-), visual quality (-)	Kenny and O'Beirne, 2009
Citric acid	spinach	↓ initial microbial population (+)	Rahman *et al*., 2010
	rocket	↓ chlorophyll (-)	Ferrante *et al*., 2004
Aqueous ozone	spinach	↓ initial microbial population (+)	Selma *et al*., 2008; Rahman *et al*., 2010
	lettuce	↑ appearance (+)	Garcia *et al*., 2003
		↓ microbial population (+)	Garcia *et al*., 2003; Beltran *et al*., 2005; Olmez and Akbas, 2009; Alexopoulos *et al*., 2013
		↑ visual quality (+)	Beltran *et al*., 2005
		↓ browning (+)	Beltran *et al*., 2005
	rocket	↓ microbial population (+)	Martinez-Sanchez *et al*., 2008a
	watercress	↓ microbial population (+)	Martinez-Sanchez *et al*., 2008a

*(+) positive response, (-) negative response, (±) neutral response

The aim for this research was, to find optimal conditions for storage of spinach leaves. Storage temperature is the most important factor that affects quality of leafy vegetables (Jacxsens *et al*., 2002; Bergquist *et al*., 2006; Luo *et al*., 2009), whereas current knowledge on the impact of light conditions during storage is scarce (Lester *et al*., 2010b). Thus, a series of experiments were conducted, where bags with spinach supplied by PDM Produce Ltd, were stored under different temperature (factor 1) and light (factor 2) conditions. These factors were chosen, based on the practicality issues, as it is not so difficult to set them up in the cold stores. It was of interest for our industrial partner to know how the quality of spinach will change when bags are stored under different conditions, and they wanted to know how these two factors affect the quality of baby leaf spinach.

There is enough evidence in the literature to suggest that hot water treatments can improve/maintain postharvest quality of leafy vegetables. Most of the studies mentioned in Table 1.1, however, have been conducted on lettuce or rocket leaves. Whilst, there was only one paper (Gomez *et al*., 2008), reporting improved shelf-life of spinach treated with hot water at 40 °C for 3.5 min prior to storage, these samples were subsequently stored at 23 °C. It is a common practice in the UK, to store spinach under refrigerated conditions, thus it was of interest to investigate whether hot water treatments can be recommended as a viable technique for improving postharvest quality of spinach if samples were to be subsequently stored under commercial conditions.

The following literature review focuses on: (i) how the quality of leafy vegetables changes during storage and how these changes are affected by temperature and light conditions during storage, (ii) the effects of pre-storage treatments on quality changes of leafy vegetables during storage.

1.3 Changes in the quality of leafy vegetables during storage

There is increasing evidence that the concentration of antioxidants in fresh produce is important to human nutrition (Stanner *et al.*, 2004, Gordon, 2012). Antioxidants such as ascorbic acid, carotenoids and flavonoids are suggested to be involved in protection against a range of chronic cardiovascular diseases in humans (Diplock, 1991, Yang *et al.*, 1996). These phytochemicals are present in leafy vegetables, including lettuce (*Lactuca sativa* sp.), spinach (*Spinacia oleracea* L.) and endive (*Cichorium intybus* L.). The antioxidant activity varies among the individual compounds, therefore not only the total concentration should be considered as being important, but rather the composition of these compounds (Singh *et al.*, 2006, Llorach *et al.*, 2008).

Several authors (Pandrangi and LaBorde, 2004, Bergquist *et al.*, 2006, Martinez-Sanchez *et al.*, 2008a, Luo *et al.*, 2009, Spinardi and Ferrante, 2012) attempted to determine the effect of storage temperature on quality maintenance during the storage of leafy vegetables. Temperature of storage was found to be the key factor affecting the rate of quality loss, thus changes during storage under different temperature regimes are reviewed.

Fresh produce is exposed to various light conditions during its displayed shelf-life. Thus, it is not surprising that in recent years, there has been an increasing interest in studying the effects of light exposure during storage on changes in the quality of chard (*Beta vulgaris* L. var. *vulgaris*) (Sanz *et al*., 2008), Chinese kale (*Brassica oleracea* var. *alboglabra*) (Noichinda *et al*., 2007), kale (*Brassica oleracea* L. var. *acephala*) (Kobori *et al*., 2011) lettuce (Martinez-Sanchez *et al.*, 2011, Zhan *et al.*, 2012, Zhan *et al.*, 2013) rocket (*Eruca sativa*) (Barbieri *et al*., 2011) and spinach (Lester *et al*., 2010b). It is clear that industry cannot ignore the role of light exposure during the storage of leafy vegetables, as a number of authors reported effects of light exposure on their texture (Sanz *et al.*, 2008, Martinez-Sanchez *et al.*, 2011, Medina *et al.*, 2012), visual quality (Sanz *et al.*, 2008, Kobori *et al.*, 2011, Martinez-Sanchez *et al.*, 2011, Medina *et al.*, 2012) and nutritional quality (Noichinda *et al.*, 2007, Lester *et al.*, 2010b, Martinez-Sanchez *et*

al., 2011, Zhan *et al.*, 2013). These effects of light exposure during storage are also reviewed.

Finally, a number of workers have reported benefits of a short, high temperature treatment prior to subsequent storage at low temperature (Delaquis *et al.*, 2004, Murata *et al.*, 2004, Martin-Diana *et al.*, 2005). Temperature treatments can be applied either in the form of air or water (Lurie, 1998); however, water has been suggested to be a better medium in terms of heat transfer efficiency (Fallik, 2004) and practicality. Thus, the effects of hot water treatments on quality changes in leafy vegetables during subsequent storage will also be discussed in this review.

1.3.1 Textural and physiological changes

Quality loss during the storage of leafy vegetables may be accelerated by changes in the metabolic activity of harvested leaves. A large and growing body of literature has reported respiratory activity and associated changes in the gas composition within the bags during the storage of lettuce (Allende *et al.*, 2004a, Del Nobile *et al.*, 2006, Escalona *et al.*, 2006, Martinez-Sanchez *et al.*, 2011), spinach (Allende *et al.*, 2004b, Pandrangi and LaBorde, 2004, Conte *et al.*, 2008, Medina *et al.*, 2012), cabbage (*Brassica oleracea* var. *capitata*) (Gomez-Lopez *et al.*, 2005, Vandenkinderen *et al.*, 2008), kale (Kobori *et al.*, 2011), mizuna (*Brassica rapa* L. ssp. *nipposinica*) (Martinez-Sanchez *et al.*, 2008a), watercress (*Nasturtium officinale*) (Martinez-Sanchez *et al.*, 2008a), salad rocket (*Eruca vesicaria*) and wild rocket (*Diplotaxis tenuifolia*) leaves (Martinez-Sanchez *et al.*, 2006, Martinez-Sanchez *et al.*, 2008a).

Respiration rate is a measure of physiological activity of leaf tissue (Pirovani *et al*., 1998) and it increases in response to tissue damage (*e.g.* during processing stage), thus it is not surprising that several authors have reported high respiration rate as a result of tissue damage in harvested leaves of lettuce (King *et al.*, 1991, McKellar *et al.*, 2004), spinach (Allende *et al.*, 2004b, Escalona *et al.*, 2010) and wild rocket (Martinez-Sanchez *et al.*, 2006). Respiration uses O_2 and produces CO_2 and this can change the gas composition inside the bag and lead to anaerobic conditions unless semi-permeable or

permeable packaging is used (Pirovani *et al.*, 1998, Allende *et al.*, 2004b, Martinez *et al.*, 2005, Del Nobile *et al.*, 2006). A high respiration rate indicates high metabolic activity and this may result in a decrease in dry matter content, due to carbohydrate and protein breakdown (Masih *et al*., 2002).

Respiration rate has been shown to increase with increasing temperature of storage of rocket, mizuna and watercress leaves (Martinez-Sanchez *et al.*, 2008a). These authors observed significant increase in the respiration rate of baby leaves stored at 8 and 12 °C, when compared with those stored at 1 °C, whereas the difference between samples stored at 1 and 4 °C was not always significant. A significantly higher respiration rate has also been reported for lettuce stored at 25 °C when compared with samples stored at 5 °C (Oliveira *et al.*, 2010). The oxygen content in the bags stored at 25 °C decreased from 20% to 0.8% after 3 days, while in lettuce stored at 5 °C oxygen concentration decreased to 8% after 10 days of storage. Similarly, carbon dioxide concentration in lettuce bags stored at 25 °C increased to 11% after 3 days, while in samples stored at 5 °C the carbon dioxide level reached 6% after 10 days of storage. In the case of spinach, the respiration rate of the leaves stored at 5 °C was lower than those stored at 8 °C (Artes-Hernandez *et al.*, 2009), and this was further confirmed by more pronounced changes in the gas composition inside the bags.

Light exposure has been reported to affect the respiration rate during the storage of chard (Sanz *et al.*, 2008) and lettuce (Martinez-Sanchez *et al.*, 2011). Continuous light during storage has been reported to support photosynthetic activity during the storage of Chinese kale (Noichinda *et al.*, 2007), lettuce (Zhan *et al.*, 2013) and spinach leaves (Toledo *et al*., 2003a) as indicated by higher sugar content in light-stored samples when compared with their dark-stored counterparts. Too much light and associated increased level of sugars, however, may be responsible for inducing the leaf senescence, most likely via hexokinase function (Yoshida, 2003).

Texture loss during storage, evaluated by the fracture of leaves, is a major problem in leafy vegetables and according to Martin-Diana *et al.* (2006) texture loss is the

main reason for tissue deterioration in lettuce. Marked reduction in texture has also been reported during the storage of spinach leaves at 10 °C (Babic *et al*., 1996). These authors suggested that pectolytic bacteria belonging to the genus *Pseudomonas* sp. were probably involved in the process of textural quality degradation. Care must be taken as cultivar differences in leaf texture have been reported by Taniwaki *et al.* (2009) who determined the texture of four different cultivars of cabbage (*Brassica oleracea* L. var. *capitata*) using a texture measurement device (AMC; Applied Vibro-Acoustics Inc., Higashi-Hiroshima, Japan).

Several authors have reported fresh weight (FW) loss during the storage of leafy vegetables (Ihl *et al.*, 2003, Noichinda *et al.*, 2007, Conte *et al.*, 2008, Manolopoulou *et al.*, 2010); however, in most of these studies only a slight (up to 4%) decrease in FW took place. Several authors reported significantly higher FW loss during the storage of chard (Sanz *et al.*, 2008), Chinese kale (Noichinda *et al.*, 2007) and lettuce (Martinez-Sanchez *et al.*, 2011, Zhan *et al.*, 2012, Zhan *et al.*, 2013) as a result of light exposure when compared with dark-stored counterparts. The above mentioned weight loss was shown to correlate with the higher numbers of stomata that remained open in light-stored leaves compared with their dark-stored counterparts (Noichinda *et al.*, 2007, Martinez-Sanchez *et al.*, 2011). Excess water loss leads to a loss of turgor and decrease in textural quality of leafy vegetables (Martin-Diana *et al.*, 2006, Wagstaff *et al.*, 2007, Aguero *et al.*, 2008).

One of the characteristics of tissue breakdown during storage is membrane disruption, which is often quantified by solute leakage assays (Marangoni *et al.*, 1996, Wagstaff *et al.*, 2007). A correlation between solute leakage and quality deterioration was found (Allende *et al.*, 2004b) during the storage of spinach leaves. This finding is supported by Wagstaff *et al*. (2010) who demonstrated that lettuce with reduced membrane permeability and modified cell wall properties exhibited improved shelf-life. Light exposure during storage has been reported to reduce solute leakage from lettuce (Martinez-Sanchez *et al.*, 2011) and spinach leaves (Kar and Choudhuri, 1986) when compared with their dark-stored counterparts. Martinez-Sanchez *et al*. (2011) suggested that this may be related to changes in gas composition inside the bags with fresh produce.

As textural and physiological changes during the storage of leafy vegetables depend on the temperature and light conditions during storage, low storage temperature (0-4 °C) and low intensity light are recommended for commercial storage of these products.

1.3.2 Development of off-odours and off-flavours

Sensory evaluation of food relies on the human perception of it. Among the attributes that can be assessed in this way are overall visual quality, taste and development of off-odours. Significant changes in all these attributes have been reported in iceberg lettuce (*Lactuca sativa* var. *capitata*) stored for 5 days at 7 °C (Gomez-Lopez *et al.*, 2005). On the other hand, Baur *et al*. (2005) have only reported a significant decrease in visual quality of iceberg lettuce stored for 7 days at 4 °C, while development of off-odours was not significant. In contrast, development of off-odours has been reported after only 3 days of storage at 7 °C (Gomez-Lopez *et al.*, 2005). Thus, it is not surprising that others reported off-odours in Romaine lettuce stored for 8 days at 5 °C, (Luo, 2007) and 10 days at 7 °C (Martinez-Sanchez *et al.*, 2011), respectively.

Other authors (Piagentini *et al.*, 2002, Allende *et al.*, 2004b) have also reported significant changes in the general appearance and development of off-odours as main problems during the storage of fresh-cut spinach. In a recent study, slight to moderate development of off-odours was reported in spinach leaves after 10 days of storage at 5 °C (Artes-Hernandez *et al.*, 2009), while these changes were already severe in the samples stored at 8 °C. Development of off-odours during the storage of spinach has also been reported after 12 days at 7 °C (Medina *et al.*, 2012). This suggests that even a small difference in storage temperature may have a major impact on the development of off-odours inside the bag with fresh produce.

To test the hypothesis that the rate of development of off-odours is temperature dependent, Piagentini *et al*. (2005) conducted a study, where they monitored the changes in this attribute in iceberg lettuce stored at four different temperature regimes. They found that development of off-odours took place after 6 days of storage at 9 °C, while at 20 °C

this commercially unacceptable change had already occurred after 2 days. No development of off-odours was observed after 10 days of storage if the samples were kept in the temperature below 5 °C. No development of off-odours was found in a similar study with cabbage (Gomez-Lopez *et al.*, 2005) or rocket leaves (Martinez-Sanchez *et al.*, 2006) after 9 days of storage at 7 °C and 14 days of storage at 4 °C, respectively.

No significant change in the development of off-flavours was found during the storage of lettuce for 7 days at 4 °C (Baur *et al*., 2005), while a significant change in this parameter was reported for iceberg lettuce (Gomez-Lopez *et al.*, 2005) and Romaine lettuce (Martinez-Sanchez *et al.*, 2011) stored at 7 °C for 5 and 10 days, respectively. On the other hand, slight to moderate development of off-flavours was reported in spinach leaves after 10 days of storage at 5 °C (Artes-Hernandez *et al.*, 2009), while these changes were already severe in the samples stored at 8 °C. What we know about the loss of sensory quality in leafy vegetables is that these changes are accelerated with increasing temperature of storage. Thus, to prevent the development of off-odours and off-flavours, leafy vegetables should always be kept at low refrigerated temperature; this should limit the decline in their sensory quality.

1.3.3 Visual quality changes

Visual quality is important as fresh leafy vegetables with a good appearance are preferred by customers. In most leafy vegetables green colour is the key quality characteristic as it adds to attractiveness and any colour alteration of the leaves might be recognised as a symptom of senescence (Ferrante *et al.*, 2004, Wagstaff *et al.*, 2007, Koukounaras *et al.*, 2009), reducing their marketability. In the case of endive which is white, however, the greening of the leaves as a result of chlorophyll formation has a negative effect on customer perception (Charles *et al*., 2008). Similarly, some leafy vegetables are supposed to be red (*e.g.* red oak leaf lettuce) due to the presence of other pigments, *e.g.* anthocyanin (Mou, 2005).

The visual quality of leafy vegetables, *e.g.* kale (Lefsrud *et al*., 2007), lettuce (Barg *et al*., 2009) and spinach (Bergquist *et al.*, 2006) has been reported to be affected by the maturity stage at harvest, mainly due to changes in the concentration of leaf pigments. Several authors have reported a decrease in visual quality during the storage of leafy vegetables (Ferrante *et al.*, 2004, Noichinda *et al.*, 2007, Gomez *et al.*, 2008, Koukounaras *et al.*, 2009, Medina *et al.*, 2012). Luo *et al*. (2009) have demonstrated that in the case of spinach leaves, the loss of visual quality is accelerated with increasing temperature of storage. A similar observation has been reported for kale (Kobori *et al.*, 2011). Leaf colour changes are not only affected by the storage temperature but also by the light conditions during storage. Martinez-Sanchez *et al*. (2011) observed stronger colour alteration in light-stored Romaine lettuce leaves when compared with their dark-stored counterparts.

Monitoring the colour changes during the storage of leafy vegetables is important in terms of determining visual quality loss. Commonly used parameters of colour in 3D colour space are: either 1. hue angle which describes the basic colour, luminance and chroma, *i.e.* colour saturation (Clydesdale, 1978) or 2. leaf lightness (from black to white), greenness (from green to red) and yellowness (from blue to yellow) values (Abbott, 1999). These techniques have been used to assess leaf colour changes in lettuce (Ihl *et al.*, 2003, Baur *et al.*, 2005, Martinez-Sanchez *et al.*, 2011), spinach (Pandrangi and LaBorde,

2004, Conte *et al.*, 2008, Artes-Hernandez *et al.*, 2009), rocket leaves (Koukounaras *et al.*, 2009) and endive (Charles *et al.*, 2008).

Seasonal differences in the colour characteristics of spinach leaves have been reported (Conte *et al.*, 2008); however, no changes were observed when spinach was stored at 5 °C for 13 days. In contrast, Tudela *et al*. (2013) reported significant changes in leaf lightness, greenness and yellowness during the storage of spinach at 7 °C for 10 days. Leaf lightness and yellowness values increased after 3 and 7 days, respectively. Changes in leaf colour characteristics have also been reported by others during refrigerated storage of lettuce (Kenny and O'Beirne, 2009, Manolopoulou *et al.*, 2010) and rocket leaves (Koukounaras *et al.*, 2009). Leaves became darker during the storage of lettuce (Kenny and O'Beirne, 2009, Manolopoulou *et al.*, 2010), while leaf lightness increased in rocket leaves over the storage period (Koukounaras *et al.*, 2009). This could be explained by tissue browning that occurs as a result of the oxidation of phenolic compounds, due to tissue damage, often observed during the storage of lettuce (Hisaminato *et al.*, 2001, Murata *et al.*, 2004) and leaf yellowing in rocket (Koukounaras *et al.*, 2009). Significant differences in leaf colour changes have been reported in lettuce leaves stored at 0 and 5 °C (Manolopoulou *et al.*, 2010), where colour was better preserved in lettuce leaves that were stored at 0 °C.

During the storage of chard leaves at 4 °C the leaf lightness value (*L**) increased in light-stored samples when the bags with high O_2 permeability were used. No change in the *L** value took place in the dark stored counterparts regardless the permeability of the bags (Sanz *et al.*, 2008). On the other hand, *L** value decreased in light-stored samples if film O_2 permeability was low. No change in leaf yellowness (*b**) value took place in the dark stored counterparts regardless the permeability of the bags (Sanz *et al.*, 2008), while *b** value decreased in the light-stored samples. Decrease in *L** and *b** values resulted in greenish-brown, darker leaves.

It has been reported (Toledo *et al.*, 2003a) that yellowing of spinach leaves stored at 8 °C was faster in the case of light-stored leaves when compared with their dark-stored

counterparts. Leaves may become more yellow (Plate 1.4) due to chlorophyll loss with simultaneous retention of carotenoids (change in the chlorophyll: carotenoids ratio).

Plate 1.4 Visual quality loss during extended storage of spinach leaves

Colour alteration usually results from chlorophyll degradation and/or tissue browning (Toivonen and Brummell, 2008). Colour changes can also occur due to changes in carotenoid content, however, because of antioxidant properties of this group of compounds these changes will be discussed in the consideration of nutritional quality changes (1.3.4.2.2 Nutritional quality changes - carotenoids).

1.3.3.1 Changes in chlorophyll content

A number of studies have found a decrease in chlorophyll concentration during the storage of Chinese kale (Noichinda *et al.*, 2007), Swiss chard (*Beta vulgaris* L.) (Ferrante *et al.*, 2004) rocket leaves (Ferrante *et al.*, 2004, Koukounaras *et al.*, 2009) and spinach (Kopas-Lane and Warthesen, 1995, Piagentini *et al.*, 2002, Gomez *et al.*, 2008). On the other hand, some authors have reported chlorophyll content to be relatively stable during the storage of endive (Ferrante *et al.*, 2004) lettuce (Spinardi and Ferrante, 2012) and spinach (Bergquist *et al.*, 2006, Conte *et al.*, 2008).

Studies on spinach (Kopas-Lane and Warthesen, 1995, Piagentini *et al.*, 2002) have reported small but significant changes in chlorophyll concentration during storage. A marked decrease in chlorophyll concentration during the storage of spinach leaves has been reported by Gomez *et al*. (2008). These authors stored spinach at 23 °C, whereas others used much lower temperatures, *e.g.* 4-5 °C (Kopas-Lane and Warthesen, 1995, Piagentini *et al.*, 2002). Chlorophyll degradation has been reported to be enhanced with increasing storage temperature (Gnanasekharan *et al.*, 1992, Pandrangi and LaBorde, 2004). On the other hand, no difference in chlorophyll retention was found between lettuce leaves stored at 4 and 10 °C (Spinardi and Ferrante, 2012). No correlation, however, was found between chlorophyll content and colour change during the storage of spinach leaves (Pandrangi and LaBorde, 2004, Bergquist *et al.*, 2006).

Decrease in chlorophyll concentration was found to be more pronounced in light-stored spinach leaves (Kopas-Lane and Warthesen, 1995). This view is supported by Ferrante *et al*. (2004) who also observed stronger colour alteration in light-stored rocket leaves when compared with their dark-stored counterparts. In contrast, higher chlorophyll retention was observed in light-stored Chinese kale (Noichinda *et al.*, 2007) and Swiss chard leaves (Ferrante *et al*., 2008).

No change in chlorophyll content was observed during dark storage of lettuce for 14 days at either 4 or 10 °C (Spinardi and Ferrante, 2012). Similar to spinach, enhanced degradation of carotenoids and chlorophyll during light storage has been reported for rocket leaves (Ferrante *et al.*, 2004), while no difference was found in the concentration of these pigments between light- and dark-stored Swiss chard leaves (Ferrante *et al.*, 2004, Ferrante *et al.*, 2008). Kopas-Lane and Warthesen (1995) have also reported enhanced chlorophyll *a* and *b* degradation in light-stored spinach, while the content of both chlorophylls remained relatively stable during dark storage. In contrast, low intensity light has recently been shown to be useful for carotenoids and chlorophyll *a* and *b* preservation during the storage of Chinese kale (Noichinda *et al.*, 2007) and Romaine lettuce (Zhan *et al.*, 2013). These differences might be due to different sensitivity to light of the plants used in these studies and/or due to different light intensities used by all these authors.

Previous studies have found that chlorophyll *a*: *b* ratio is relatively stable during the storage of leafy vegetables (Pandrangi and LaBorde, 2004, Bergquist *et al.*, 2006, Noichinda *et al.*, 2007, Conte *et al.*, 2008). In both Chinese kale (Noichinda *et al.*, 2007) and spinach (Conte *et al.*, 2008) this ratio increased during storage after 2 to 4 days. This change took place due to an increase in chlorophyll *a* with a simultaneous decrease in chlorophyll *b.* After that change, both chlorophyll *a* and *b*, and thus total chlorophyll content were quite stable. It has been suggested that chlorophyll *b* was transformed to chlorophyll *a* prior to its degradation (Noichinda *et al.*, 2007), and this might explain why chlorophyll *b* catabolites were not found during the storage of spinach (Piagentini *et al.*, 2002). This view is supported by Rudiger (2002) who reviewed the biosynthetic pathway of chlorophylls. Based on structural similarities of both chlorophyll *a* and *b*, it has been suggested that both compounds are synthesized through the same pathway with an additional step of chlorophyll *a* to chlorophyll *b* transformation (Rudiger, 2002). Furthermore, Rudiger (2002) claimed that chlorophyll *b* can be reduced back to chlorophyll *a*, and has proposed a “chlorophyll cycle”. Over the years, significant progress has been made in the understanding of chlorophyll degradation and this process has been reviewed elsewhere (Matile *et al.*, 1999, Thomas *et al.*, 2001, Hortensteiner, 2006) and thus will not be discussed in more detail here.

1.3.3.2 Tissue browning

Tissue browning is one of the main causes of quality loss during the storage of leafy vegetables (Hisaminato *et al.*, 2001, Ferrante *et al.*, 2004, Degl'Innocenti *et al.*, 2007). The key factors involved in this process are tissue integrity, phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) activity, polyphenol content, and polyphenol oxidase (PPO; EC 1.14.18.1) activity (Martinez and Whitaker, 1995, Hisaminato *et al.*, 2001, Kang and Saltveit, 2003, Degl'Innocenti *et al.*, 2005, Degl'Innocenti *et al.*, 2007).

A considerable amount of literature dealing with polyphenol biochemistry and subsequent reactions leading to tissue browning has been published (Martinez and Whitaker, 1995, Sheptovitsky and Brudvig, 1996, Hisaminato *et al.*, 2001, Murata *et al.*, 2004). Briefly, the shikimate pathway leads to L-phenylalanine synthesis, which then enters the phenylpropanoid pathway. L-phenylalanine is transformed by PAL into cinnamic acid that is subsequently further converted into hydroxyphenols. Finally, PPO transforms them into quinones, which then polymerize resulting in tissue browning. Both PAL and PPO require molecular oxygen for their activity (Martinez and Whitaker, 1995, Degl'Innocenti *et al.*, 2007, Toivonen and Brummell, 2008), which might decrease during storage due to oxygen depletion inside the bag.

A correlation between PAL activity and tissue browning has been reported during the storage of lettuce (Hisaminato *et al.*, 2001, Murata *et al.*, 2004), and this suggests that *de novo* biosynthesis of polyphenols is necessary to cause tissue browning. Furthermore, in lettuce the activity of PAL and polyphenol content has been observed to increase after 3 days of storage, while PPO activity was relatively stable during storage (Hisaminato *et al.*, 2001, Baur *et al.*, 2004). This shows that if the concentration of phenolic substrates is not high enough even the high activity of PPO cannot cause an immediate tissue browning.

Degl'Innocenti *et al*. (2005) have attempted to explain the biochemistry behind tissue browning of lettuce leaves during storage. They investigated the activities of all enzymes potentially involved in the browning process in two lettuce cultivars differing in

their susceptibility to browning. PAL activity was induced earlier in the less susceptible cultivar (Red Salade Bowl, RSB), reaching a maximum value after 3 h, while in the same time a decrease in PAL activity was observed in the more susceptible cultivar (Green Salade Bowl, GSB). After 6 h, however, this pattern reversed, a decrease in PAL activity being observed in RSB, while the activity of this enzyme increased in GSB. These authors also reported only a slight increase in the overall concentration of phenolic compounds during storage and they did not find any significant difference in phenolic content between both cultivars. In contrast to other authors (Hisaminato *et al.*, 2001, Baur *et al.*, 2004), they reported a strong decrease in PPO activity during the storage of both cultivars of lettuce. On the other hand, significant difference between cultivars has been reported for the activity of peroxidase (POD; EC 1.11.1.7), and it has been suggested (Degl'Innocenti *et al.*, 2005) that this enzyme may also play a role in tissue browning. This view is supported by other authors (Martin-Diana *et al.*, 2005, Rico *et al.*, 2008) who found that with decreasing POD activity the extent of tissue browning decreases.

In the study of Pereyra *et al*. (2005) the peak in PAL activity during the storage of lettuce correlated well with the intensity of tissue damage and these authors observed tissue browning to occur first in the most highly damaged leaves. This is somewhat different from the findings of Degl'Innocenti *et al*. (2005) who observed that the peak in PAL activity in lettuce after 3 h, did not lead to intensive tissue browning. Furthermore, in a subsequent study where Degl'Innocenti *et al*. (2007) investigated the sensitivity of different crops (endive, lettuce, and rocket) to tissue browning, they reported peaks in PAL activity after approximately 6 h in lettuce, 12 h in rocket leaves, and 72 h in endive but there was no correlation between peak timing and tissue browning. Both endive and lettuce (belonging to *Asteraceae* family) exhibited some browning already after 72 h of storage, while no visible symptoms of tissue browning could be seen in the case of rocket leaves (*Brassicaceae* family). It can be concluded from those studies (Degl'Innocenti *et al.*, 2005, Degl'Innocenti *et al.*, 2007) that timing of peak in PAL activity cannot be used consistently as an indicator of subsequent browning. Furthermore, it may also suggest significant differences between crops belonging to different families.

It has recently been suggested that leafy vegetables with high concentration of ascorbic acid (AsA) are less susceptible to enzymatic browning (Degl'Innocenti *et al.*, 2007, Bottino *et al.*, 2009). This claim has been made based on the results obtained from studies where the sensitivity of different leafy vegetables to tissue browning was investigated (Degl'Innocenti *et al.*, 2007). These authors reported tissue browning during the storage of lettuce and endive, while no browning occurred during the storage of rocket leaves. An absence of tissue browning has also been observed during the storage of spinach (Bottino *et al.*, 2009). Both authors (Degl'Innocenti *et al.*, 2007, Bottino *et al.*, 2009) suggested that it was due to the action of AsA on PPO activity, which led to lowered concentration of quinones, thus delaying the polymerization reaction. However, it does not fully fit with the previous findings, where Degl'Innocenti *et al*. (2005) demonstrated higher susceptibility to browning in GSB lettuce, while GSB was the cultivar that had significantly higher AsA content at harvest compared to RSB, which was less susceptible to browning. Furthermore, it has previously been suggested by these authors and others (Hisaminato *et al.*, 2001, Baur *et al.*, 2004) that PPO cannot cause severe tissue browning if the concentration of phenolic substrates is not sufficient.

Light exposure during storage reduced visual quality and accelerated tissue browning of Romaine lettuce (Martinez-Sanchez *et al.*, 2011) and chard (Sanz *et al.*, 2008). In a recent study light exposure during storage has been reported to affect the activity of enzymes involved in tissue browning – PAL, PPO and POD (Zhan *et al.*, 2012). Both low (7 μmol m^{-2} s^{-1}) and high (34 μmol m^{-2} s^{-1}) intensity light increased the activity of PAL, during the storage of Romaine lettuce, when compared with their dark-stored counterparts. Interestingly, the activity of PPO and POD was increased and decreased when samples were exposed to either low or high intensity light, respectively.

1.3.4 Nutritional quality changes

Plants produce active oxygen species (AOS) during cellular metabolism; however, in response to environmental stresses, *e.g.* temperature stress, AOS production, as well as the activity of antioxidant enzymes - ascorbate peroxidase (APX; EC 1.11.1.11),

catalase (CAT; EC 1.11.1.6), and superoxide dismutase (SOD; EC 1.15.1.1) may increase (Tsang *et al.*, 1991, Eraslan *et al.*, 2007). AOS include such compounds as superoxide radicals (O_2^-), singlet oxygen ($^1O_2$) and highly reactive hydroxyl radicals (OH^-). SOD catalyses the dismutation of superoxide (O_2^-) to H_2O_2 which is then transformed to H_2O and O_2 by simultaneous action of APX and CAT. To mitigate AOS, plants may also induce the biosynthesis of antioxidants, including AsA which is involved in the reduction of AOS through the ascorbate-glutathione cycle (Figure 1.1) (Mittler, 2002, Potters *et al.*, 2002, Meyer, 2008). Other antioxidants, like carotenoids and flavonoids have also been suggested to play an important role as AOS scavengers (Chu *et al.*, 2000, Fraser and Bramley, 2004, Hernandez *et al.*, 2009). AOS may also play a role of signalling molecules. Thus, as a result of cross-talk, they may induce different defence responses within plants, *e.g.* in response to pathogens or to multiple stresses (Mittler, 2002, de Pinto *et al.*, 2006, Fujita *et al.*, 2006). Senescence of broccoli (*Brassica oleracea* var. *italica*) has been found to be delayed as a result of enhanced activity of the ascorbate-glutathione cycle (Shigenaga *et al.*, 2005), thus suggesting its role in extending the shelf-life of fresh produce.

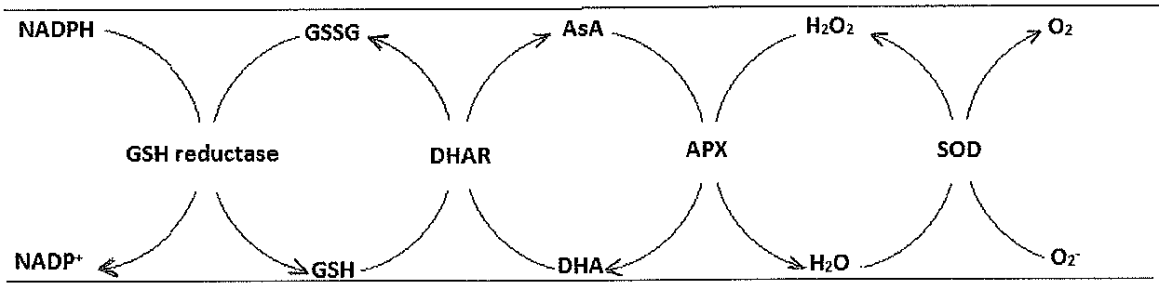

Figure 1.1 Overview of ascorbate-glutathione cycle in plants. NADPH - nicotinamide adenine dinucleotide phosphate (reduced form); $NADP^+$- nicotinamide adenine dinucleotide phosphate (oxidised form); GSH – reduced glutathione; GSSG - oxidised glutathione; DHAR - dehydroascorbate reductase; DHA – dehydroascorbic acid; AsA – ascorbic acid; APX - ascorbate peroxidase; SOD - superoxide dismutase (based on Mittler, 2002).

Light quantity may have an impact on the activity of antioxidant enzymes as the activity of the following enzymes - SOD, CAT, APX, monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), and glutathione

reductase (GR; EC 1.6.4.2) - was found to be increased in lettuce grown at high intensity light (Zhou *et al*., 2009). The increase in the activity of MDHAR and DHAR with increasing light intensity has previously been reported during the growth of *Arabidopsis thaliana* (Bartoli *et al*., 2006), while Toledo *et al*. (2003b) did not observe any differences in the activity of these enzymes in spinach leaves stored under low intensity light when compared with their dark-stored counterparts. This can be explained by different responses of leaves to light exposure during growth and during storage; alternatively, it may also be due to a different plants and/or different light intensities used in those studies. In their studies, Bartoli *et al*. (2006) and Zhou *et al*. (2009) used irradiances in the range of 50-250 and 200-1200 μmol m^{-2} s^{-1}, respectively, and these are relatively higher than 20-25 μmol m^{-2} s^{-1} used by Toledo *et al*. (2003b).

1.3.4.1 Biochemistry of selected antioxidants

1.3.4.1.1 Ascorbic acid (AsA)

In the last decade, there has been an increasing interest in AsA biosynthesis and its role in plants (Conklin, 2001, Hancock and Viola, 2005, Ishikawa *et al.*, 2006). Progress has been made in understanding the pathways and key enzymes involved have been identified. Multiple pathways have been suggested to be involved in the biosynthesis of AsA (Hancock and Viola, 2005). It is worth noting that the concentration of this bioactive compound in plant tissue can be regulated not only by inducing its biosynthesis, but also through limitation of its turnover, which might actually be more important in terms of antioxidant content manipulation during the storage of leafy vegetables.

Several reviews have described the biosynthesis of AsA (Smirnoff, 1996, Smirnoff and Wheeler, 2000, Hancock and Viola, 2005, Ishikawa and Shigeoka, 2008), and will be used as a main source of information regarding this section.

Multiple biosynthetic pathways may exist, however D-glucose is recognised as a precursor of AsA. There is strong evidence that the biosynthesis of AsA proceeds via a D-mannose/L-galactose pathway and that this route is the most significant source of AsA in plants (Conklin *et al.*, 1999, Smirnoff and Wheeler, 2000, Hancock and Viola, 2005,

Ishikawa *et al.*, 2006, Ishikawa and Shigeoka, 2008). This pathway proceeds via GDP-D-mannose and L-galactose, and the final aldonolactone precursor of AsA is L-galactono-1, 4-lactone. This last step of AsA biosynthesis is catalysed by L-galactono-1, 4-lactone dehydrogenase located on the mitochondrial membrane (Millar *et al*., 2003).

In the pathway proposed by Ishikawa and Shigeoka (2008) no inversion of carbon skeleton occurs, while the pathway with inversion of carbon skeleton has previously been proposed by Smirnoff (1996). Ishikawa and Shigeoka (2008) reviewed the available information on other enzymes, which may play a role in AsA biosynthesis. These enzymes are as follows: L-galactose dehydrogenase, GDP-mannose pyrophosphorylase, GDP-mannose 3‘, 5‘-epimerase and L-galactose-1- phosphate phosphatase. The activity of L-galactose dehydrogenase has been reported to be regulated by AsA (Mieda *et al*., 2004), thus suggesting that biosynthesis of AsA is regulated by its concentration in the tissue.

In 1990, a group of scientists from Washington State University (Loewus *et al.*, 1990, Saito *et al.*, 1990) proposed a non-inversion pathway of AsA biosynthesis that starts from D-glucose, which is then converted to D-glucosone, L-sorbosone and finally to AsA. There is, however, no recent information available to support the role of this pathway in plants, thus this pathway remains debated.

Ascorbic acid concentration in the plant tissue can also be increased through enhanced turnover of this compound. The level of AsA is regulated by MDHAR and DHAR (Smirnoff, 1996, Conklin, 2001, Chen *et al.*, 2003, Ishikawa and Shigeoka, 2008). These two enzymes are responsible for the turnover of AsA. This process follows two steps, first dehydroascorbic acid (DHA) is reduced to monodehydroascorbic acid (MDHA), and then further to ascorbic acid (AsA) (Davey *et al.*, 2000, Potters *et al.*, 2002, Ishikawa *et al.*, 2006). The reaction catalysed by DHAR uses glutathione (GSH) as a reductant. While DHA is converted back to AsA, GSH is oxidised to GSSG (oxidised glutathione). Furthermore, the redox state of AsA has been shown to be associated with the redox state of GSH, and these components together contribute to the ascorbate-glutathione cycle (Smirnoff, 1996, Potters *et al.*, 2002, Chen *et al.*, 2003). Ascorbic acid peroxidases

(APXs) play a key role in H_2O_2 metabolism in this cycle. APX uses AsA as an electron donor to reduce H_2O_2 (generated by SOD from AOS) to water, while AsA is converted into MDHA. AsA is regenerated in the ascorbate-glutathione cycle by DHAR and GSSG is regenerated by GR (Davey *et al.*, 2000, Shigenaga *et al.*, 2005, Ishikawa and Shigeoka, 2008, Meyer, 2008). If this is not the case, MDHA can be oxidised further to DHA which may then undergo a reduction to AsA, catalysed by DHAR, or it may undergo an irreversible hydrolysis to 2, 3-diketogulonic acid (Yang and Loewus, 1975, Davey *et al.*, 2000, Chen *et al.*, 2003).

The oxidised form of ascorbic acid can be converted back into reduced form in the human body. Thus, both forms have to be considered when determining the concentration of total AsA (AsA + DHA). The activity of both reduced and oxidised forms of ascorbic acid has been investigated in spinach (Gil *et al*., 1999). These authors have suggested that when AsA is oxidised it loses most of its antioxidative activity. This suggests that determining the AsA: DHA ratio might be important in terms of human nutrition.

In addition, AsA seems to be metabolised into oxalic and tartaric acid (Yang and Loewus, 1975, Smirnoff, 1996, Conklin, 2001, Nakata, 2003, Ishikawa *et al.*, 2006). The formation of calcium oxalate crystals occurs in idioblasts and has been reported to be regulated by calcium itself (Nakata, 2003).

1.3.4.1.2 Carotenoids

It has been suggested that to enhance carotenoid concentrations in crops a good understanding of their biochemistry is needed (Kopsell and Kopsell, 2006). The information on carotenoid biosynthesis has recently been reviewed (Botella-Pavia and Rodriguez-Concepcion, 2006, Kopsell and Kopsell, 2006) and this pathway seems to be conservative among plants.

Like all isoprenoids, carotenoids are synthesized from the 5-carbon isoprene units, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Addition of three IPP molecules to one DMAPP unit generates geranylgeranyl diphosphate (GGPP), a common precursor for several groups of plastid isoprenoids. In the first step of carotenoid biosynthesis, phytoene synthase (PSY; EC 2.5.1.32) catalyses the two-step conversion of two molecules of GGPP into pre-phytoene pyrophosphate (PPPP) and then into phytoene. In this process two molecules of GGPP are joined in a condensation reaction with the loss of hydrogen and the diphosphate group from C-1' of the same molecule. Cleavage of the C-1 diphosphate group of the resulting PPPP, followed by a 1-1' rearrangement, results in the formation of phytoene. Phytoene has the basic C40 skeleton of carotenoids, and all subsequent reactions in the pathway involve chemical conversions of this basic structure. A series of desaturation reactions convert colourless compound phytoene into yellow, orange, and red carotenoids by creating the conjugated double bonds that form the chromophore. There are four desaturation steps between phytoene and lycopene.

A branching point in the plant carotenoid pathway is marked by the cyclization of lycopene, which is converted into either *α*-carotene or *β*-carotene by the enzyme lycopene cyclase (LYC; EC 5.5.1.19). Xanthophylls are hydroxyl-, epoxy-, furanoxy- and oxy-derivatives of the carotenes formed in the late stages of the pathway. The first xanthophylls are formed from cyclic carotenoids such as *α*-carotene (lutein) and *β*-carotene (zeaxanthin, violaxanthin and neoxanthin) by the introduction of hydroxy groups in positions C3 and C3' of the ionone rings, followed by epoxidation at the 5, 6 and 5', 6' positions. Xanthophylls undergo light-dependent epoxidation/de-epoxidation cycles by the

interconversion of zeaxanthin and violaxanthin and this conversion is known as xanthophyll cycle, and plays a key role in response to excess light (Muller *et al*., 2001). Zeaxanthin is readily converted to violaxanthin via antheraxanthin by introducing 5, 6-epoxy groups into the 3-hydroxy rings, a reaction catalyzed by the enzyme zeaxanthin epoxidase (ZEP; EC 1.14.13.90). In the leaves under excess light, violaxanthin deepoxidase (VDE; EC 1.10.99.3) catalyses the two-step de-epoxidation reaction that transforms violaxanthin back into zeaxanthin, which is much more efficient in dissipating the excess excitation energy (Demmig-Adams *et al*., 1996). Return to low-light conditions results in the transformation of zeaxanthin into violaxanthin (Muller *et al.*, 2001). In the last step of the pathway, violaxanthin is transformed into neoxanthin by the activity of neoxanthin synthase (NSY; EC 5.3.99.9) (Botella-Pavia and Rodriguez-Concepcion, 2006).

Knowledge of changes in the concentrations of individual antioxidants during the storage of leafy vegetables is important (DuPont *et al.*, 2000, Martinez-Sanchez *et al.*, 2006, Llorach *et al.*, 2008, Martinez-Sanchez *et al.*, 2008b) due to their health promoting properties. It has recently been suggested that antioxidants may also improve the shelf-life of fresh produce (Bergquist *et al.*, 2006). Several authors have investigated the effect of different storage temperatures (Chu *et al.*, 2000, Pandrangi and LaBorde, 2004, Bergquist *et al.*, 2005, Bergquist *et al.*, 2006, Bergquist *et al.*, 2007) and light exposure (Noichinda *et al.*, 2007, Lester *et al.*, 2010b, Kobori *et al.*, 2011, Martinez-Sanchez *et al.*, 2011, Zhan *et al.*, 2013) on antioxidant content in leafy vegetables. The main antioxidants in leafy vegetables are AsA, carotenoids, and flavonoids. Information on changes in the concentration of these compounds is reviewed.

1.3.4.2 Antioxidant content of leafy vegetables

1.3.4.2.1 Total ascorbic acid (ascorbic acid and dehydroascorbic acid)

The concentration of AsA generally declines during the storage of curly kale (*Brassica oleracea* L. var. *acephala*) (Hagen *et al.*, 2009), Chinese kale (Noichinda *et al.*, 2007), lettuce (Martinez-Sanchez *et al.*, 2012, Spinardi and Ferrante, 2012), Swiss chard (Gil *et al*., 1998) wild rocket leaves (Martinez-Sanchez *et al.*, 2006) and spinach (Gil *et al.*, 1999, Hodges *et al.*, 2001, Hodges and Forney, 2003, Bergquist *et al.*, 2006, Bergquist *et al.*, 2007). Bergquist *et al*. (2007) reported, however, that AsA concentration may also increase during storage, thus suggesting that AsA biosynthesis can occur after harvest. This view is supported by Lester *et al*. (2010b) who demonstrated that AsA was well preserved in mid- and bottom-canopy spinach leaves but not in the top-canopy leaves stored under continuous light for 9 days at 5 °C. Initial AsA content in top-canopy leaves was higher than in medium- or bottom-canopy leaves, thus regardless the decline, AsA content in these leaves after 9 days of storage was not lower when compared with older leaves. Higher AsA content in baby spinach leaves compared to mature leaves has previously been reported (Bergquist *et al.*, 2006). In the case of lamb's lettuce (*Valerianella olitoria*) leaves, both AsA and DHA content increased after 1 day of dark storage at 4 °C and then declined (Ferrante *et al*., 2009). Most of the studies mentioned above have also reported a decrease in AsA: DHA ratio during storage; this decrease occurs through AsA oxidation and/or degradation. On the other hand, the ratio actually increased (cut leaves) or decreased (whole leaves) after 1 day of storage in lamb's lettuce leaves (Ferrante *et al.*, 2009) and then declined (cut leaves) or increased (whole leaves) after 5 days of storage. After 8 days of storage AsA: DHA ratio (in both cut and whole leaves) was not significantly different from the initial value. Due to the fact, that DHA can be converted back to AsA, it is important to quantify the concentration of both forms of ascorbic acid (Gil *et al.*, 1999, Lee and Kader, 2000) which contribute to the total AsA pool.

In contrast to spinach (Gil *et al.*, 1999, Bergquist *et al.*, 2006, Bergquist *et al.*, 2007, Bottino *et al.*, 2009), curly kale (Hagen *et al.*, 2009), mizuna and watercress

(Martinez-Sanchez *et al.*, 2008b), different lettuce varieties (iceberg, Romaine, continental, red oak leaf and lollo rosso) have been reported to contain more DHA, and lower AsA: DHA ratio (Beltran *et al.*, 2005, Degl'Innocenti *et al.*, 2005, Llorach *et al.*, 2008, Martinez-Sanchez *et al.*, 2011), which varies between cultivars (Llorach *et al.*, 2008). In some studies, no DHA at all was found in iceberg lettuce (Kenny and O'Beirne, 2009). On the other hand, Singh *et al*. (2006) reported significant differences in AsA content between different cultivars of cabbage.

Ascorbic acid content in lettuce declines during storage (Kenny and O'Beirne, 2009, Aguero *et al.*, 2011, Martinez-Sanchez *et al.*, 2012, Spinardi and Ferrante, 2012). Degl'Innocenti *et al*. (2005) demonstrated the differences between lettuce cultivars in the way AsA and DHA behave during storage. In their study, the concentration of DHA increased (AsA remained unchanged) in the case of RSB cultivar, while a decrease in both AsA and DHA was observed in GSB cultivar, where AsA disappeared within 3 days of storage. This inconsistent pattern (increase/decrease) in AsA content changes during storage has also been observed by other authors (Murata *et al.*, 2004, Beltran *et al.*, 2005, Martinez-Sanchez *et al.*, 2011) in experiments with iceberg lettuce. In addition to AsA to DHA conversion, AsA can also be transformed into oxalic acid (Yang and Loewus, 1975) and this has been observed during the storage of spinach (Toledo *et al*., 2003b).

The effect of storage temperature of 2 and 10 °C on AsA content in harvested spinach has been investigated (Bergquist *et al.*, 2006, Bergquist *et al.*, 2007). They found a smaller loss of AsA at the lower storage temperature. Furthermore, they observed a decrease in AsA: DHA ratio during storage, suggesting faster degradation or metabolism, or higher oxidation of AsA. This process was more pronounced at higher storage temperature. Overall, loss of AsA during storage occurs faster with increasing storage temperature (Davey *et al.*, 2000, Lee and Kader, 2000), no difference in AsA retention, however, was found between lettuce stored at 4 and 10 °C (Spinardi and Ferrante, 2012).

The increase in AsA has been observed during growth of lettuce (Zhou *et al.*, 2009) and *Arabidopsis* (Bartoli *et al.*, 2006, Yabuta *et al.*, 2007) in response to high intensity light, while Yoshimura *et al*. (2000) reported that light had no effect on AsA

content in spinach. On the other hand, a decrease in AsA : DHA ratio in response to high intensity light has been found both in spinach (Yoshimura *et al*., 2000) and lettuce (Zhou *et al.*, 2009). In the case of spinach, it was clearly due to increased DHA content in response to high intensity light.

It has been demonstrated that light manipulation during storage may be used to improve nutritional quality of leafy vegetables (Toledo *et al.*, 2003b, Noichinda *et al.*, 2007, Lester *et al.*, 2010b). Several authors have found slower AsA loss during light-storage of Chinese kale (Noichinda *et al.*, 2007), lettuce (Zhan *et al.*, 2012, Zhan *et al.*, 2013) and spinach leaves (Toledo *et al.*, 2003b, Lester *et al.*, 2010b) when compared with their dark-stored counterparts. Better retention of AsA has also been observed in light-stored spring-grown rocket leaves, whereas no difference between two storage conditions was observed for summer-grown leaves (Barbieri *et al.*, 2011). Lester *et al*. (2010b) observed higher AsA content in light-stored spinach in cultivar Lazio, but not in the case of cultivar Samish, where no difference in AsA content between two storage conditions was found for top- and medium-canopy leaves after 9 days of storage. Toledo *et al*. (2003b) found no difference in the activity of enzymes involved in AsA biosynthesis and metabolism (L-galactono-1, 4-lactone dehydrogenase, MDHAR and DHAR) between light- and dark-stored leaves. These authors suggested that higher biosynthesis of AsA could be due to increased availability of soluble carbohydrates in light-stored leaves.

On the other hand, a significantly higher increase in DHA content was found in dark-stored lettuce (Zhan *et al.*, 2012, Zhan *et al.*, 2013), rocket (Barbieri *et al.*, 2011) and spinach leaves (Lester *et al.*, 2010b). The increase in DHA compensated the loss in AsA content and resulted in similar total AsA content between dark and light-stored samples. No difference in total AsA between dark and light-stored lettuce has been reported by Martinez-Sanchez *et al*. (2011). It is also worth noting that no difference in total AsA content between dark and light-stored spinach leaves was reported for cultivar Lazio while differences were observed in cultivar Samish (Lester *et al.*, 2010b), thus highlighting different responses among cultivars.

Another important factor that may influence the effect of light exposure on AsA and other bioactive compounds (*e.g.* carotenoids) is leaf age as the response to light has been shown to be different in spinach leaves of different maturity (Lester *et al.*, 2010b). Furthermore, the initial concentration of AsA and carotenoids in plants of different age varies (Bergquist *et al.*, 2006, Bergquist *et al.*, 2007). AsA concentration is higher in younger plants (Bergquist *et al.*, 2006, Bergquist *et al.*, 2007, Lester *et al.*, 2010b), while carotenoid concentration increases with plant maturity (Bergquist *et al.*, 2006).

1.3.4.2.2 Carotenoids

The carotenoid concentration of leafy vegetables prior to storage is affected by environmental conditions during growth, *e.g.* light intensity (Eskling and Akerlund, 1998, Lefsrud *et al.*, 2006) and air temperature (Lefsrud *et al*., 2005). Thus, it is not surprising that plant pigments concentration differs between seasons (Kopsell *et al.*, 2004, Mou, 2005) and plants of different maturity (Lefsrud *et al.*, 2007). Furthermore, differences among cultivars, types and genotypes in *β*-carotene and lutein content have been reported for *Lactuca* sp. (Mou, 2005) and *Brassica* sp. (Kopsell *et al.*, 2004, Singh *et al.*, 2006).

Postharvest storage may cause changes in the concentration of these bioactive compounds. Several studies have reported changes in carotenoids during the storage of spinach leaves under different conditions (Pandrangi and LaBorde, 2004, Bergquist *et al.*, 2006, Bergquist *et al.*, 2007, Bunea *et al.*, 2008). The findings, however, have been inconsistent and contradictory.

A decrease in carotenoid content during storage has been reported in endive, Swiss chard and rocket leaves (Ferrante *et al.*, 2004), and spinach (Pandrangi and LaBorde, 2004, Bunea *et al.*, 2008, Tudela *et al.*, 2013). In contrast, an increase in carotenoid content has been reported during the storage of spinach leaves (Bergquist *et al.*, 2006, Bergquist *et al.*, 2007), which suggests that synthesis of carotenoids may occur; however, this change was not always significant. They concluded that carotenoids are quite well preserved during storage. Bergquist *et al*. (2006) suggested that these

differences could occur due to different cultivars used in these studies or due to different growth stages of harvested plants that were used.

The major carotenoids found in spinach are lutein, *β*-carotene, violaxanthin and neoxanthin (Kopas-Lane and Warthesen, 1995, Pandrangi and LaBorde, 2004, Bergquist *et al.*, 2006, Bergquist *et al.*, 2007, Bunea *et al.*, 2008). Both Bergquist *et al*. (2006) and Bunea *et al*. (2008) observed that lutein comprised around 40% of total carotenoids. Lutein has also been found to be the main carotenoid in kale (Lefsrud *et al.*, 2005, Lefsrud *et al.*, 2006, Nilsson *et al.*, 2006, Lefsrud *et al.*, 2007) and white cabbage (Nilsson *et al.*, 2006).

The composition of individual carotenoids changes during storage. The concentrations of lutein and violaxanthin have been reported to decrease during the storage of spinach leaves (Pandrangi and LaBorde, 2004, Bunea *et al.*, 2008), while Bergquist *et al*. (2006) observed an increase in these carotenoids after 9 d of storage. In the case of *β*-carotene, a decrease in its concentration in stored spinach has been observed (Kopas-Lane and Warthesen, 1995, Pandrangi and LaBorde, 2004, Bunea *et al.*, 2008), while the results were inconsistent in another study (Bergquist *et al.*, 2006), where *β*-carotene concentration was relatively stable, and in some cases it increased.

Storage temperature may influence the concentration of carotenoids (Pandrangi and LaBorde, 2004, Bergquist *et al.*, 2006, Yang *et al.*, 2010). Higher loss in carotenoid content with increasing temperature of storage has been reported in kale (Kobori *et al.*, 2011) when authors compared samples stored at 1 and 11 °C. On the other hand, no difference in carotenoid content was reported between lettuce stored at 4 and 10 °C (Spinardi and Ferrante, 2012).

Higher loss of carotenoids was also observed in spinach with increasing storage time and storage temperature (Pandrangi and LaBorde, 2004). These results are contradictory to Bergquist *et al*. (2006) who reported higher concentration of lutein and violaxanthin in harvested spinach stored at 10 °C. In their research, the only carotenoid that was better preserved at 2 °C was *β*-carotene, while neoxanthin was not affected by

storage temperature. Pandrangi and LaBorde (2004), however, did not observe significant differences in lutein and neoxanthin concentration between spinach leaves stored for 6 days at 10 °C and those stored for 8 days at 4 °C, respectively. The retention of these carotenoids might be less sensitive to changes in temperature during storage. Furthermore, *all-trans β*-carotene has been found to be more stable than 9-*cis β*-carotene when spinach was stored at 4 °C (Pandrangi and LaBorde, 2004), while retention of both isomers was not significantly different at higher storage temperature. This may explain better retention of *β*-carotene at lower storage temperatures (Bergquist *et al.*, 2006).

Kobori *et al*. (2011) observed no changes in neoxanthin, violaxanthin, lutein and *β*-carotene content during dark storage of kale at 1 °C. The content of all investigated carotenoids, however, declined during dark storage at 11 °C. The same pattern was observed for violaxanthin and *β*-carotene in light-stored samples, whereas the content of lutein and neoxanthin remained relatively stable. No significant difference in the content of neoxanthin and *β*-carotene was found between two storage conditions, while lutein content was significantly higher in light-stored samples. The content of violaxanthin was higher in the dark-stored counterparts due to the fact that in response to light exposure during storage violaxanthin was transformed to zeaxanthin. This transformation takes place within the “xanthophyll cycle” that is involved in photoprotection by responding to changes in the light intensity (Muller *et al.*, 2001).

Kopas-Lane and Warthesen (1995) have reported enhanced carotenoids degradation in light-stored spinach. These authors found that all investigated carotenoids (lutein, neoxanthin, violaxanthin, *β*-carotene) were quite stable in dark-stored spinach, while a decrease in their concentration was apparent after 4 days in light-stored samples. Only *all-trans β*-carotene decreased in dark stored samples, however, its decrease in the light was stronger. All investigated carotenoids (lutein, neoxanthin, violaxanthin, *all-trans β*-carotene, 9-*cis β*-carotene and 13-*cis β*-carotene) decreased in the light stored samples. This finding is partially supported by Lester *et al*. (2010b), who reported carotenoids (lutein/zeaxanthin, *β*-carotene and violaxanthin) to be relatively stable or even increase during dark storage of two spinach cultivars - Lazio and Samish. In cultivar Lazio,

β-carotene and violaxanthin content was significantly higher in mid- and bottom-canopy leaves stored in the dark, while no difference between two storage conditions was observed in top-canopy leaves. On the other hand, in cultivar Samish significantly higher *β*-carotene and violaxanthin content was reported for top- and mid-canopy leaves, while there was no difference in bottom-canopy leaves. This suggests that quality changes during the storage of spinach leaves of different cultivars and maturity follow a different pattern. Furthermore, changes in carotenoid content during light- and dark-storage are influenced by growing conditions as Barbieri *et al*. (2011) observed higher carotenoids content in the spring-grown rocket leaves stored in the light, while no difference between two storage conditions was found in the summer-grown leaves. This could be explained by different light intensity over two growing seasons.

The pigment concentration in leafy vegetables may also be affected by irradiance level (Lefsrud *et al.*, 2006). These authors found that irradiance of 335 µmol m^{-2} s^{-1} significantly increased lutein and *β*-carotene concentration in kale, while none of the irradiance levels investigated (125, 200, 335, 460, and 620 µmol m^{-2} s^{-1}) had any effect on the concentration of these compounds in spinach, when values were given on FW basis; however, on a dry weight (DW) basis significant differences in lutein and *β*-carotene concentration were reported in spinach, whereas they were not significant in kale. The different outcome based on DW and FW reported by these authors can be explained by the change in moisture content in response to different irradiance levels. The change in moisture content in response to increasing irradiance was found to be higher in kale than in spinach resulting in higher dilution of plant pigments in kale.

1.3.4.2.3 Flavonoids

Flavonoids are a group of metabolites that play a role in plants protection against damage induced by abiotic (*e.g.* UV radiation, wounding) and biotic (*e.g.* pathogens and predators) stressors (Harborne and Williams, 2000, Pourcel *et al.*, 2007). These compounds have also been suggested to be important for human health. Their medicinal and nutritional properties have been reviewed elsewhere (Harborne and Williams, 2000, Nijveldt *et al.*, 2001).

In the last decade, there has been an increasing interest in the flavonoid content of fresh produce because of their antioxidant properties (Gil *et al.*, 1999, DuPont *et al.*, 2000, Bergquist *et al.*, 2005, Llorach *et al.*, 2008). The most common flavonoids in leafy vegetables are quercetin, kaempferol and luteolin, and their glycosides (Crozier *et al.*, 1997, DuPont *et al.*, 2000, Llorach *et al.*, 2008, Martinez-Sanchez *et al.*, 2008b).

In lettuce, flavonoids have been found to be present mainly in the form of quercetin conjugates (Crozier *et al.*, 1997, DuPont *et al.*, 2000, Arabbi *et al.*, 2004, Llorach *et al.*, 2008). The presence of five quercetin conjugates in lettuce has been reported (DuPont *et al.*, 2000). These authors have identified following compounds: quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, quercetin 3-*O*-(6-*O*-malonyl) glucoside and quercetin 3-*O*-rhamnoside. The quercetin 3-*O*-glucoside was the dominant form of flavonoids found in this study. In the recent study, Llorach *et al*. (2008) identified the presence of new flavonoids in red lettuce varieties (red oak leaf and lollo rosso). These were: quercetin-7-*O*-glucuronide-3-*O*-(6”-*O*-malonyl)-glucoside and quercetin-7-*O*-glucoside-3-*O*-(6”-*O*-malonyl)-glucoside. The concentration of quercetin conjugates was lower in the inner leaves of lettuce when compared with outer leaves (Crozier *et al.*, 1997). Moreover, the concentration of quercetin was shown to be significantly different between lettuce varieties (Crozier *et al.*, 1997, DuPont *et al.*, 2000, Llorach *et al.*, 2008). In addition to quercetin conjugates, DuPont *et al*. (2000) described the presence of luteolin 7-*O*-glucuronide and cyanidin conjugates [cyanidin 3-*O*-glucoside and cyanidin 3-*O*-[(6-*O*-malonyl) glucoside] in the green- and red-leafed varieties of lettuce, respectively. The presence of quercetin and luteolin conjugates in lettuce has been confirmed in more

recent studies (Arabbi *et al.*, 2004, Heimler *et al.*, 2007, Llorach *et al.*, 2008). The presence of cyanidin in red lettuce has also been reported by others (Ferreres *et al.*, 1997b, Arabbi *et al.*, 2004, Llorach *et al.*, 2008).

In endive, DuPont *et al*. (2000) reported the presence of three kaempferol conjugates, kaempferol 3-*O*-glucoside, kaempferol 3-*O*-glucuronide, and kaempferol 3-*O*-[(6-*O*-malonyl) glucoside]. The presence of kaempferol conjugates in endive has been confirmed by Llorach *et al*. (2008). In contrast, the presence of kaempferol, quercetin, luteolin and apigenin has been reported by Arabbi *et al*. (2004) when these authors analysed the flavonoid content of endive, and this finding is supported by others (Heimler *et al.*, 2007, Heimler *et al.*, 2009). Furthermore, seasonal variation in the concentration of individual flavonoids has been reported (Arabbi *et al.*, 2004).

Significant difference in flavonoid content between salad rocket and wild rocket leaves has been reported (Heimler *et al.*, 2007, Martinez-Sanchez *et al.*, 2007). In the case of wild rocket leaves, both kaempferol and quercetin conjugates were found (Martinez-Sanchez *et al.*, 2006, Heimler *et al.*, 2007, Martinez-Sanchez *et al.*, 2008b). Even though they were both present, the concentration of quercetin conjugates was relatively high, while that of kaempferol was very low. In contrast, salad rocket leaves have been reported to contain a relatively high concentration of kaempferol, while quercetin content was much lower (Martinez-Sanchez *et al.*, 2007, Martinez-Sanchez *et al.*, 2008b). In addition, Martinez-Sanchez *et al*. (2007) identified the presence of isorhamnetin-glucosides in both rocket and wild rocket leaves and this finding has been confirmed (Martinez-Sanchez *et al.*, 2008b, Jin *et al.*, 2009).

Both kaempferol and quercetin conjugates have been reported to be present in mizuna and watercress leaves (Martinez-Sanchez *et al.*, 2008b). In addition, mizuna leaves also contained isorhamnetin 3, 7-di-*O*-glucoside. All compounds mentioned above (quercetin, kaempferol and isorhamnetin) have been reported to be present in pak choi (*Brassica rapa* L. ssp. *chinensis* L. (Hanelt.)) leaves (Rochfort *et al*., 2006). The composition of individual flavonoids varied among 11 cultivars investigated in their study.

Harbaum *et al*. (2007) also observed significant differences among different pak choi cultivars. In both studies kaempferol was the main flavonoid present in pak choi leaves followed by isorhamnetin, whereas the concentration of quercetin was very low in all cultivars.

Kaempferol and quercetin derivatives have also been confirmed to be the main flavonoids present in kale (Olsen *et al.*, 2009, Olsen *et al.*, 2010, Schmidt *et al.*, 2010a, Schmidt *et al.*, 2010b, Zietz *et al.*, 2010, Kobori *et al.*, 2011). In addition, Schmidt *et al*. (2010a, b) have also reported the presence of isorhamnetin in some kale cultivars, while in some other cultivars isorhamnetin was not found. Furthermore, similar to other red leafy vegetables, cyanidin derivatives were also found in the red variety of curly kale (Olsen *et al.*, 2010). Recently, the list of identified flavonoids that are present in kale leaves expanded up to 71 (Schmidt *et al.*, 2010b). Schmidt *et al*. (2010a) have also observed significant differences between different genotypes of kale in terms of both total flavonoids and individual flavonoids composition.

Spinach lacks flavonoids common among other leafy vegetables, *e.g.* kaempferol, quercetin and luteolin (Ferreres *et al*., 1997a). While these compounds are absent, some other flavonoids have been found to be present, and these are unique for spinach (Ferreres *et al.*, 1997a, Edenharder *et al.*, 2001). A number of flavonoids (Table.1.2) have been identified in spinach leaves (Ferreres *et al.*, 1997a, Gil *et al.*, 1999, Bergquist *et al.*, 2005, Cho *et al.*, 2008) and they include glucuronides and glycosides of oxygenated flavonols (Ferreres *et al.*, 1997a, Gil *et al.*, 1999).

Analysis of spinach extracts (Ferreres *et al.*, 1997a, Gil *et al.*, 1999) confirmed the presence of ten flavonoids. Among them Ferreres *et al*. (1997a) identified some previously unknown compounds. These were compounds number 3, 7, 11 and 16 from Table 1.2. These flavonoids were confirmed in a subsequent study by Gil *et al*. (1999). Both papers (Ferreres *et al.*, 1997a, Gil *et al.*, 1999) have also confirmed the presence of other flavonoids in spinach leaves; these were compounds number 1, 9, 10, 18, 19, and 21 (Table 1.2). The presence of some flavonoids previously reported (Ferreres *et al.*, 1997a,

Gil *et al.*, 1999) has also been confirmed by other authors (Kidmose *et al.*, 2001, Howard *et al.*, 2002, Bergquist *et al.*, 2005, Pandjaitan *et al.*, 2005). In addition to those previously described, Bergquist *et al*. (2005) identified another two compounds, patuletin-3-*O*-*β*-D-(2-*p*-coumaroylglucopyranosyl-(1→6)-[*β*-D-apiofuranosyl-(1→2)]-*β*-D-glucopyranoside and patuletin-3-*O*-*β*-D-(2''-feruloylglucopyranosyl)-(1→6)-*β*-D-glucopyranoside. Recently, in their study on flavonoid content in spinach, Cho *et al*. (2008) confirmed the presence of another 9 flavonoids, extending the list of flavonoids in spinach leaves up to 21 (Table 1.2).

Table 1.2 List of flavonoids identified in spinach extracts.

Peak	Compound	Reference
1	Patuletin-3-*O*-*β*-D-glucopyranosyl-(1 → 6)-[*β*-D-apiofuranosyl-(1 → 2)]-*β*-D-glucopyranoside	Ferreres *et al.*, 1997; Gil *et al.*, 1999; Kidmose *et al.*, 2001; Howard *et al.*, 2002; Bergquist *et al.*, 2005; Pandjaitan *et al.*, 2005; Cho *et al.*, 2008; Bottino *et al.*, 2009
2	Patuletin-3-*O*-*β*-D-glucopyranosyl-(1 → 6)-*β*-D-glucopyranoside	Cho *et al.*, 2008
3	Spinacetin-3-*O*-*β*-D-glucopyranosyl-(1 → 6)-[*β*-D-apiofuranosyl-(1 → 2)]-*β*-D-glucopyranoside	Ferreres *et al.*, 1997; Gil *et al.*, 1999; Kidmose *et al.*, 2001; Howard *et al.*, 2002; Bergquist *et al.*, 2005; Pandjaitan *et al.*, 2005; Cho *et al.*, 2008; Bottino *et al.*, 2009
4	Compound 6 isomer	Cho *et al.*, 2008
5	Compound 7 isomer	Cho *et al.*, 2008
6	Patuletin-3-*O*-*β*-D-(2 -*p*-coumaroylglucopyranosyl-(1 → 6)-[*β*-D-apiofuranosyl-(1 → 2)]-*β*-D-glucopyranoside	Bergquist *et al.*, 2005; Cho *et al.*, 2008
7	Patuletin-3-*O*-*β*-D-(2''-feruloylglucopyranosyl)-(1 → 6)-[*β*-D-apiofuranosyl-(1 → 2)]-*β*-D-glucopyranoside	Ferreres *et al.*, 1997; Gil *et al.*, 1999; Howard *et al.*, 2002; Bergquist *et al.*, 2005; Pandjaitan *et al.*, 2005; Cho *et al.*, 2008
8	Compound 1 isomer	Cho *et al.*, 2008
9	Spinacetin-3-*O*-*β*-D-glucopyranosyl-(1 → 6)-*β*-D-glucopyranoside	Ferreres *et al.*, 1997; Gil *et al.*, 1999; Howard *et al.*, 2002; Pandjaitan *et al.*, 2005; Cho *et al.*, 2008
10	Spinacetin-3-*O*-*β*-D-(2''-ρ-coumaroylglucopyranosyl-(1 → 6)-[*β*-D-apiofuranosyl-(1 → 2)]-*β*-D-glucopyranoside	Ferreres *et al.*, 1997; Gil *et al.*, 1999; Howard *et al.*, 2002; Pandjaitan *et al.*, 2005; Cho *et al.*, 2008
11	Spinacetin-3-*O*-*β*-D-(2''-feruloylglucopyranosyl)-(1 → 6)-[*β*-D-apiofuranosyl-(1 → 2)]-*β*-D-glucopyranoside	Ferreres *et al.*, 1997; Gil *et al.*, 1999; Kidmose *et al.*, 2001; Howard *et al.*, 2002; Bergquist *et al.*, 2005; Pandjaitan *et al.*, 2005; Cho *et al.*, 2008; Bottino *et al.*, 2009
12	Patuletin-3-*O*-*β*-D-(2''-ρ-coumaroylglucopyranosyl-(1 → 6)-*β*-D-glucopyranoside	Cho *et al.*, 2008; Bottino *et al.*, 2009
13	Patuletin-3-*O*-*β*-D-(2''-feruloylglucopyranosyl)-(1 → 6)-*β*-D-glucopyranoside	Bergquist *et al.*, 2005; Cho *et al.*, 2008
14	Patuletin derivative	Cho *et al.*, 2008
15	Patuletin derivative	Cho *et al.*, 2008
16	Spinacetin-3-*O*-*β*-D-(2''-feruloylglucopyranosyl)-(1 → 6)-*β*-D-glucopyranoside	Ferreres *et al.*, 1997; Gil *et al.*, 1999; Howard *et al.*, 2002; Bergquist *et al.*, 2005; Cho *et al.*, 2008; Bottino *et al.*, 2009
17	Spinatoside	Kidmose *et al.*, 2001; Bergquist *et al.*, 2005; Cho *et al.*, 2008
18	Jaceidin-4'-*β*-D-glucuronide	Ferreres *et al.*, 1997; Gil *et al.*, 1999; Kidmose *et al.*, 2001; Howard *et al.*, 2002; Pandjaitan *et al.*, 2005; Cho *et al.*, 2008; Bottino *et al.*, 2009
19	5,3',4'-Trihydroxy-3-methoxy-6:7-methylendioxyflavone-4' -*β*-D-glucuronide	Ferreres *et al.*, 1997; Gil *et al.*, 1999; Kidmose *et al.*, 2001; Howard *et al.*, 2002; Bergquist *et al.*, 2005; Cho *et al.*, 2008; Bottino *et al.*, 2009
20	5,4'-Dihydroxy-3-methoxy-6:7-methylendioxyflavone-4' -*β*-D-glucuronide	Cho *et al.*, 2008
21	5,4'-Dihydroxy-3,3'-dimethoxy-6:7-methylendioxyflavone-4'-*β*-D-glucuronide	Ferreres *et al.*, 1997; Gil *et al.*, 1999; Kidmose *et al.*, 2001; Howard *et al.*, 2002; Bergquist *et al.*, 2005; Pandjaitan *et al.*, 2005; Cho *et al.*, 2008; Bottino *et al.*, 2009

In a study of flavonoid content in commercial cultivars and advanced breeding lines of spinach, Howard *et al*. (2002) found a significant difference in total flavonoid content between them. A difference in flavonoid content between different genotypes of spinach has also been reported by Cho *et al*. (2008). Furthermore, differences in individual flavonoids reported in both studies (Howard *et al.*, 2002, Cho *et al.*, 2008) were even more significant; some compounds were present in high concentrations in some of the cultivars, while the concentrations of the same compounds were very low in others.

A decrease in flavonoid content in lettuce and endive after 7 days of dark storage at 1 °C has been observed (DuPont *et al.*, 2000). In contrast, Ferreres *et al*. (1997b) did not observe significant changes in flavonoid content of lollo rosso lettuce leaves during 14 days of storage at 5 °C. In the case of wild rocket leaves, the concentration of flavonoids increased or was stable during 14 days of storage at 4 °C in the leaves stored in controlled atmosphere, while it decreased during air storage (Martinez-Sanchez *et al.*, 2006). Flavonoid concentrations have also been reported to be relatively stable by Jin *et al*. (2009) who monitored the changes in quercetin, kaempferol, isorhamnetin and cyanidin content in salad rocket and wild rocket leaves during 14 days of storage at 4 °C. These authors suggested that changes in flavonoid content may be affected by pre-harvest conditions (*e.g.* light). This view is supported by Harbaum-Piayda *et al*. (2010) who observed significant changes in flavonoid composition in pak choi leaves cultivated at two different temperatures (9 and 22 °C) with or without UV light exposure. Flavonoid content in pak choi leaves cultivar Hangzhou You Dong Er was relatively stable during 20 days of storage at 2 °C (Harbaum-Piayda *et al*., 2010), which is in agreement with the previous study, where these authors (Harbaum *et al*., 2008) did not observe significant changes in flavonoid content during the storage of the same pak choi cultivar at either 4 or 20 °C. In the same study (Harbaum *et al.*, 2008) one of the cultivars used (Xue Li Hong) showed a significant increase in flavonoid content during 6 days of storage followed by a decline on day 8. In the case of two cultivars (Hangzhou You Dong Er and Shanghai Qing) flavonoid content was higher after 6 days of storage at 4 °C when compared with leaves stored at 20 °C. On the other hand, no difference between two storage temperatures was observed

for two other cultivars used in that study. The flavonoid content was also reported to be stable during the storage of curly kale for 6 weeks at 1 °C (Hagen *et al.*, 2009) and kale for 15 days at 1 and 10 °C (Kobori *et al.*, 2011).

The concentration of total flavonoids in spinach seems to be stable during storage (Gil *et al.*, 1999, Bergquist *et al.*, 2005), but Bottino *et al*. (2009) found that this was only the case for intact spinach leaves, not in fresh-cut spinach. Moreover the concentration of individual compounds such as spinacetin-3-(2”-feroylglucosyl)(1-6)[apiosyl(1-2)]-glucoside, jaceidin glucuronide, 5,3’,4’-trihydroxy-3-methoxy-6:7-methylenedioxiflavone-4’-glucuronide, patuletin-3-glucosyl-(1-6)[apiosyl(1-2)]-glucoside, patuletin-3-(2”-feroylglucosyl)-(1-6)[apiosyl(1-2)]-glucoside, patuletin-3-gentiobioside, and spinatoside-4’-glucuronide, and thus the composition of flavonoids, has been reported to change significantly during storage (Bergquist *et al.*, 2005, Bottino *et al.*, 2009). The increase in total flavonoids resulted from increased concentration of the following compounds: spinacetin-glucuronide, jaceidin-glucuronide, patuletin-3-(2”-feroylglucosyl)-(1-6)[apiosyl(1-2)]-glucoside, 5,4’-dihydroxy-3,3’-dimethoxy-6:7-methylenedioxyflavone-4’-glucuronide. Simultaneously the concentration of some other compounds (patuletin-3-glucosyl-(1-6)[apiosyl(1-2)]-glucoside, patuletin-3-gentiobioside, and spinacetin-glucuronide) decreased during storage (Bergquist *et al.*, 2005, Bottino *et al.*, 2009). In addition, 5,3’4’-trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4’glucuronide has been found to be the main flavonoid in baby leaf spinach (Bergquist *et al.*, 2005) and its concentration was found to be rather stable during storage (Bergquist *et al.*, 2005, Bottino *et al.*, 2009).

The effect of different storage temperatures on flavonoid content has also been investigated (Chu *et al.*, 2000, Bergquist *et al.*, 2005). No significant difference in flavonoid content was observed between kale samples stored at 1 and 10 °C (Kobori *et al.*, 2011). On the other hand, significant decrease in flavonoid content in sweet potato leaves during storage has been reported (Chu *et al.*, 2000). They found that flavonoid losses were much higher at 25 °C when compared with leaves stored at 4 °C. Flavonoids seem to be quite

stable during the storage of spinach, as no significant difference has been observed when leaves were stored at 2 and 10 °C (Bergquist *et al.*, 2005).

Knowledge of changes in the concentrations of individual antioxidants, *e.g.* carotenoids and flavonoids, during the storage of leafy vegetables is important because changes in these compounds affect antioxidant capacity of fresh produce. The changes in the concentration of carotenoids reported by different groups are inconsistent. A number of studies (Kopas-Lane and Warthesen, 1995, Pandrangi and LaBorde, 2004, Bunea *et al.*, 2008) reported a decrease in lutein and *β*-carotene content during the storage of spinach leaves, whereas Bergquist *et al*. (2006) observed an increase in the concentration of these carotenoids. Nonetheless, both Bergquist *et al*. (2006) and Pandrangi and LaBorde (2004) noticed that changes in the concentrations of individual compounds are temperature dependent.

Total flavonoid content seems to be relatively stable during refrigerated storage of salad rocket (Jin *et al.*, 2009), wild rocket (Jin *et al.*, 2009), pak choi (Harbaum *et al.*, 2008), curly kale (Hagen *et al.*, 2009) and spinach (Gil *et al.*, 1999, Bergquist *et al.*, 2005, Bottino *et al.*, 2009). Although total flavonoid content may remain stable, the composition of individual compounds may change (Bergquist *et al.*, 2005, Bottino *et al.*, 2009). This is mainly due to the fact that the concentrations of some compounds may increase while the concentrations of others may decrease at the same time, and vice versa. Furthermore, the changes in these compounds vary among different plant commodities and cultivars.

1.3.5 Changes in microbial counts

There is a substantial number of papers dealing with the aspect of quality loss of leafy vegetables during storage due to microbial action (Babic *et al.*, 1996, Babic and Watada, 1996, Garcia-Gimeno and Zurera-Cosano, 1997, Allende *et al.*, 2004a, Allende *et al.*, 2004b, Ragaert *et al.*, 2007, Conte *et al.*, 2008, Medina *et al.*, 2012). Raw plant material is never free from microorganisms and even the washing treatments are not sufficient to remove all of them from the leaf surface.

The end of shelf-life for fresh produce is not only defined by the loss of sensory and nutritional quality; it may also be compromised by high microbial counts (Jacxsens *et al*., 2002), since consumption of fresh produce contaminated with microorganisms may pose health risk to humans. Quality loss during storage due to the presence of spoilage microorganisms should not be underestimated. The knowledge of the microbial populations that may be present on various leafy vegetables is of key importance for the fresh produce industry. Furthermore, there is considerable interest in determining how these microbial counts change during storage under different conditions.

1.3.5.1 Identified microflora

Native microbial populations have been identified for lettuce (King *et al.*, 1991); spinach (Babic *et al.*, 1996) and Swiss chard leaves (Ponce *et al*., 2002). In the case of lettuce the microflora was dominated by bacteria belonging to the genera *Pseudomonas* sp., *Erwinia* sp. and *Serratia* sp. (King *et al.*, 1991). Bacteria that were also present, although in much lower numbers, belong to *Flavobacterium* sp., *Xanthomonas* sp., *Janthinobacterium* sp. and *Alcaligenes* sp. King *et al*. (1991) have also reported a populations of yeasts that belong to the genera *Cryptococcus* sp., *Pichia* sp., *Torulaspora* sp. and *Trichosporon* sp. In addition, small number of moulds belonging to *Penicillium* sp., *Rhizopus* sp., *Cladosporium* sp., *Phoma* sp. and *Aspergillus* sp. were also found on lettuce leaves (King *et al.*, 1991).

In spinach, the microflora identified consisted mainly of the families *Pseudomonadaceae* (*Pseudomonas fluorescens*), *Enterobacteriaceae* (*Citrobacter*

freundii, *Serratia* sp.), *Micrococcaceae* (*Staphylococcus xylosus*), lactic acid bacteria and yeasts (*Cryptococcus* sp.)(Babic *et al.*, 1996). A high percentage of *Pseudomonas* isolates, which were the dominant population, had pectolytic, proteolytic and lipolytic activity. Thus, Babic *et al*. (1996) suggested that they could possibly be involved in the process of leaf tissue deterioration.

In comparison with lettuce (King *et al.*, 1991) and spinach (Babic *et al.*, 1996), different groups of microorganisms were identified on Swiss chard leaves (Ponce *et al.*, 2002). The bacteria that were found belong to the genera *Micrococcus* sp. (*M. sedentarius* and *M. roseus*), *Bacillus* sp. (*B. pumilus* and *B. subtilis*) and *Lactobacillus* sp. (*L. paracesei*). In addition, several genera of moulds including *Alternaria* sp., *Botrytis* sp., *Aspergillus* sp., *Penicillium* sp., *Microsporium* sp. and *Fusarium* sp. were also found to be present on Swiss chard leaves (Ponce *et al.*, 2002). This suggests that different microorganisms may be present on the leafy vegetables already on the harvesting day, due to environmental conditions during growth and differences among leaf characteristics (*e.g.* leaf surface and shape) among crops.

In addition to spoilage microorganisms that are always present on leafy vegetables, there is a great concern about human pathogens, *e.g. Escherichia coli* O157: H7, *Listeria monocytogenes* or *Salmonella* sp., which may also be found on/in fresh produce (Luo *et al.*, 2009, Caponigro *et al.*, 2010, Khalil and Frank, 2010, Oliveira *et al.*, 2010). Although contamination of leafy vegetables with human pathogens will not be discussed in detail within this review, care must be taken within the supply chain due to the fact that temperature of storage affects their growth.

Several authors (Li *et al.*, 2001a, Luo *et al.*, 2009, Khalil and Frank, 2010, Oliveira *et al.*, 2010) have investigated the behaviour of *E. coli* O157:H7 on leafy vegetables during storage at different temperatures. Khalil and Frank (2010) reported that *E. coli* O157:H7 did not grow on lettuce stored at either 8 or 12 °C; though pathogen growth was observed at 15 °C. This finding is in agreement with other authors (Li *et al.*, 2001a, Oliveira *et al.*, 2010), who observed a decline in *E. coli* O157:H7 population on lettuce

stored at 5 °C. Furthermore, these authors also observed *E. coli* O157:H7 growth on lettuce stored at 15 °C (Li *et al.*, 2001a) and 25 °C (Oliveira *et al.*, 2010), respectively. In the case of spinach leaves, *E. coli* O157:H7 growth was observed at 8 and 12 °C (Luo *et al.*, 2009, Khalil and Frank, 2010); being significantly faster at 12 °C. If the spinach leaves were stored at either 1 or 5 °C, the population of pathogen decreased (Luo *et al.*, 2009).

Similar to *E. coli* O157:H7, the population of *Salmonella* sp. decreased on lettuce stored at 5 °C, while an increase was observed at 25 °C (Oliveira *et al.*, 2010). On the other hand, *L. monocytogenes* was able to grow on lettuce at both 5 and 25 °C (Oliveira *et al.*, 2010); the growth was faster with increasing temperature. This is in agreement with Jacxsens *et al*. (2002) who reported that *L. monocytogenes* managed to survive, as expected, on lettuce stored at 2 °C, while the growth of this pathogen was only observed at 4, 7 and 10 °C. Again, growth was significantly faster at 7 and 10 °C, when compared with samples stored at 4 °C. These findings give evidence that pathogens do not behave in the same way on lettuce and spinach and that low storage temperature is important.

1.3.5.1.1 Mesophilic and psychrotrophic microorganisms

Mesophilic (bacteria with optimum growing conditions at moderate temperature, neither too hot nor too cold, typically between 10 and 40 °C) and psychrotrophic (bacteria with optimum growing conditions at low temperature, *e.g*. below 10 °C) bacteria are commonly found on lettuce (King *et al.*, 1991, Delaquis *et al.*, 1999, Li *et al.*, 2001b), spinach (Babic *et al.*, 1996, Allende *et al.*, 2004b, Luo *et al.*, 2009), cabbage (Gomez-Lopez *et al.*, 2005), endive (Allende *et al.*, 2008a, Allende *et al.*, 2008b), wild rocket (Martinez-Sanchez *et al.*, 2006) and Swiss chard leaves (Ponce *et al.*, 2002) during refrigerated storage. The populations of mesophilic and psychrotrophic bacteria increase during storage due to the fact that even low storage temperature does not inhibit their growth (Babic *et al.*, 1996, Jacxsens *et al.*, 2002, Allende *et al.*, 2004b). A number of studies (Babic and Watada, 1996, Jacxsens *et al.*, 2002, Pandrangi and LaBorde, 2004, Luo *et al.*, 2009) have demonstrated that microbial counts were lower when leafy vegetables were kept at lower temperatures. This can be explained by changes in the lag-

phase length of spoilage microorganisms, which increases with decreasing storage temperature (Jacxsens *et al.*, 2002). On the other hand, no difference in bacterial growth on lettuce leaves was observed when samples were stored at 2, 5 or 7.5 °C (King *et al.*, 1991). This discrepancy might be due to the fact that the range of temperatures used by King *et al*. (1991) was relatively low when compared with those used by Jacxsens *et al*. (2002).

The growth of mesophilic and psychrotrophic microorganisms may also be affected by the gas composition inside the bags (Babic and Watada, 1996). These authors have demonstrated that low oxygen content inside the bag reduces their numbers when compared with perforated bags; but, this was only observed when samples were stored at 5 °C, while at 10 °C this effect was lost.

It has been suggested that the growth of psychrotrophic bacteria could be enhanced by tissue damage (King *et al.*, 1991, Babic *et al.*, 1996, Allende and Artes, 2003) due to solute leakage that provides nutrients for bacterial growth. Babic *et al*. (1996) used low temperature scanning electron microscopy to identify areas covered with microorganisms. Leaf areas with severe damage were covered with bacteria, while only low numbers of bacteria were found on healthy unbroken leaves.

McKellar *et al*. (2004) found that loss of quality during the storage of lettuce at 4 °C correlated with increasing numbers of psychrotrophic bacteria and *Pseudomonas* sp. On the other hand, others (Jacxsens *et al.*, 2002, Allende *et al.*, 2008a) have not found an association between microbial counts and quality loss because the shelf-life of fresh produce is often compromised by the loss of its sensory quality before microbial counts reach high numbers. This suggests that high microbial counts are not a reason for leaf tissue deterioration but rather the result of it, as tissue damage needs to occur first to support their growth.

1.3.5.1.2 Lactic acid bacteria

The population of lactic acid bacteria on leafy vegetables on the day of harvest is often very small compared with mesophilic and psychrophilic bacteria (Li *et al.*, 2001b, Ponce *et al.*, 2002, Luo, 2007, Conte *et al.*, 2008) and sometimes it may be so small that this group of microorganisms may not be detected (Babic and Watada, 1996, Delaquis *et al.*, 1999). Lactic acid bacteria counts usually remain low during refrigerated storage of spinach (Babic *et al.*, 1996, Allende *et al.*, 2004b, Allende *et al.*, 2006, Conte *et al.*, 2008), while an increase has been observed during the storage of lettuce (Luo, 2007). This could be due to differences in packaging films (in terms of oxygen transmission rate - OTR) used for storage of spinach and lettuce leaves. It has been observed (McKellar *et al.*, 2004) that gas composition changes (reduced O_2, increased CO_2 content) in packages with low OTR support the growth of lactic acid bacteria. This may explain why the population of lactic acid bacteria in lettuce did not change during 6 days of storage at 0-2 °C and then started to increase (Aguero *et al.*, 2011). Overall, lactic acid bacteria do not seem to play an important role during microbial spoilage of spinach (Babic *et al.*, 1996) or lettuce (Jacxsens *et al.*, 2002), due to low numbers during the storage of these commodities.

1.3.5.1.3 Yeasts and moulds

Although yeasts and moulds probably do not play an important role during microbial spoilage of leafy vegetables (Babic *et al.*, 1996, Babic and Watada, 1996, Jacxsens *et al.*, 2002) their growth is promoted when the gas composition inside the bag becomes anaerobic (King *et al.*, 1991). Changes in the atmosphere inside the bag often take place when OTR of the film used for storage is not sufficient or when samples are stored at inappropriate temperature.

The yeast and mould counts on lettuce (King *et al.*, 1991, Li *et al.*, 2001b, Allende *et al.*, 2004a, Allende *et al.*, 2006) and spinach (Babic and Watada, 1996, Conte *et al.*, 2008, Luo *et al.*, 2009, Escalona *et al.*, 2010) are usually low; but the slow increase may still take place during refrigerated storage. The population of yeasts and moulds on lettuce

increased during 6 days of storage at 0-2 °C (Aguero *et al.*, 2011) and 5 °C (Allende and Artes, 2003, Allende *et al.*, 2006) and then remained stable (Allende *et al.*, 2006, Aguero *et al.*, 2011) and eventually declined in the end of storage (Aguero *et al.*, 2011). In the case of spinach leaves the population of yeasts and moulds increased during 3 days of storage at 1, 5, 8 and 12 °C, and then remained stable (Luo *et al.*, 2009), whereas other authors observed slow growth during the storage at 5 °C for 6 days (Artes-Hernandez *et al.*, 2009), 10 days (Conte *et al.*, 2008), 12 days (Allende *et al.*, 2004b) and 14 days (Escalona *et al.*, 2010), respectively.

No significant difference was found for growth of yeasts on lettuce stored at 2, 5 and 7.5 °C (King *et al.*, 1991) and only slight difference in their counts was observed between spinach leaves stored at 5 and 8 °C (Artes-Hernandez *et al.*, 2009). On the other hand, significantly faster growth of these microorganisms was reported when Li *et al*. (2001b) compared yeasts and moulds populations on lettuce stored at 5 and 15 °C. This suggests that small difference in storage temperature does not lead to a significant increase of yeast and mould counts.

Low temperature is essential during the storage of leafy vegetables. It is clear that the growth of different groups of microorganisms is enhanced with increasing temperature of storage. Low temperature not only decreases the growth of spoilage microorganisms, but it also reduces the survival of human pathogens, *e.g. E.coli* O157:H7 and *Salmonella* sp. that may be present on the raw plant material. Thus, to preserve the quality of leafy vegetables and meet food safety requirements (FSA, 2011), the temperature along the supply chain should be kept low, preferably below 5 °C.

1.4 High temperature treatment

Temperature may also affect the quality of leafy vegetables during storage if applied as a treatment prior to storage. Temperature treatments can be applied either in the form of air or water (Lurie, 1998). Water has been suggested to be a better medium in terms of heat transfer efficiency (Fallik, 2004) and practicality. The effects of hot water treatments on quality changes in leafy vegetables during subsequent storage are reviewed below.

1.4.1 Effect of hot water treatment on quality maintenance

Heat treatment (50 °C) of lettuce with either water for 90 s (Murata *et al.*, 2004) or calcium lactate (15g/l, w/v in water) for 60 s (Martin-Diana *et al.*, 2006) improved organoleptic quality and texture maintenance during storage, thus extending shelf-life. Calcium lactate is a firming agent used as a food additive in order to precipitate residual pectin, thus strengthening the structure of the food (Luna-Guzman and Barrett, 2000; Martin-Diana *et al*., 2006). Texture of lettuce was also maintained when hot (47 °C) chlorinated (100 ppm) water was applied for 3 min prior to storage (Delaquis *et al.*, 1999, Delaquis *et al.*, 2004). Thermal treatment reduced the loss of turgor during storage, possibly due to higher activity of pectin methylesterase (PME; EC 3.1.1.11), an enzyme related to textural changes in the tissue. This resulted in improved crispiness of lettuce (Martin-Diana *et al.*, 2006). This effect is not consistently observed; no difference in texture was found between lettuce treated with hot (50 °C for 60 s) and cold (4 °C for 60 s) water prior to storage (Baur *et al.*, 2005, Martin-Diana *et al.*, 2005) and a decrease in textural quality of lettuce was observed when leaves were treated with hot water (50 °C) for 2 min prior to storage (Moreira *et al*., 2006). This decrease in texture may be a consequence of too long treatment time at this high (50 °C) temperature, justifying why others (Baur *et al.*, 2005, Martin-Diana *et al.*, 2005) treated the lettuce for only 60 s instead of 2 min used by Moreira *et al*. (2006). This also supports the findings of others (Delaquis *et al.*, 1999, Delaquis *et al.*, 2004) who observed that with the temperature of treatment and treatment time being too high and too long, leaf tissue might be damaged as measured by increased solute leakage. Interestingly, lower solute leakage during

storage was observed in spinach leaves treated with hot (40 °C) water for 3.5 min prior to storage, when compared with untreated leaves (Gomez *et al.*, 2008). Overall, to reduce the chance of damage resulting from thermal treatment, the time of treatment should be reduced with increasing temperature (Fallik, 2004).

Several authors (Gomez *et al.*, 2008, Martinez-Sanchez *et al.*, 2008a) have made an attempt to determine the effect of hot water treatment on the respiration rate of fresh produce. Hot water treatment (50 °C for 60 s) of rocket, mizuna and watercress baby leaves increased the respiration rate if samples were subsequently stored at 8 °C (Martinez-Sanchez *et al.*, 2008a) but no change in the respiration rate in response to hot water treatment was observed, however, if the leaves were stored at 4 °C. In contrast, hot water (40 °C for 3.5 min) treatment had no effect on respiration rate of spinach leaves subsequently stored at 23 °C (Gomez *et al.*, 2008).

Others (Odumeru *et al.*, 2003, McKellar *et al.*, 2004, Baur *et al.*, 2005, Martinez-Sanchez *et al.*, 2008a) have also compared respiration rates in response to hot and cold water treatment. The treatments used in those studies differed. Baur *et al*. (2005) and Martinez-Sanchez *et al*. (2008a) used hot water treatment at 50 °C for 60 s, while McKellar *et al*. (2004) and Odumeru *et al*. (2003) used chlorinated water (100 ppm) in their studies.

No significant difference in the gas composition in bags containing lettuce leaves treated with either cold (4 °C for 90 s) or hot (50 °C for 60 s) water prior to storage was observed during 5 days of storage at 4 °C (Baur *et al.*, 2005). From day 6, the oxygen content was lower and carbon dioxide content was higher in the case of lettuce treated with hot water prior to storage and it was concluded that respiration rate increases with increasing temperature of treatment.

Other studies (Odumeru *et al.*, 2003, McKellar *et al.*, 2004) investigated the effect of cold and hot water treatment on respiration rate of lettuce stored at 4 °C. In contrast to the studies mentioned above, these authors used chlorinated water (100 ppm) applied at 47±2 °C for 30, 60 or 180 s (Odumeru *et al.*, 2003) and at 48 °C for 30 s (McKellar *et al.*,

2004), respectively. McKellar *et al*. (2004) observed significantly higher CO_2 production during the storage of fresh-cut lettuce washed with hot (48 °C for 30 s) chlorinated water when compared with samples washed with cold (4 °C) chlorinated water prior to storage. In contrast, lettuce washed with hot (47±2 °C for 30, 60 or 180 s) chlorinated water had a lower oxygen consumption and carbon dioxide production rate when compared with lettuce washed with cold (4 °C for 30 s) chlorinated water (Odumeru *et al.*, 2003).

No difference in the development of off-odours (Delaquis *et al.*, 2004, Murata *et al.*, 2004, Baur *et al.*, 2005) and off-flavours (Baur *et al.*, 2005) has been reported for lettuce treated with hot water at 50 °C for 60 s prior to storage. Care must be taken because sensory analyses of food often rely on public perception, thus small differences in food quality may not be detected.

1.4.2 Effect of hot water treatment on visual quality

The decrease in visual quality of leafy vegetables may occur due to chlorophyll loss or tissue browning (Toivonen and Brummell, 2008). Chlorophyll loss during storage was slower in spinach treated with hot water (40 °C) for 3.5 min (Gomez *et al.*, 2008) and in rocket leaves treated with hot water (50 °C) for 30 s prior to storage (Koukounaras *et al.*, 2009), but no effect on colour changes in response to hot water (50 °C) treatment for 60 s has been reported in lettuce (Baur *et al.*, 2005). The yellowing process of rocket leaves was reduced by hot water (50 °C) treatment for 30 s prior to storage (Koukounaras *et al.*, 2009).

Other studies have shown that hot water (50 °C) treatment for either 60 s (Baur *et al.*, 2005, Martin-Diana *et al.*, 2005), 90 s (Murata *et al.*, 2004) or 120 s (Moreira *et al.*, 2006) reduced the browning process in lettuce during storage. Browning has also been reported to be delayed when lettuce was treated with hot (47 °C) chlorinated (100 ppm) water for 3 min (Delaquis *et al.*, 1999, Delaquis *et al.*, 2004) or hot (50 °C) chlorinated (20 ppm) water for 90 s (Li *et al.*, 2001b) prior to storage. Delaquis *et al*. (2004) reported that the temperature of chlorinated water used for washing treatments can be increased from 47 °C up to 51 °C with a simultaneous reduction in exposure time from 180 down to 60 s.

Martin-Diana *et al*. (2005) suggested that the reduced browning process in lettuce in response to heat treatment is due to reduced activity of PPO and POD, while other authors believe that it is due to suppression of PAL activity (Delaquis *et al.*, 1999, Saltveit, 2000, Delaquis *et al.*, 2004). This latter view is supported by the study of Murata *et al*. (2004) who found that hot water treatment blocked the induction of PAL, affected the accumulation of phenolic compounds and reduced tissue browning. In another study, it has been reported that the content of small heat shock proteins increased in hot water treated spinach leaves (Gomez *et al.*, 2008), thus suggesting their role in quality maintenance during storage.

1.4.3 Effect of hot water treatment on nutritional quality

Temperature treatment prior to storage may affect the concentration of antioxidants, including AsA, carotenoids and flavonoids, when it is applied as a stressor, such as a rapid change in temperature of the environment, at least 10 °C from the optimal growth temperature (Li *et al*., 1999). It is important to identify the optimal dose, temperature and time of exposure (Schoffl *et al*., 1998). Sublethal dose may induce positive responses in plants, which later protect them from the damage due to oxidative stress. Unfortunately, information on the effects of heat treatment on antioxidant content in leafy vegetables is scarce. There are only a few papers reporting the effect of high temperature treatment on AsA content in leafy vegetables during storage (Murata *et al.*, 2004, Moreira *et al.*, 2006, Gomez *et al.*, 2008), while little information exists on the effects of heat treatment on carotenoids (Updike and Schwartz, 2003, Aman *et al.*, 2005) and flavonoids.

Hot water (40 °C) treatment for 3.5 min and hot water (50 °C) treatment for 90 s had no effect on AsA content in spinach (Gomez *et al.*, 2008) and lettuce (Murata *et al.*, 2004), respectively. In contrast, Moreira *et al*. (2006) observed a decrease in AsA content in lettuce in response to hot water treatments. AsA loss was enhanced with increasing temperature of treatment and with longer treatment time. Heat treatment resulted in a reduction in carotenoid concentration in spinach (Aman *et al.*, 2005). It has also been

reported to cause *trans* to *cis* isomerisation of *β*-carotene and lutein in spinach (Updike and Schwartz, 2003, Aman *et al.*, 2005), and kale (Updike and Schwartz, 2003).

1.4.4 Effect of hot water treatment on changes in microbial populations

Microorganisms are usually found on the leaf surface and/or within a few outer cell layers. Delaquis *et al*. (1999) reported that chlorinated (100 ppm) water applied for 3 min at 47 °C reduced the initial microbial population in lettuce by 3 log cfu g^{-1}. Only 1 log reduction was observed when the water temperature was 4 °C. Numbers of psychrotrophic microorganisms, yeasts and moulds were significantly reduced by both cold (4 °C) and hot (47 °C) water treatments during 10 days of storage, when compared with untreated samples. In a later study, Delaquis *et al*. (2004) found that 50 °C treatment for 1 min might be a good alternative to 47 °C treatment for 3 min. This finding is supported by Baur *et al*. (2005) who found that this treatment gave better reduction in microbial counts on lettuce (total bacteria, *Pseudomonads* and *Enterobacteriaceae*) when compared with a cold water wash (4 °C) for 1 min.

The decrease in mesophilic and psychrotrophic microorganisms, *Enterobacteriaceae* counts, yeast, and mould populations in iceberg lettuce in response to warm (20 °C) and hot (50 °C) water treatments applied for 90 s has also been reported (Li *et al.*, 2001b). Counts of aerobic bacteria, coliforms and *Enterobacteriaceae* were also reduced on lettuce washed with either hot water at 47 °C for 2 min (followed by rinsing in cold water at 4 °C for 1 min) or cold water at 5 °C for 3 min (Rajkowski and Fan, 2008). A number of studies (Delaquis *et al.*, 1999, Li *et al.*, 2001b, Odumeru *et al.*, 2003, Delaquis *et al.*, 2004) have reported that hot water treatment was more efficient than cold water in reducing microbial counts. In contrast, no difference in washing efficiency between cold and hot water was observed by Rajkowski and Fan (2008).

Other authors have reported no effect of heat shock treatments on microbial counts in lettuce (Murata *et al.*, 2004), rocket leaves, mizuna and watercress (Martinez-Sanchez *et al.*, 2008a). Furthermore, microbial counts after 12 days of storage at 4 °C were significantly higher on lettuce treated with hot water prior to storage when compared

with control (Murata *et al.*, 2004). Similarly, the reduction in microbial counts (mesophilic bacteria, psychrophilic bacteria, yeasts and moulds) in lettuce in response to hot water treatment (50 °C for 1 min) was lost after 4 days and in counts of *Enterobacteriaceae* after 10 days of storage at 5 °C (Li *et al.*, 2001b), respectively. This might be due to the tissue damage, as a result of treatment application, and may lead to solute leakage from the leaves, thus supporting the growth of microorganisms (Moreira *et al.*, 2006), and in this way limiting the effectiveness of a hot water treatment.

1.5 Conclusions

This review highlights the importance of keeping fresh leafy vegetables under refrigerated temperature to reduce a loss in their quality. All determinants of quality, the loss of texture, development of off-odours and off-flavours, visual quality (leaf colour, chlorophyll degradation and/or tissue browning), changes in AsA, carotenoids and flavonoids are temperature dependent.

Textural and physiological changes have been shown in several studies to be accelerated with increasing temperature of storage. Similarly, development of off-odours and off-flavours was noticed early during storage in the bags (containing leafy vegetables) that were kept at high temperatures, whereas in counterparts stored at low storage temperature (0-4 °C) these changes were often not significant.

Loss of visual quality in leafy vegetables is accelerated with increasing temperature of storage. These changes are often related to chlorophyll degradation and the process of enzymatic browning. Tissue browning takes place faster as a result of texture loss. The substrates and enzymes involved in it are localized in different cell compartments, thus tissue disruption needs to occur prior enzymatic tissue browning. As texture loss is accelerated with increasing temperature of storage, tissue browning is observed earlier in the leafy vegetables (*e.g.* lettuce, endive) stored at higher temperatures. In some species (*e.g.* spinach, rocket leaves) no tissue browning has been observed during storage. This might be due to differences in polyphenol biochemistry.

In terms of nutritional quality changes, these are also affected by temperature during storage. AsA loss is accelerated at higher temperatures. Although, total carotenoids and flavonoids concentrations may be quite stable during storage, the concentrations of individual compounds are usually different at various temperatures.

Furthermore, the growth of microorganisms that are present on/in leafy vegetables is also faster at higher temperature. Low temperature during storage is recommended to reduce the risk of pathogen development in bags containing fresh produce, an issue of importance in the light of recent outbreaks of *E. coli* bacteria.

It has been reported that light exposure during storage improves texture of the leaves, as indicated by lower solute leakage. On the other hand, visual quality of leafy vegetables is often reduced in light-stored samples. Leaves are lighter and more yellow when stored under high intensity light conditions.

The effect of light exposure during storage on nutritional quality of leafy vegetables is not clear. Some authors have reported a decline in carotenoid content in spinach leaves stored in the light, while others found carotenoids to be either relatively stable or increase during storage. Similarly, changes in AsA content varied between spinach cultivars. Differences between leaves in response to light conditions during storage were also reported between seasons and due to plant maturity.

A number of studies have reported positive effects of pre-storage hot water treatments on shelf-life of lettuce, spinach and rocket leaves. However, positive effects of hot water treatment were not always convincing as the differences observed early in the storage between hot water treated and control samples were usually lost after several days of storage. Furthermore, microorganisms' counts that were initially reduced as an effect of washing (hot water being more efficient than cold water) were similar or even higher in the end of storage, thus suggesting that microbial growth occurs faster on heat-treated leaves.

The recent knowledge on the effects of hot water treatments on nutritional quality of leafy vegetables is scarce and requires further investigation. It is worth noting that

although different aspects of quality loss during storage have been discussed separately for the purpose of this review, the interactions between them take place during the shelf-life of fresh produce.

Objectives for this study are:

- To determine the effect of storage temperature on quality maintenance during the storage of baby leaf spinach;
- To determine the effect of light exposure during storage on quality maintenance of baby leaf spinach;
- To determine the effect of a combination of temperature and light conditions on quality maintenance during the storage of baby leaf spinach;
- To determine the effect of hot water treatment prior to storage on quality changes of baby leaf spinach during refrigerated storage.

Hypotheses to be tested:

- Storage temperature manipulation does not affect the concentration of bioactive compounds (AsA and carotenoids) of baby leaf spinach;
- Storage temperature manipulation does not affect visual or textual quality of spinach leaves;
- Manipulating light conditions (light quantity) of postharvest environment does not affect nutritional quality (AsA and carotenoids) of harvested spinach leaves;
- Manipulating light conditions (light quantity) of postharvest environment does not affect visual or textural quality of baby leaf spinach;
- Temperature treatment applied immediately after harvest does not affect nutritional quality (AsA and carotenoids) of baby leaf spinach;
- Temperature treatment prior to storage does not affect visual or textural quality of baby leaf spinach;
- The stability of particular compounds (AsA and those different from AsA, *e.g.* carotenoids, chlorophylls) during storage is important in terms of maintaining both nutritional and visual quality of baby leaf spinach, thus extending its shelf-life.

Chapter 2 General materials and methods

The aim of this study was to determine the effect of temperature and light exposure during storage on quality characteristics of baby leaf spinach. Furthermore, the response of spinach leaves to hot water treatments prior to storage was investigated. The experiments reported within this thesis are listed below (each experiment was repeated twice with similar results):

- **In Experiment 1**, 81 bags with baby leaf spinach were kept at three different temperatures (1±1, 10±1, 20±2 °C) for 9 days under low intensity light (30-35 µmol m^{-2} s^{-1}) conditions.
- **In Experiment 2**, the range of temperatures used was smaller than in experiment 1; 12 bags with baby leaf spinach were kept at 1±1 and 6±1 °C for 7 days under low intensity light (30-35 µmol m^{-2} s^{-1}) conditions.
- **In Experiment 3**, 18 bags with baby leaf spinach were kept at 1±1 °C for 7 days under three different light conditions – in the dark, under low intensity light (30-35 µmol m^{-2} s^{-1}) or high intensity light (130-140 µmol m^{-2} s^{-1}) conditions.
- **In Experiment 4**, 24 bags with baby leaf spinach were kept at 1±1 °C for 10 days under continuous (24 h) low intensity light (30-35 µmol m^{-2} s^{-1}) or photoperiod of 6 h high intensity light (130-140 µmol m^{-2} s^{-1})/18 h dark conditions.
- **In Experiment 5**, 24 bags with baby leaf spinach were kept at 1±1 °C or 10±1 °C for 7 days under two different light conditions - low intensity light (30-35 µmol m^{-2} s^{-1}) or high intensity light (130-140 µmol m^{-2} s^{-1}).
- **In Experiment 6**, baby leaf spinach was subjected to hot water (40, 45 or 50 °C) treatment for 0, 30, 60 or 120 s. Leaves were subsequently washed with cold water (4 °C) for 120 s and then carefully blotted with absorbent paper, before storage at 4 °C in the dark for 10 days.
- **In Experiment 7**, baby leaf spinach was subjected to hot water treatment at 45 °C for 0 or 60 s and were then subsequently washed in cold (4 °C) distilled water containing 100 µL L^{-1} active chlorine (Cl) (Koukounaras *et al*., 2009) for 120 s.

Leaves were carefully blotted with absorbent paper, before storage at 4 °C in the dark for 10 days.

In all experiments spinach samples were stored in Sanyo Versatile Environmental Test Chambers (SANYO Electric Co., Ltd, Japan). Samples were blocked across the shelves, so that corresponding treatments were exposed to similar conditions.

2.1 Plant material and handling

For experiments 1-5 baby leaf spinach (*Spinacia oleracea* L.) (in 180-200 g bags; the weight of a single leaf would normally be between 0.5-1.0 g), that was commercially bagged in 35 µm single layer of biaxially oriented polypropylene film (ASP Packaging Ltd, UK), designed for commercial storage of baby leaf salads, was supplied by PDM Produce Ltd, Shropshire, TF10 9BN, UK. Packaging thickness was assumed to be the same between experiments; however, it was not measured. Thus, the data obtained in different experiments cannot be directly compared due to the fact that changes in film thickness may affect O_2, CO_2 and water vapour permeability and light transmission (Del Nobile *et al*., 2006) and in this way changing the gas composition inside packages, which may have further consequences, *e.g.* by affecting leaf texture (Kar and Choudhuri, 1986, Martinez-Sanchez *et al*., 2011). These leaves were washed in cold (4 °C) water and subsequently dried with ambient (18-20 °C) air prior to the bagging step; bags were flushed with ambient air and sealed at PDM to avoid the situation where gas composition/volume of air in the bags would differ between the samples. The bags with baby leaf spinach that were used came directly from the production line (*i.e.* this was the spinach that would otherwise be send to the Supermarkets, *e.g.* ASDA, Sainsbury's or Co-operative).

For experiments 6-7, baby leaf spinach (*Spinacia oleracea* L.) cultivar Toucan was commercially grown at PDM Produce Ltd. For each experiment, approximately 3 kg of leaves were collected at harvest and transported to the laboratory (~15 minutes) in insulated opaque containers and treated immediately on arrival.

In experiment 6, spinach leaves (20.0±1.0 g) were subjected to hot water (40, 45 or 50 °C) treatment for 0, 30, 60 or 120 s (using different shaking water baths (Grant

Instruments Ltd, UK) with distilled water and baskets made of stainless steel) prior to storage. Leaves were subsequently washed with cold water (4 °C) for 120 s and then carefully blotted with absorbent paper, before storage at 4 °C. For packaging, leaves were placed in polypropylene trays 15 cm × 10cm × 4 cm and lids were covered with a 35 µm film (ASP Packaging Ltd, UK) designed for commercial storage of baby leaf salads. In experiment 6, spinach leaves were stored under dark conditions and were analysed after storage at 4 °C for 10 days.

In experiment 7, spinach leaves (80.0±1.0 g) were subjected to hot water treatment at 45 °C for 0 or 60 s and were then subsequently washed in cold (4 °C) distilled water containing 100 µL L^{-1} active chlorine (Cl) (Koukounaras *et al*., 2009) for 120 s. Chlorinated water was prepared by mixing 4 ml of 10-15% sodium hypochlorite solution (Sigma-Aldrich, UK) with 5 l of deionised water, pH of the solution was measured at 8.9. Chlorine was chosen as it is most common chemical sanitising solution used in the fresh produce industry (Table 1.1.). Leaves were carefully blotted with absorbent paper, before storage at 4 °C. For packaging, leaves were placed in polypropylene trays 20 cm × 13cm × 5 cm (Plate 2.1) and lids were covered with a 35 µm film (ASP Packaging Ltd, UK) designed for commercial storage of baby leaf salads. In experiment 7, spinach leaves were stored under dark conditions and were analysed on the day of harvest and after storage at 4 °C for 5 and 10 days.

Plate 2.1 Spinach leaves placed in polypropylene tray (20 cm × 13cm × 5 cm) with lid covered with a 35 µm film (ASP Packaging Ltd, UK).

The photosynthetically active radiation (PAR) was measured with quantum sensor (Skye Instruments Ltd, UK). Storage temperature was recorded continuously with Tinytag^{TM} temperature loggers (Gemini Data Loggers Ltd, UK). Spinach leaves were prepared for further analyses (gas composition analyses, solute leakage measurement, total ascorbic acid, and total carotenoids and chlorophylls content determination, leaf colour evaluation) on the harvesting day and after being collected from storage.

2.2 Measurements

2.2.1 Gas composition analyses

Gas composition (O_2 and CO_2) in individual bags was monitored using a MAP test 3050 analyser (HITECH Instruments, UK). The volume of 0.20 ml of gas from each package was analysed when bags were removed from storage.

2.2.2 Solute leakage determination

Solute leakage was determined according to the method of Wagstaff *et al.* (2007). Approximately 5.0 g of spinach leaves, with an even distribution of size and stage of development, were transferred from each bag to 500 ml beakers, to which 200 ml of deionised water was added. This step was followed by 3 h incubation at ambient

temperature (20.0±2.0 °C). Conductivity was subsequently measured in micro Siemens (µS) using a Jenway model 4510 conductivity meter (Bibby Scientific Ltd, UK) and calculated per g FW. Samples were removed from the bathing solution and slowly frozen at −20.0±2.0 °C to ensure maximum disruption of membranes prior to re-measuring the conductivity using the same method as for fresh tissue. Solute leakage was then expressed as a percentage of maximum conductivity.

2.2.3 Total ascorbic acid extraction and determination

Ascorbic acid was extracted and analysed using a method described by Bergquist *et al*. (2006) with some modifications. Spinach leaves (50.0±2.0 g) were chopped with a sharp knife. Spinach tissue (5.00±0.01 g) was placed in a 50 ml tube to which 25 ml of cold (4 °C) 1.5% (15 g/l w/v in H_2O) *meta*-phosphoric acid (HPO_3) (Acros Organics, UK) was added. Samples were immediately homogenized with a Silverson SL2 mixer (Silverson Machines Ltd, UK), and then put on ice. Processed samples were transferred to a freezer (-70 °C) for storage. Prior to analysis, spinach extracts were thawed in lukewarm water, in the dark. The extracts were centrifuged at 3,480 x g (g – force of gravity) for 40 min at 4 °C. Supernatants were filtered with Sep Pak filters (Phenomenex, UK) and 1.5 ml was collected in Eppendorf tubes. Following filtration, extracts were microfuged at 9,300 x g for 5 min. Finally, 500 µl were transferred into HPLC vials for AsA determination. Another 500 µl were transferred to new 1.5 ml Eppendorf tubes and mixed thoroughly using a vortex, with an equal volume of 1% (11 mg/ml w/v in 1 M K_2HPO_4/H_2O (1/4, v/v)) DTT solution (DL-Dithiothreitol)(Fisher Scientific, UK). The DTT solution samples were left for 40 min at room temperature (20.0±2.0 °C), and then microfuged at 9,300 x g for 5 min. Samples were transferred into HPLC vials for total AsA (AsA + DHA) determination.

Samples were analysed using an Agilent 1100 HPLC (Agilent, UK) with a Luna 5 µm NH2 100 A column (250 mm × 4.6 mm) (Phenomenex, UK) at a flow rate of 1.2 ml min^{-1} and pressure in the range of 70-80 bars. The mobile phase consisted of 25% 15 mmol l^{-1} (1.725 g/l w/v in H_2O) of $NH_4H_2PO_4$ (mono ammonium phosphate) (Sigma-

Aldrich, UK) and 75% of acetonitrile (Fisher Scientific, UK); pH was adjusted to 3.9 with 1 M *ortho*-phosphoric acid (H_3PO_4) (Acros Organics, UK). Freshly prepared eluent was degassed for 30 min in an ultrasonic bath. The concentration of AsA and DHA was determined according to external AsA standards (Acros Organics, UK) of 10, 25, 50 and 100 ppm. The volume of 20 μl of each sample was analysed in this process.

2.2.4 Chlorophyll and total carotenoid determination

The concentration of chlorophylls and total carotenoids was determined using the method of Lichtenthaler and Wellburn (1983), with modifications of Edwards *et al*. (1998). A portion (50.0±1.0 g) of the leaf material was freeze-dried and ground into powder. The volume of 0.50±0.01 g was transferred to a 50 ml tube and 40 ml of 80% (acetone/H_2O (4/1, v/v)) acetone (Fisher Scientific, UK) containing 1 mg of magnesium carbonate (Fisons Scientific Apparatus Ltd, UK), to stabilize pigments, and 0.5 mg of sodium bisulfite (Sigma-Aldrich, UK) - drying agent, was added. Each tube was covered with a lid, and left for 24 hours in the dark for the extraction to proceed. Extracts were subsequently centrifuged at 15,000 x g for 15 min. Approximately 4.5 ml of supernatant was transferred to a quartz cuvette and the absorbance of extracts was measured spectrophotometrically at 480, 645, 663 and 710 nm. The concentrations of chlorophyll *a*, *b* and total carotenoids were determined using spectrophotometer readings in equations given by Lichtenthaler and Wellburn (1983).

Equations used:

Chlorophyll *a*

chl *a* (μg ml^{-1}) = [12.21 * (A663 – A710)]-[2.81 * (A645-A710)]

Chlorophyll *b*

chl *b* (μg ml^{-1}) = [20.13 * (A645-A710)]-[5.03 * (A663-A710)]

Total carotenoids

Total carotenoids (μg ml^{-1}) = ([1000 * (A480-A710)]-[3.27 * chl *a* (μg ml^{-1})]-[104 * chl *b* (μg ml^{-1})])/200

2.2.5 Fresh and dry weight determination

Aluminium cups were weighed on an electronic balance and filled with approximately 4.0 g of chopped spinach leaves for fresh weight (FW) determination. The cups were transferred to an oven (75±2 °C) for 48 h, and then placed in 105±2 °C for 60 min. The weight of the aluminium cups with fresh and dried spinach tissue was quantified and moisture content was determined.

2.2.6 Leaf colour measurements

Leaf colour was measured with a Minolta CR-300 chroma meter (Minolta, Japan) calibrated using the manufacturer's standard white plate (Plate 2.2). Leaf colour changes were quantified for 10 leaves (Plate 2.3 A, B) from each sample in the *L**, *a** and *b** colour space (Abbott, 1999). The chroma meter was frequently calibrated to give the accurate readings.

Plate 2.2 Minolta being calibrated using the manufacturer's standard white plate.

Plate 2.3 (A) Sample of spinach leaves showing uniformity of leaf colour. (B) Taking measurement of spinach leaf colour using Minolta.

2.2.7 Statistical analyses

Data are presented as mean values. Results were analysed using one-way ANOVA to identify significant differences between the treatments and two-way ANOVA to identify factors that had significant effect on quality changes during the storage of baby leaf spinach. Tukey's test was used to allow comparisons between individual treatments. All statistical analyses were performed using GenStat 14th Edition software (Payne *et al*., 2010).

Chapter 3 Effect of storage temperature on quality changes of baby leaf spinach

3.1 Introduction

The shelf-life of leafy vegetables, such as spinach is relatively short. Thus, it is not surprising that in recent years, there has been an increasing interest in finding the way to extend it. As reviewed in Chapter 1, one approach is to establish the optimum storage conditions that will reduce quality loss during storage. The quality of spinach is defined by its appearance (visual quality), texture, nutritional quality (level of antioxidants) and microbial contamination. It is not clear, however, which of these parameters are best describing the shelf-life.

The quality of spinach (visual, textural and/or nutritional quality) has been reported to decline during storage (Pandrangi and LaBorde, 2004; Bergquist *et al*., 2006; Luo *et al*., 2009). A significant decline in visual quality of the leaves was found with increasing storage temperature from 4 to 20 °C (Pandrangi and LaBorde, 2004), from 2 to 10 °C (Bergquist *et al*., 2006) and from 1 to 12 °C (Luo *et al*., 2009). Bergquist *et al*. (2006) have also reported a significant decline in AsA with increasing temperature of storage. Luo *et al*. (2009) observed higher respiration rate and increased solute leakage in samples stored at 12 °C when comparing with those stored at 1 °C. On the other hand, no significant differences in chlorophyll and carotenoid content were found between spinach leaves stored at 2 and 10 °C (Bergquist *et al*., 2006), while a significant decline in pigment content, when compared with samples stored at 4 °C, was reported in samples stored at 10 and 20 °C already after 4 days of storage (Pandrangi and LaBorde, 2004). Available information suggests that visual quality, texture (as indicated by solute leakage) and AsA content are good indicators of shelf-life, while the use of plant pigment content as a quality measure is not clear. Thus, it is necessary to investigate and identify the best indicator of shelf-life.

Experiment 1 was conducted to better understand physiological/biochemical changes of spinach leaves during storage and the effect of temperature. Bagged spinach was stored at three different temperatures of 1, 10 and 20 °C. In Experiment 2, the range of temperatures was narrowed and spinach leaves were stored at 1 and 6 °C,

respectively. This range of temperatures was chosen to determine the best indicators of shelf-life.

The following null hypothesis was tested: storage temperature does not affect the maintenance of nutritional, textural and/or visual quality characteristics of spinach leaves. The aim of this study was: (i) to investigate the effect of storage temperature on quality changes of spinach leaves, (ii) to identify which quality measures can potentially be used as good indicators of shelf-life.

3.2 Materials and Methods

3.2.1 Plant material and handling

Spinach used in Experiment 1 was harvested on 25th of July and 9th of August 2011. Spinach was bagged at PDM Produce Ltd and transported to the laboratory (~15 minutes) in insulated opaque containers as described in section 2.1.

In Experiment 1, bags with baby leaf spinach were kept at three different temperatures (1±1, 10±1 and 20±2 °C) under continuous (24 hours) low intensity light (30-35 μmol m^{-2} s^{-1}) conditions for 9 days. These temperatures have been used by other research groups (Pandrangi and LaBorde, 2004; Bergquist *et al*., 2006; Luo *et al*., 2009). The actual observed average temperatures were 1.3, 10.3 and 20.8 °C as recorded with Tinytag™ temperature loggers (Gemini Data Loggers Ltd, UK). All measurements (gas composition, solute leakage, total ascorbic acid, and total carotenoids and chlorophylls content, leaf colour) were taken on the harvesting day and then when samples were collected from storage.

Based on the results from Experiment 1, a decision was made to narrow the temperature range to be used in Experiment 2. Spinach used in Experiment 2 was harvested on 2nd and 12th of September 2011. Spinach was bagged at PDM Produce Ltd and transported to the laboratory (~15 minutes) in insulated opaque containers as described in section 2.1. Bags with baby leaf spinach were kept at two different temperatures (1±1 and 6±1 °C) under continuous (24 hours) low intensity light (30-35 μmol m^{-2} s^{-1}) conditions for 7 days. These temperatures were used to see whether significant differences in the quality of spinach leaves that were observed between samples stored at 1 and 10 °C would still be there. Temperatures of 1 and 5 °C have previously been used by Luo *et al*. (2009). A storage temperature of 5 °C was also used by Allende *et al*. (2004b), while others (Medina *et al*., 2012; Tudela *et al*., 2013) kept spinach at 7 °C. The actual observed average temperatures were 1.2 and 5.8 °C as recorded with Tinytag™ temperature loggers (Gemini Data Loggers Ltd, UK). All measurements (solute leakage assay, total ascorbic acid, total carotenoids, chlorophylls

content determination, leaf colour evaluation) were taken on the harvesting day and then when samples were collected from storage after 3 and 7 days.

In Experiment 1, nutritional quality of spinach declined significantly after only 1 day of storage at 10 °C. Consequently decision was made to analyse the samples early during storage at 1 and 6 °C. Thus, spinach leaves were sampled after 3 days of storage; a sampling time previously used by others (*e.g*. Gil *et al*., 1999; Allende *et al*., 2004b; Pandrangi and LaBorde, 2004; Luo *et al*., 2009; Lester *et al*., 2010b). Although significant differences in visual, textural and nutritional quality of spinach leaves between samples stored at 1 and 10 °C were already observed after 5 days of storage, decision was made to analyse samples after 7 days of storage. This sampling time was previously used by others (*e.g*. Gil *et al*., 1999; Tudela *et al*., 2013) and is justified by the smaller range of temperatures (1 and 6 °C) used in Experiment 2.

3.2.2 Measurements

All measurements were taken following the methods described in Chapter 2.

3.2.3 Statistical analyses

Each experiment was repeated twice with very similar results (verified by the Bartlett's homogeneity test and CV (%) values). Data are presented as mean values from two experiments that were used as blocks. Results were analysed using one-way ANOVA to identify the factors/treatments that had a significant effect on quality changes during the storage of baby leaf spinach. Tukey's test was used to allow the comparisons between individual treatments. All statistical analyses were performed using GenStat 14th Edition software (Payne *et al*., 2010).

3.3 Results

3.3.1 Leaf dry matter

In Experiment 1, leaf dry matter at harvest was 5.4%. It did not change significantly throughout the storage period in spinach samples stored at 1 (5.4-5.6%) and at 10 °C (5.2-5.4%). A significant (P<0.001) increase in dry matter (6.1%), however, was observed after 3 days of storage at 20 °C, which was associated with enhanced water loss from these samples.

In Experiment 2, leaf dry matter at harvest was 5.7%. It did not change significantly throughout the storage period in spinach samples stored at 1 (5.8-6.0%) and at 6 °C (5.6-6.0%).

3.3.2 Gas composition

Experiment 1

Temperature of storage had a significant (P<0.001) effect on gas composition inside the bags with spinach leaves. In the case of spinach leaves stored at 1 °C, the oxygen level remained high and carbon dioxide was not detected during the 7 day storage period (Table 3.1), suggesting low respiration. A small decrease in oxygen level with simultaneous increase in carbon dioxide throughout the storage period was found in the bags stored at 10 °C, whereas a substantial drop in oxygen content associated with an increase in carbon dioxide took place in bags with spinach that were stored at 20 °C already after 1 day of storage (Table 3.1). After 4 days of storage at 20 °C, gas composition inside these bags was close to anaerobic conditions. It is clear from the Table 3.1 that not in all bags with spinach the sum of O_2 and CO_2 gives around 21%. This may be explained by the fact that film permeability to O_2 and CO_2 and thus diffusion of these gases is temperature dependent (Siracusa, 2012), with CO_2 diffusion through the film (out of the bag) being faster with increasing temperature of storage. Gas composition in the bags stored at 20 °C may be further affected by anaerobic respiration (Saenmuang *et al*., 2012).

Table 3.1 Changes in the gas composition inside the bags with baby leaf spinach stored at different temperatures (1, 10 and 20 °C) for 7 days. Data represent mean values from 6 replicates.

Time of storage	Storage temperature	O_2	CO_2
Day 0		19.4%	0.0%
Day 1	1 °C	20.9% a	0.0% b
	10 °C	19.0% a	0.7% b
	20 °C	12.2% b	6.8% a
Day 2	1 °C	20.9% a	0.0% c
	10 °C	18.8% b	1.1% b
	20 °C	6.6% c	10.6% a
Day 3	1 °C	20.9% a	0.0% b
	10 °C	19.3% a	0.3% b
	20 °C	4.0% b	12.0% a
Day 4	1 °C	20.9% a	0.0% c
	10 °C	17.6% b	2.3% b
	20 °C	1.9% c	13.4% a
Day 5	1 °C	20.9% a	0.0% c
	10 °C	18.4% b	0.6% b
	20 °C	1.3% c	13.5% a
Day 6	1 °C	19.8% a	0.0% c
	10 °C	13.9% b	4.6% b
	20 °C	1.6% c	14.0% a
Day 7	1 °C	20.9% a	0.0% c
	10 °C	17.3% b	2.4% b
	20 °C	1.6% c	14.0% a

within columns, for each day, different letters indicate that values are significantly different (P<0.05). O_2: 1 d (P<0.001; SEM=0.387, CV=2.0%), 2 d (P<0.001; SEM=0.318, CV=3.0%), 3 d (P<0.001; SEM=1.184, CV=6.0%), 4 d (P<0.001; SEM=0.207, CV=2.4%), 5 d (P<0.001; SEM=0.448, CV=4.3%), 6 d (P=0.008; SEM=0.856, CV=12.8%), 7 d (P=0.031; SEM=0.347, CV=2.2%). CO_2: 1 d (P<0.001; SEM=0.365, CV=13.4%), 2 d (P<0.001; SEM=0.365, CV=9.7%), 3 d (P<0.001; SEM=0.746, CV=12.4%), 4 d (P=0.006; SEM=0.568, CV=6.8%), 5 d (P<0.001; SEM=0.401, CV=14.4%), 6 d (P=0.023; SEM=1.097, CV=10.3%), 7 d (P=0.031; SEM=1.052, CV=9.1%).

3.3.3 Solute leakage

Experiment 1

After 3 days of storage, solute leakage decreased from 2.7% (initial value) to 2.3% in samples stored at 1 and 10 °C (Figure 3.1), whereas a significant (P=0.005) increase to 6.3% was observed in samples stored at 20 °C. From that point, samples stored at 20 °C were not suitable for solute leakage determination. Their texture was already lost and high

amount of solute was present in the bag, which is why the data for those samples do not go beyond 3 days of storage. From day 5, solute leakage increased in the case of spinach leaves stored at 10 °C, while it remained at similar level during storage at 1 °C (Figure 3.1). This led to significant (P<0.05) differences in solute leakage being observed between spinach samples stored at 1 and 10 °C.

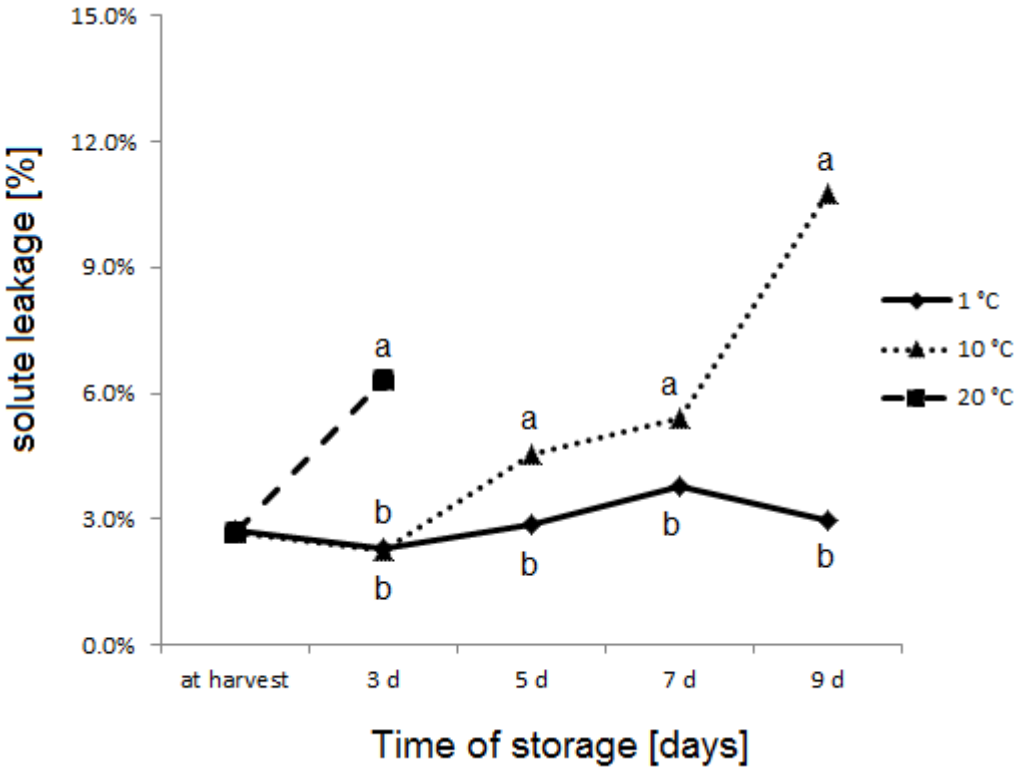

Figure 3.1 Solute leakage from spinach leaves stored for 9 days (d) at three different temperatures (1, 10 and 20 °C). Solute leakage: 3 d (P=0.005; SEM=0.888, CV=30.6%), 5 d (P=0.039; SEM=0.522, CV=20.1%), 7 d (P=0.047; SEM=0.521, CV=3.0%), 9 d (P<0.001; SEM=1.197, CV=4.2%). Different letters indicate that values are significantly different (P<0.05). Each data point is the mean of 6 replicates.

Experiment 2

In the subsequent study, where spinach leaves were stored at 1 and 6 °C, no significant difference in solute leakage was observed after 3 days of storage. After 7 days, however, solute leakage from spinach leaves stored at 6 °C (1.0%) was significantly (P<0.001) higher (Figure 3.2) when compared with those stored at 1 °C (0.6%).

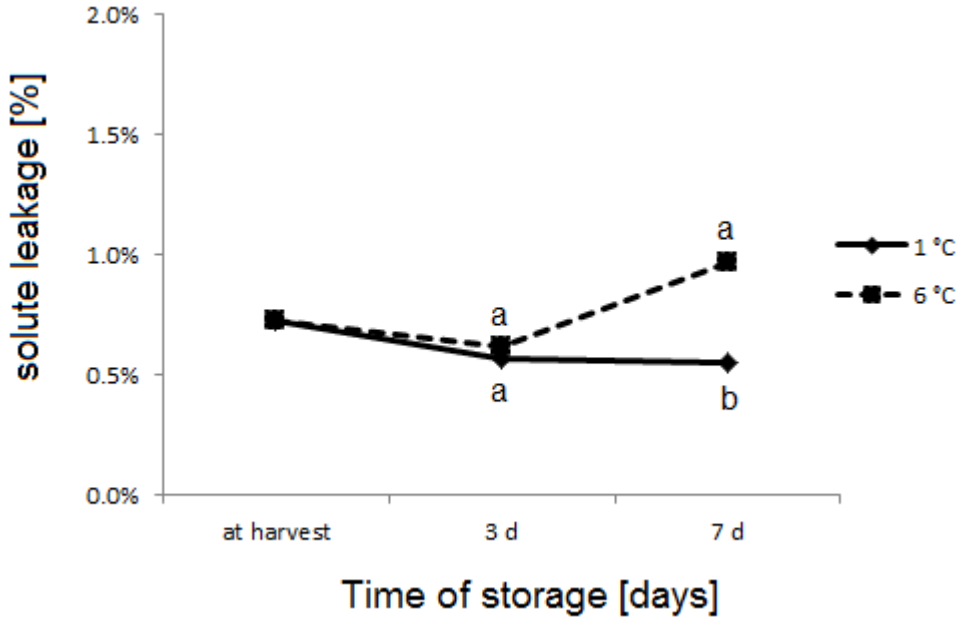

Figure 3.2 Solute leakage from spinach leaves stored for 7 days (d) at two different temperatures (1 and 6 °C). Solute leakage: 3 d (P=0.736; SEM=0.096, CV=31.1%), 7 d (P=0.002; SEM=0.079, CV=31.4%). Different letters indicate that values are significantly different (P<0.05). Each data point is the mean of 6 replicates.

3.3.4 Total ascorbic acid (ascorbic acid (AsA) + dehydroascorbic acid (DHA))

Experiment 1

A significant (P<0.001) decrease from 3.14 mg g^{-1} DW (at harvest) to 2.50 and 1.05 mg g^{-1} DW in AsA content with increasing temperature of storage was already observed after 1 day of storage at 10 and 20 °C, respectively (Table 3.2). AsA content was relatively stable during 9 days of storage in spinach stored at 1 °C, whereas its content significantly (P<0.001) decreased in samples stored at 10 and 20 °C; AsA loss being more pronounced at 20 °C (Table 3.2).

Table 3.2 Effect of storage temperature on ascorbic acid (AsA) and dehydroascorbic acid (DHA) content on a dry weight (DW) basis in spinach leaves stored for 9 days at three different temperatures (1, 10 and 20 °C). Data represent mean values from 6 replicates.

Storage temperature [°C]	**AsA [mg/g DW]**					
	storage time [days]					
	0	1	3	5	7	9
1	3.49	3.14 a	3.28 a	3.05 a	2.85 a	3.36 a
10		2.50 b	1.65 b	1.33 b	0.92 b	0.47 b
20		1.05 c	0.84 c	X	X	X
Significance:						
Treatment		<0.001	<0.001	<0.001	<0.001	<0.001
SEM		0.075	0.096	0.095	0.107	0.102
CV		3.2%	4.8%	8.6%	3.7%	4.3%
Storage temperature [°C]	**DHA [mg/g DW]**					
	storage time [days]					
	0	1	3	5	7	9
1	0.27	0.25 b	0.63 b	0.50 a	0.53 a	0.20 b
10		0.35 a	0.29 b	0.52 a	0.32 a	0.82 a
20		0.38 a	1.54 a	X	X	X
Significance:						
Treatment		<0.001	0.006	0.926	0.319	<0.001
SEM		0.021	0.254	0.170	0.148	0.098
CV		7.3%	58.3%	26.6%	79.4%	43.7%

within columns, for each day, different letters indicate that values are significantly different (P<0.05).

X-samples were not suitable for further analysis.

The highest DHA content was observed in spinach leaves stored at 20 °C (Table 3.2). After 1 day of storage, DHA content in samples stored at 20 °C was not significantly different from counterparts stored at 10 °C; nonetheless, DHA content in these samples was significantly (P<0.001) higher than in samples stored at 1 °C. After 3 days of storage DHA content in samples stored at 20 °C was 1.54 mg g^{-1} DW and was significantly (P=0.006) higher than in other samples. No significant difference was observed between samples stored at 1 and 10 °C until 9 days of storage. After 9 days, DHA content increased to 0.82 mg g^{-1} DW in samples stored at 10 °C and was then significantly (P<0.001) higher than 0.20 mg g^{-1} DW observed in samples stored at 1 °C.

In the case of total AsA content (AsA + DHA), the pattern was similar to the one observed for AsA content (Figure 3.3). After 1 day of storage, total AsA content decreased from 3.76 mg g^{-1} DW (at harvest) to 2.85 and 1.43 mg g^{-1} DW in samples stored at 10 and 20 °C, respectively. Total AsA content was relatively stable throughout the storage period in samples stored at 1 °C (Figure 3.3). This led to significantly (P<0.001) higher total AsA content being observed in those samples when compared with their counterparts stored at 10 and 20 °C. Overall, total AsA content decreased with increasing temperature of storage, with an exception of day 3, when no significant difference was observed between samples stored at 10 and 20 °C. This was due to a significant increase in DHA content in samples stored at 20 °C.

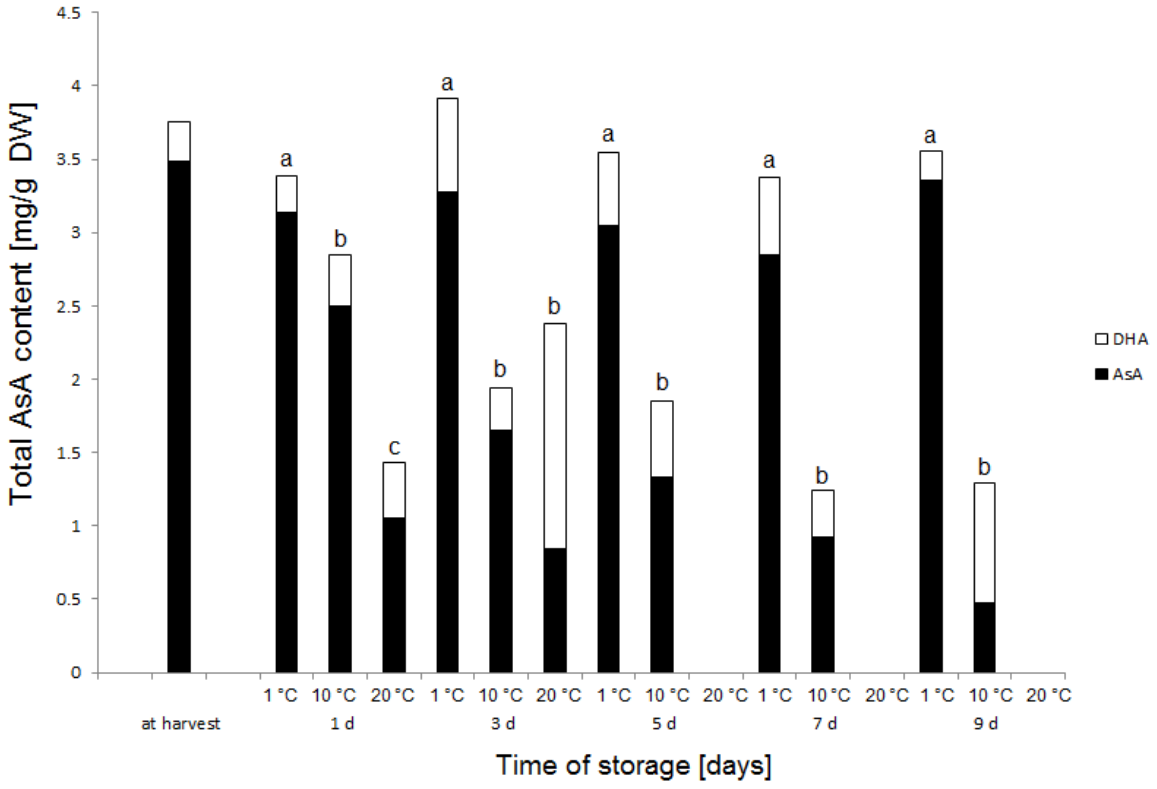

Figure 3.3 Changes in total AsA (AsA + DHA) content (■ – coloured bar (AsA) and □ – uncoloured bar (DHA)), on a dry weight (DW) basis in spinach leaves stored for 9 days (d) at 1, 10 and 20 °C. Total AsA: 1 d (P<0.001; SEM=0.082, CV=2.0%), 3 d (P<0.001; SEM=0.286 CV=20.2%), 5 d (P<0.001; SEM=0.199, CV=4.6%), 7 d (P<0.001; SEM=0.147, CV=11.9%), 9 d (P<0.001; SEM=0.132, CV=5.8%). Different letters indicate that values are significantly different (P<0.05). Each data point is the mean of 6 replicates.

Experiment 2

After 3 days of storage, AsA content in spinach stored at 1 °C was 3.67 mg g^{-1} DW and was significantly (P=0.006) higher than 3.43 mg g^{-1} DW in samples stored at 6 °C. After 7 days, however, no difference in AsA content was observed between these samples. On the other hand, after 3 days of storage, there was no difference in DHA content between spinach leaves stored at 1 and 6 °C, while the difference became significant (P<0.001) after 7 days of storage; DHA content was significantly higher at 1 °C when compared with the samples stored at 6 °C (Table 3.3).

Table 3.3 Effect of storage temperature on ascorbic acid (AsA) and dehydroascorbic acid (DHA) content on a dry weight (DW) basis in spinach leaves stored for 7 days at two different temperatures (1 and 6 °C). Data represent mean values from 6 replicates.

Storage temperature [°C]	**AsA [mg/g DW]**			**DHA [mg/g DW]**		
	storage time [days]					
	0	3	7	0	3	7
1	4.23	3.67 a	3.62 a	0.15	0.16 a	0.21 a
6		3.43 b	3.46 a		0.17 a	0.13 b
Significance:						
Treatment		0.006	0.472		0.663	<0.001
SEM		0.053	0.145		0.012	0.008
CV		35.5%	58.6%		14.2%	39.6%

within columns, for each day, values with different letters are significantly different (P<0.05).

After 3 days of storage total AsA content decreased; a stronger decrease was observed in samples stored at 6 °C when compared with those stored at 1 °C (Figure 3.4). After 7 days of storage, total AsA content remained at similar level but was no longer significantly higher at 1 °C, when compared with counterparts stored at 6 °C.

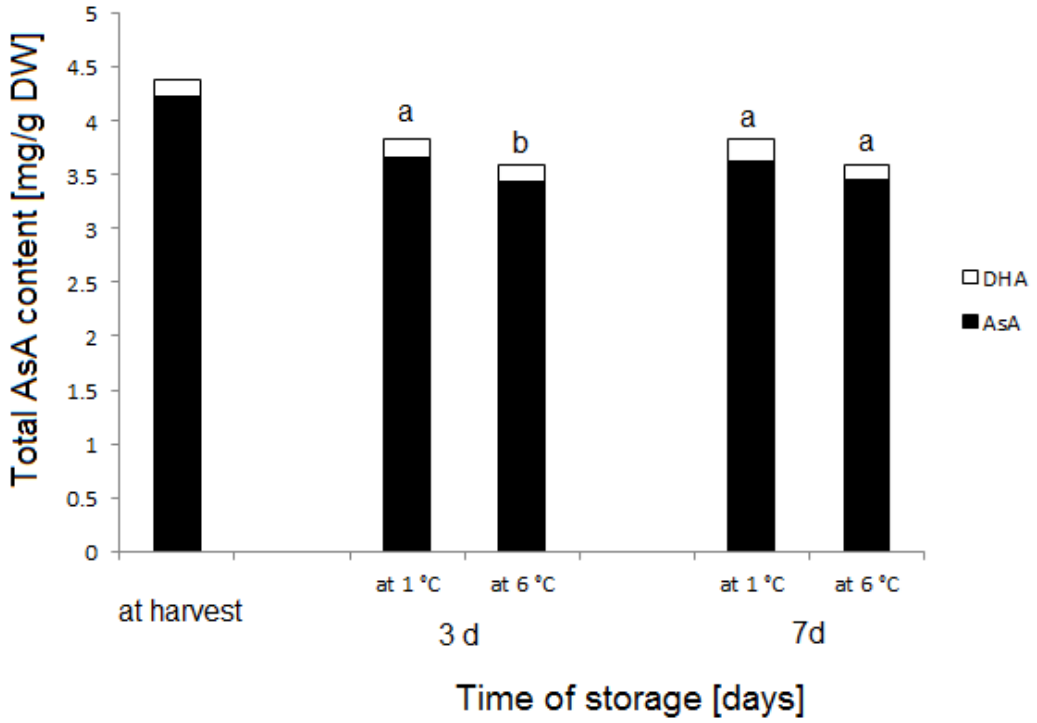

Figure 3.4 Changes in total AsA (AsA + DHA) content on a dry weight (DW) basis in spinach leaves stored for 7 days (d) at 1 and 6 °C. Total AsA: 3d (*P*=0.005, SEM=0.051, CV=4.1%), 7 d (*P*=0.265, SEM=0.143, CV=11.6%). Different letters indicate that values are significantly different (*P*<0.05). Each data point is the mean of 6 replicates.

3.3.5 Total carotenoid and chlorophyll content

Experiment 1

There was no significant difference in total carotenoid content between spinach leaves stored at 1, 10 and 20 °C during 3 days of storage. From day 5, however, total carotenoid content in spinach leaves stored at 1 °C was significantly higher when compared with samples stored at 10 °C, where total carotenoid content declined (Table 3.4).

There was no significant difference in chlorophyll *a* and *b* content between spinach leaves stored at 1, 10 and 20 °C during 3 days of storage. The concentration of both chlorophyll *a* and *b* declined in the samples stored at 10 °C. Thus, from day 5, the content of chlorophyll *a* and *b* in spinach stored at 1 °C was significantly higher than in spinach stored at 10 °C (Table 3.4), with an exception of day 7, when no significant difference

between storage conditions was observed in chlorophyll *b* content (Table 3.4). Chlorophyll *a*: *b* ratio decreased with increasing temperature of storage.

Table 3.4 Changes in chlorophyll *a*, *b*, *a*:*b* ratio and on total carotenoid content on a dry weight (DW) basis in spinach leaves stored for 9 days at three different temperatures (1, 10 and 20 °C).

Storage temperature [°C]	**chlorophyll *a* [mg/g DW]**					
	storage time [days]					
	0	1	3	5	7	9
1	19.56	17.87 a	20.58 a	21.32 a	17.67 a	20.72 a
10		22.19 a	19.66 a	17.05 b	15.19 b	14.38 b
20		22.05 a	18.99 a	X	X	X
Significance:						
Treatment		0.079	0.491	<0.001	0.014	<0.001
SEM		1.394	0.915	0.593	0.561	0.356
CV		8.7%	11.4%	9.3%	7.0%	6.8%
Storage temperature [°C]	**chlorophyll *b* [mg/g DW]**					
	storage time [days]					
	0	1	3	5	7	9
1	5.43	4.71 a	5.32 a	5.52 a	4.71 a	5.56 a
10		5.92 a	5.34 a	4.48 b	4.36 a	4.38 b
20		6.15 a	5.86 a	X	X	X
Significance:						
Treatment		0.044	0.467	0.002	0.123	<0.001
SEM		0.389	0.340	0.165	0.143	0.093
CV		8.8%	12.9%	8.8%	5.7%	7.2%
Storage temperature [°C]	**chlorophyll *a*: *b* ratio**					
	storage time [days]					
	0	1	3	5	7	9
1	3.6	3.79 a	3.87 a	3.86 a	3.75 a	3.73 a
10		3.74 b	3.68 a	3.81 b	3.48 b	3.28 b
20		3.59 c	3.24 b	X	X	X
Significance:						
Treatment		<0.001	<0.001	0.031	<0.001	<0.001
SEM		0.014	0.053	0.017	0.015	0.013
CV		0.5%	1.2%	0.7%	1.4%	0.5%
Storage temperature [°C]	**total carotenoids [mg/g DW]**					
	storage time [days]					
	0	1	3	5	7	9
1	4.98	4.73 a	5.78 a	5.80 a	5.01 a	5.86 a
10		5.57 a	5.40 a	4.74 b	4.37 b	4.78 b
20		5.60 a	5.50 a	X	X	X
Significance:						
Treatment		0.186	0.518	0.007	0.016	<0.001
SEM		0.358	0.239	0.211	0.148	0.148
CV		8.3%	11.5%	12.5%	8.9%	5.6%

within columns, for each day, different letters indicate that values are significantly different (P<0.05). X – samples were not suitable for further analysis.

Experiment 2

Total carotenoid content remained relatively stable during storage and was only slightly but not significantly higher in spinach leaves stored at 1 °C when compared with samples stored at 6 °C (Table 3.5).

Table 3.5 Changes in chlorophyll *a*, chlorophyll *b*, ratio and total carotenoid content on a dry weight (DW) basis in spinach leaves stored for 7 days at two different temperatures (1 and 6 °C). Data represent mean values from 6 replicates.

Storage temperature [°C]	**chlorophyll *a* [mg/g DW]**			**chlorophyll *b* [mg/g DW]**		
	storage time [days]					
	0	3	7	0	3	7
1	10.32	11.64 a	8.98 a	2.25	2.33 a	2.13 a
6		10.86 b	7.99 b		2.43 a	1.75 b
Significance:						
Treatment		0.003	0.002		0.37	0.011
SEM		0.167	0.201		0.077	0.096
CV		5.4%	8.5%		11.7%	17.7%
Storage temperature [°C]	**chlorophyll *a*: *b* ratio**			**total carotenoids [mg/g DW]**		
	storage time [days]					
	0	3	7	0	3	7
1	4.59	5.13 a	4.29 a	2.05	2.46 a	1.95 a
6		4.51 b	4.89 a		2.33 a	1.86 a
Significance:						
Treatment		0.030	0.236		0.059	0.202
SEM		0.188	0.345		0.099	0.102
CV		14.1%	27.1%		7.5%	9.6%

within columns, for each day, different letters indicate that values are significantly different (P<0.05).

After 3 days of storage, chlorophyll *a* content was significantly (P=0.003) higher in spinach leaves stored at 1 °C when compared with the samples stored at 6 °C (Table 3.5). Its content decreased after 7 days of storage, however the content of chlorophyll *a* was still significantly (P=0.002) higher in spinach leaves stored at 1 °C. In the case of chlorophyll *b*, no difference in its content was observed after 3 days of storage, whereas after 7 days chlorophyll *b* content decreased in spinach samples stored at 6 °C. This led to significantly (P=0.011) lower chlorophyll *b* content being observed in those samples (Table 3.5). After 3 days of storage, chlorophyll *a*: *b* ratio was significantly higher in

spinach leaves stored at 1 °C (Table 3.5). The ratio, however, declined during storage at 1 °C while it did not change at 6 °C. Thus, after 7 days of storage there was no significant difference between the two storage temperature regimes.

3.3.6 Leaf colour changes

Experiment 1

There was no significant difference (between storage temperatures of 1 and 10 °C) in leaf lightness value during 3 days of storage; however from day 5, spinach leaves stored at 10 °C were significantly lighter when compared with their counterparts stored at 1 °C (Table 3.6). Leaf lightness value did not change much throughout 9 day storage period at 1 °C (Figure 3.5), while an increase was observed in the case of spinach leaves stored at 10 °C.

There was no difference (between storage temperatures) in greenness value throughout the storage period (Table 3.6), with an exception of day 5, when spinach leaves stored at 10 °C were significantly greener than counterparts stored at 1 °C.

Table 3.6 Leaf colour changes during the storage of spinach leaves at three different temperatures (1, 10 and 20 °C) for 9 days. Data represent mean values from 6 replicates.

Storage temperature [°C]	***L* (lightness)***					
	storage time [days]					
	0	1	3	5	7	9
1	46.31	47.08 a	47.22 ab	45.99 b	47.04 b	46.53 b
10		47.26 a	46.11 b	47.31 a	48.41 a	51.31 a
20		47.31 a	47.78 a	X	X	X
Significance:						
Treatment		0.883	0.043	0.019	0.030	<0.001
SEM		0.351	0.470	0.385	0.436	0.500
CV		1.0%	0.4%	1.1%	0.8%	0.2%
Storage temperature [°C]	***a* (greenness)***					
	storage time [days]					
	0	1	3	5	7	9
1	-16.18	-16.89 a	-16.46 a	-16.23 a	-16.79 a	-16.97 a
10		-16.92 a	-16.66 a	-16.98 b	-16.66 a	-17.22 a
20		-16.46 a	-16.84 a	X	X	X
Significance:						
Treatment		0.342	0.524	0.045	0.716	0.557
SEM		0.243	0.233	0.258	0.246	0.293
CV		2.3%	0.7%	2.6%	2.4%	1.8%
Storage temperature [°C]	***b* (yellowness)***					
	storage time [days]					
	0	1	3	5	7	9
1	26.63	28.09 a	27.27 b	26.49 b	28.27 b	28.7 b
10		28.34 a	28.20 b	29.85 a	30.62 a	35.68 a
20		27.65 a	31.39 a	X	X	X
Significance:						
Treatment		0.702	<0.001	<0.001	0.029	<0.001
SEM		0.591	0.630	0.599	0.741	0.802
CV		3.2%	1.0%	3.0%	3.1%	3.5%

within columns, for each day, different letters indicate that values are significantly different (P<0.05). X – samples were not suitable for further analysis.

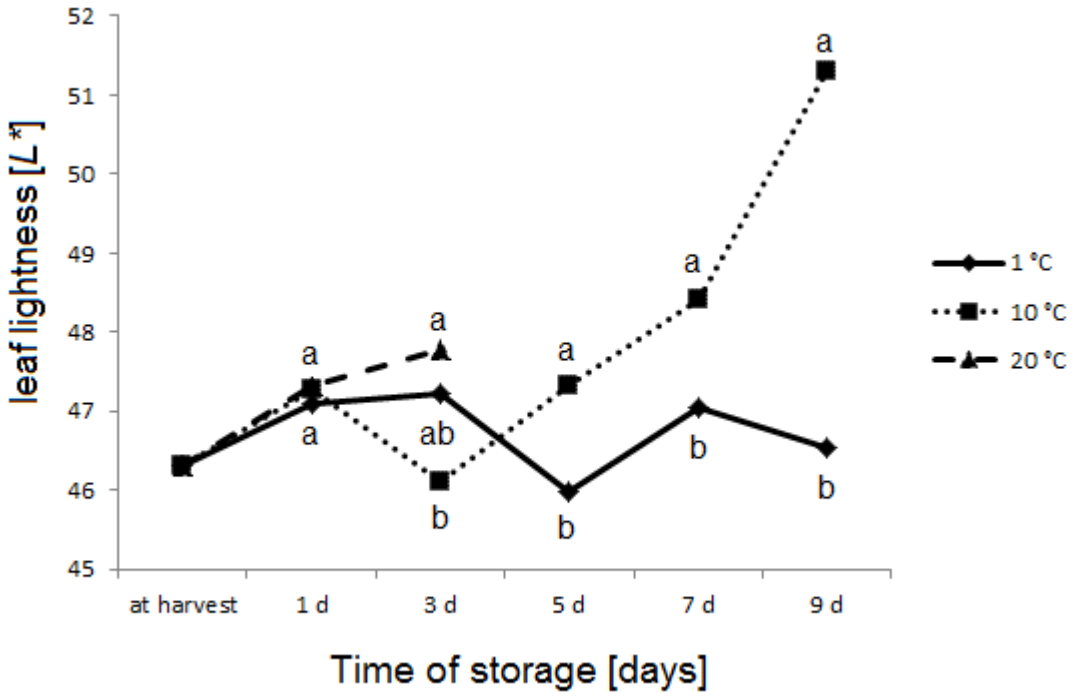

Figure 3.5 Changes in leaf lightness value (*L**) during the storage of spinach leaves for 9 days (d) at three different temperatures (1, 10 and 20 °C). *L**: 1 d (*P*=0.883; SEM=0.351, CV=1.0%), 3 d (*P*=0.043; SEM=0.470 CV=0.4%), 5 d (*P*=0.019; SEM=0.385, CV=1.1%), 7 d (*P*=0.030; SEM=0.436, CV=0.8%), 9 d (*P*<0.001; SEM=0.500, CV=0.2%). Different letters indicate that values are significantly different (*P*<0.05). Each data point is the mean of 6 replicates.

Leaf yellowness increased with increasing temperature of storage (Table 3.6). Yellowing of the leaves was already observed after 3 days of storage at 20 °C. These samples were significantly (*P*<0.05) more yellow than those stored at 1 and 10 °C (Table 3.6). No significant difference (between storage temperatures of 1 and 10 °C) in leaf yellowness was found during 3 days of storage. From day 5, however, spinach leaves stored at 10 °C were significantly (*P*<0.05) more yellow when compared with their counterparts stored at 1 °C (Table 3.6). Leaf yellowness value did not change much throughout 9 day storage period at 1 °C (Figure 3.6), while an increase was observed in the case of spinach leaves stored at 10 °C.

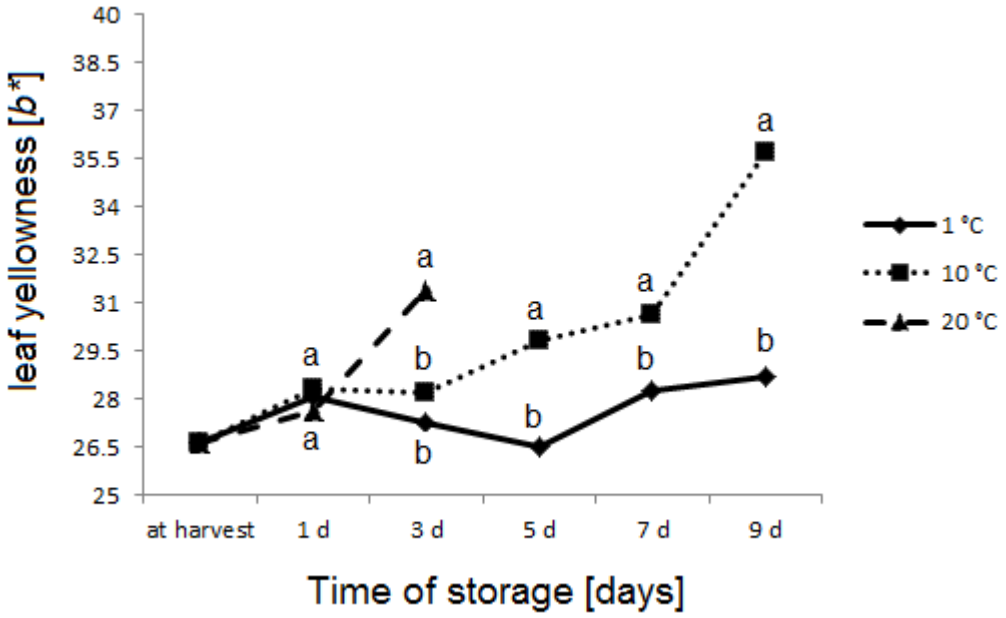

Figure 3.6 Changes in leaf yellowness value (*b**) during the storage of spinach leaves at three different temperatures (1, 10 and 20 °C) for 9 days (d). *b**: 1 d (*P*=0.702; SEM=0.591, CV=3.2%), 3 d (*P*<0.001; SEM=0.630 CV=1.0%), 5 d (*P*<0.001; SEM=0.599, CV=3.0%), 7 d (*P*=0.029; SEM=0.741, CV=3.1%), 9 d (*P*<0.001; SEM=0.802, CV=3.5%). Different letters indicate that values are significantly different (*P*<0.05). Each data point is the mean of 6 replicates.

Experiment 2

No difference was observed in leaf lightness between spinach leaves stored at 1 and 6 °C throughout 7 day storage period (Table 3.7). No difference was also observed in leaf greenness and yellowness after 3 days of storage; however, after 7 days of storage spinach leaves stored at 6 °C were significantly (*P*=0.003) greener and more yellow than those stored at 1 °C (Table 3.7).

Table 3.7 Leaf colour changes during the storage of spinach leaves for 7 days at two different temperatures (1 and 6 °C). Data represent mean values from 6 replicates.

Storage temperature [°C]	***L* (lightness)***		
	storage time [days]		
	0	3	7
1	44.82	46.84 a	45.93 a
6		46.72 a	47.83 a
Significance:			
Treatment		0.797	0.057
SEM		0.338	0.576
CV		0.6%	7.2%
Storage temperature [°C]	***a* (greenness)***		
	storage time [days]		
	0	3	7
1	-15.06	-16.19 a	-15.51 a
6		-16.02 a	-16.59 b
Significance:			
Treatment		0.614	0.003
SEM		0.225	0.243
CV		1.7%	7.3%
Storage temperature [°C]	***b* (yellowness)***		
	storage time [days]		
	0	3	7
1	24.71	27.61 a	26.45 b
6		27.03 a	30.19 a
Significance:			
Treatment		0.461	<0.001
SEM		0.546	0.717
CV		2.5%	12.9%

within columns, for each day, different letters indicate that values are significantly different (P<0.05).

3.4 Combined analysis

In both experiments, samples stored at 1 °C behaved in a similar way. Although solute leakage values at harvest differed between experiments, being 2.7% in Experiment 1 and 0.8% in Experiment 2, no significant increase during storage was observed in these samples.

Only small differences in AsA, DHA and total AsA content of spinach was observed between both experiments. In Experiment 1, the content of AsA was 3.49 mg g^{-1} DW, DHA 0.27 and total AsA 3.76 mg g^{-1} DW, while in Experiment 2, these values were 4.23, 0.15 and 4.38 mg g^{-1} DW, respectively. In both experiments, AsA and total AsA content remained relatively stable in the samples stored at 1 °C. In the case of DHA, in Experiment 1, its content remained relatively stable, while an increase was observed in Experiment 2.

Although plant pigment content (chlorophyll *a* and *b*, total carotenoids) was significantly higher in spinach leaves used in Experiment 1, their content remained relatively stable throughout the storage period in both experiments, with the exception that chlorophyll *a* declined after 7 days of storage at 1 °C (Experiment 2). Similarly, no differences between experiments were observed in leaf colour characteristics. Visual quality of spinach leaves was well maintained in the samples stored at 1 °C.

To find out, whether it is possible to observe any trends, combined analyses were conducted, where mean values obtained at 6, 10 and 20 °C were compared with corresponding values obtained at 1 °C. One must be aware that in the case of samples stored at 20 °C, the end of shelf-life was already observed after 3 days of storage, thus the data after 7 days of storage at 20 °C are not included in the combined analysis.

After 3 days of storage, solute leakage increased with increasing temperature of storage from 1 to 6 °C; however, no significant difference was observed between samples stored at 1 and 10 °C. Substantial increase in solute leakage was observed in samples stored at 20 °C. After 7 days of storage, solute leakage from samples stored at 6 and 10 °C was significantly higher than from those stored at 1 °C.

After 3 days of storage, AsA content decreased with increasing storage temperature (Table 3.8). The AsA content in spinach leaves stored at 6, 10 and 20 °C were 93.5, 50.3 and 25.6% of those stored at 1 °C, respectively. After 7 days of storage, there was a slight (4.4%) non-significant decrease in AsA content in samples stored at 6 °C when compared with those stored at 1 °C (Table 3.8). A significant decrease in AsA content was observed in samples stored at 10 °C, where in comparison with the samples stored at 1 °C, AsA content decreased by 67.7%.

After 3 days of storage, inconsistent changes in DHA content were observed. In the samples stored at 6 °C, DHA content did not change significantly (increased by 6%) when compared with samples stored at 1 °C, while a decrease by 54% and increase by 144% was observed in the samples stored at 10 and 20 °C, respectively. After 7 days of storage, DHA content decreased with the increase in temperature of storage from 1 to 6 and from 1 to 10 °C (Table 3.8), where in comparison with the samples stored at 1 °C, DHA content decreased by 38 and 39%, respectively.

After 3 days of storage, total AsA content decreased by 6, 50 and 39 % in spinach samples stored at 6, 10 and 20 °C, respectively. After 7 days of storage, the pattern was very similar to the one observed for AsA (Table 3.8); however, due to a significant decrease in DHA content at 6 °C, total AsA content in these samples was significantly lower when compared with those stored at 1 °C.

Table 3.8 Changes in the concentration of bioactive compounds (AsA, DHA, total AsA, and total carotenoids) in spinach leaves stored at three different temperatures (6, 10 and 20 °C) in comparison with samples stored at 1 °C.

AsA (% of 1 °C)		
Storage temperature	3 days	7 days
6 °C	93.5%	95.6%
10 °C	50.3%	32.3%
20 °C	25.6%	X
DHA (% of 1 °C)		
Storage temperature	3 days	7 days
6 °C	106.0%	61.9%
10 °C	46.0%	60.4%
20 °C	244.0%	X
Total AsA (% of 1 °C)		
Storage temperature	3 days	7 days
6 °C	94.0%	93.7%
10 °C	49.6%	36.7%
20 °C	60.9%	X
Total carotenoids (% of 1 °C)		
Storage temperature	3 days	7 days
6 °C	94.7%	95.4%
10 °C	93.4%	87.2%
20 °C	95.2%	X

X – samples were not suitable for further analysis.

After 3 days of storage, there was no significant difference in total carotenoid content between the samples stored at 1, 6, 10 and 20 °C. After 7 days of storage, however, total carotenoid content decreased with the increase in temperature of storage from 1 to 10 °C (Table 3.8), while no significant difference was observed between samples stored at 1 and 6 °C.

After 3 days of storage, there was no significant difference in chlorophyll *a* content between the samples stored at 1, 6, 10 and 20 °C. After 7 days of storage, chlorophyll *a* content decreased by 11 and 14% in the samples stored at 6 and 10 °C, respectively. In the case of chlorophyll *b*, no significant difference between the samples was observed after 3 days of storage. After 7 days of storage, however, a significant decrease was observed at 6 °C, where – in comparison with the samples stored at 1 °C - chlorophyll *b* content decreased by 18%.

After 3 days of storage, a significant increase in leaf yellowness was observed in the samples stored at 20 °C, while no difference was found between those stored at 1 and 6 and 1 and 10 °C. After 7 days of storage, leaves became significantly more yellow at both 6 and 10 °C, when compared with the samples stored at 1°C.

3.5 Discussion

Two experiments were conducted to determine the effect of storage temperature on quality changes of spinach. In Experiment 1, a broad range of temperatures (1, 10 and 20 °C) was used, while in the subsequent experiments the temperature range was narrowed to 1 and 6 °C. Experiment 1 was conducted in July and August, while Experiment 2 was conducted in September. This can account for the seasonal differences in the quality of the leaves. No difference was observed in dry matter content between leaves used in both experiments. The initial quality of the leaves used in Experiment 1, however, was lower when compared with those used in Experiment 2. The texture of the leaves used in Experiment 1 (indicated by higher solute leakage at harvest) was worse than those used in Experiment 2. Plant pigment content was higher in spinach leaves used in Experiment 1; however, these leaves were significantly lighter and more yellow (at harvest) when compared with those used in Experiment 2. Finally, nutritional quality of the leaves used in Experiment 1 was also a bit lower than those used in Experiment 2.

The replication number in Experiments 1 and 2 was low – 6 replicates, thus the validity of the data is limited. The findings from these preliminary experiments, however, may still be used as an indication of how the quality of baby leaf spinach responds to temperature changes during storage.

Gas composition

Previous studies have found a decrease in O_2 concentration with simultaneous increase in CO_2 concentration after 3 days of storage of spinach at 5 °C (Allende *et al.*, 2004b; Conte *et al*., 2008) and 7 °C (Tudela *et al*., 2013) in the dark. The key issue with high respiration rate is its inverse relationship with shelf-life.

The studies mentioned above (Allende *et al*., 2004b; Conte *et al*., 2008; Tudela *et al*., 2013) reported an increase in CO_2 development inside the bags with spinach during storage. In agreement with those studies CO_2 development was also observed in our study, in bags with spinach stored at 10 and 20 °C. This was, however, not the case in the bags that were stored at 1 °C, where respiration was very low and was balanced by the photosynthetic activity of the leaves (Toledo *et al*., 2003a) and/or oxygen transmission rate (OTR) of the bag.

Artes-Hernandez *et al*. (2009) observed an increase in the respiration rate when comparing spinach leaves stored in the dark at 8 °C with those stored at 5 °C. Increased respiration rate of spinach with increasing temperature of storage from 1 to 12 °C has also been reported by Luo *et al*. (2009). In agreement with those studies, respiration rate also increased with increasing temperature of storage in our study. A significant increase in the respiration rate in the samples stored at 20 °C was observed already after 1 day of storage. From day 2, respiration rate of spinach stored at 10 °C was significantly higher when compared with samples stored at 1 °C. Findings from this study and those of others (Artes-Hernandez *et al*., 2009; Luo *et al*., 2009) give clear evidence that respiration rate is sensitive to changes in the storage temperature.

Texture

Tissue breakdown during storage is often quantified by measuring solute leakage (Marangoni *et al.*, 1996, Wagstaff *et al.*, 2007). An increase in solute leakage has previously been reported during the storage of spinach (Hodges *et al.*, 2001, Allende *et al.*, 2004b, Gomez *et al.*, 2008). Similar to others, an increase in solute leakage over time was observed when spinach leaves were stored at 6, 10 and 20 °C. The values observed were in the same range as those reported for spinach by Allende *et al*. (2004b) and others (Medina *et al.*, 2012, Tudela *et al.*, 2013), but lower than reported by Gomez *et al*. (2008). This is not surprising, as Gomez *et al*. (2008) stored spinach leaves at 23 °C which is a higher temperature than 5 °C used by Allende *et al*. (2004b) and 7 °C used by others (Medina *et al.*, 2012, Tudela *et al.*, 2013). Furthermore, 23 °C is higher than the range of

temperatures used here (1, 6, 10 and 20 °C) and a substantial increase in solute leakage was already observed after 3 days of storage at 20 °C. A significant increase in solute leakage was also observed in the samples stored at 10 °C, which is in agreement with Babic *et al*. (1996) who observed a marked decrease in textural quality of spinach leaves when stored at 10 °C.

Luo *et al*. (2009) determined the effect of storage temperature on solute leakage from spinach leaves. They found a significant increase in solute leakage in samples stored at 12 °C, while no difference was observed between those stored at 1, 5 and 8 °C. In agreement with their study, solute leakage significantly increased with increasing temperature of storage from 1 to 20 °C. Interestingly, in this study, even a small difference in storage temperature (1 vs. 6 °C) significantly reduced the textural quality of spinach leaves as indicated by increased solute leakage.

In the case of spinach leaves stored at 1 °C, solute leakage values did not change over the storage period and remained relatively low. A significant increase in solute leakage, however, was observed after 4, 5 and 7 days of storage at 20, 10 and 6 °C, respectively. This is in agreement with Allende *et al*. (2004b) who observed an increase in solute leakage from spinach leaves after 6 days of storage at 5 °C, however, in contrast with others (Medina *et al.*, 2012, Tudela *et al.*, 2013) who observed an increase in solute leakage after 12 days of storage at 7 °C.

Nutritional quality

AsA content has previously been reported to decrease during the storage of spinach (Gil *et al.*, 1999, Bergquist *et al.*, 2006, Bergquist *et al.*, 2007, Bottino *et al.*, 2009). In agreement with these studies, AsA content decreased over time in our study. Bergquist *et al*. (2006) has previously observed better retention of AsA in spinach leaves stored at 2 °C when compared with samples stored at 10 °C. In agreement with these studies, the content of AsA was significantly higher in the samples stored at 1 °C when compared with those stored at 10 °C.

Changes in the content of DHA in spinach leaves were inconsistent as previously reported by Bergquist *et al*. (2006). This is in contrast with other studies that reported an increase in DHA content during the storage of spinach (Gil *et al.*, 1999, Bottino *et al.*, 2009).

A number of studies (Hodges *et al*., 2001; Bergquist *et al*., 2006, 2007; Bottino *et al*., 2009) have reported a decline in total AsA content during dark storage of spinach. This was, however, not the case when spinach was stored under continuous light (26.9 μmol m^{-2} s^{-1}) at 4 °C (Lester *et al*., 2010b). AsA content remained relatively stable during the storage at 1 and 6 °C under continuous light (30-35 μmol m^{-2} s^{-1}) conditions in our study. A decrease in total AsA content, however, was already observed after 1 day of storage in samples stored at 10 and 20 °C. Better retention of AsA at lower storage temperatures has previously been reported by Bergquist *et al*. (2006). This suggests that at higher storage temperatures, total AsA content is rapidly lost.

Visual quality

Leaf colour changes may be related to chlorophyll degradation (Pandrangi and LaBorde, 2004). Chlorophyll content was reported to decrease during the storage of spinach at 4, 10 and 20 °C (Pandrangi and LaBorde, 2004), while others (Bergquist *et al.*, 2006, Conte *et al.*, 2008) reported chlorophyll concentration to be relatively stable during the storage of spinach leaves at 2 and 10 °C (Bergquist *et al*., 2006) and 5 °C (Conte *et al*., 2008). This could be explained by the fact that both Bergquist *et al*. (2006) and Conte *et al*. (2008) stored spinach leaves in the dark, whereas chlorophyll degradation is enhanced by light (Kopas-Lane and Warthesen, 1995, Ferrante *et al.*, 2004). Chlorophyll content in spinach leaves declined in the samples stored at 6 and 10 °C in our study. Inconsistent changes were observed in samples stored at 1 °C; where chlorophyll content either declined (Experiment 2) or remained relatively stable (Experiment 1). No change in chlorophyll content was observed during 3 days of storage at 20 °C, which suggests that changes in pigment content are relatively slow. Low intensity light was used in this study, thus it is not clear whether this can be used as the only explanation.

An increase in chlorophyll degradation rate with increasing storage temperature from 4 to 20 °C has previously been observed by others (Pandrangi and LaBorde, 2004). On the other hand, Bergquist *et al*. (2006) have not reported significant differences between spinach leaves stored at 2 and 10 °C. In agreement with Pandrangi and LaBorde (2004) the loss of chlorophyll *a* and *b* was accelerated with increasing temperature of storage from 1 to 10 °C in our study. Pandrangi and LaBorde (2004) observed a decrease in chlorophyll content after 2 days of storage at 10 and 20 °C, and 6 days of storage at 4 °C, respectively. In our study, decline in chlorophyll content was observed after 5 and 7 days of storage at 10 and 6 °C, respectively. The difference in chlorophyll retention may be a consequence of studying a different cultivar to other studies, or else seasonal differences previously reported by Bergquist *et al*. (2006) and Conte *et al*. (2008).

Several authors have reported a decrease in visual quality during the storage of spinach (Luo *et al*., 2009; Medina *et al*., 2012; Tudela *et al*., 2013). In agreement with these studies, visual quality of spinach declined during storage at 1, 6, 10 and 20 °C. Conte *et al*. (2008), however, did not observe any changes in visual quality of spinach leaves stored at 5 °C for 13 days.

Luo *et al*. (2009) have demonstrated that in spinach leaves, the loss of visual quality is accelerated with increasing temperature of storage from 1 to 12 °C. These authors observed only a small decline in visual quality of spinach stored for 12 days at 1 and 5 °C, while visual quality of spinach was maintained during 13 days storage at 5 °C (Conte *et al*., 2008). In agreement with those studies, only a small colour alteration was observed in spinach stored at 1 °C. Furthermore, spinach leaves in our study became significantly lighter and more yellow with increasing temperature of storage.

Visual quality loss was reported after 7 days (Tudela *et al*., 2013) and 9 days (Medina *et al*., 2012) of storage at 7 °C, while Luo *et al*. (2009) observed visual quality loss after 9 and 12 days of storage at 12 and 8 °C, respectively. In our study, visual quality declined after 3, 5 and 7 days of storage at 20, 10 and 6 °C, respectively.

Care must be taken though, when comparing different studies as seasonal

differences in colour parameters of spinach leaves have been reported by Conte *et al*. (2008). Furthermore, leaf colour changes are not only affected by the storage temperature but also by the light conditions during storage. Martinez-Sanchez *et al*. (2011) observed stronger colour alteration in light-stored Romaine lettuce leaves when compared with dark-stored counterparts, that may explain why the difference in leaf colour was already observed when storage temperature increased from 1 to 6 °C.

Based on the results obtained in this study, the suggestion can be made that leaf textural and visual quality are the best indicators of shelf-life. These parameters were found to respond rapidly to changes in the temperature of storage. Both, solute leakage and leaf colour changed with increasing temperature of storage, which resulted in textural and visual quality loss. Differences in textural and visual quality of the leaves were observed even between samples stored at 1 and 6 °C, which confirms that changes in these parameters are sensitive to environmental changes. On the other hand, significant differences in plant biochemistry – the content of AsA, total carotenoids and chlorophylls were only observed between samples stored at 1 and 10 °C but not between those stored at 1 and 6 °C. This limits the use of these parameters as indicators of shelf-life as they are not sensitive enough to environmental changes.

The null hypothesis tested in this research has to be rejected as it has been found that all parameters that were measured were affected by changes in the temperature of storage.

3.6 Conclusions

Quality loss of spinach leaves is accelerated with increasing temperature of storage. This can be associated with the visual and textural quality loss that has been reported to occur already at 6 °C. Nutritional quality was less sensitive to changes in storage temperature. After 7 days of storage, no significant difference in AsA and total carotenoid content was observed between samples stored at 1 and 6 °C. At 10 °C, however, nutritional quality of spinach was significantly reduced. The data obtained in this study suggest that to maintain the quality of spinach leaves during storage, bags with

baby leaf spinach should be kept at refrigerated temperature below 6 °C. If that is not an option, the time of exposure to abusive temperature, throughout the supply chain, should be reduced to a minimum.

As mentioned above, textural quality loss, as indicated by increased solute leakage from the leaves is a good measure of shelf-life as it was very sensitive to changes in temperature of storage. The same can be said about visual quality loss, which is mainly associated with yellowing of spinach leaves. Both parameters indicated quality loss after 3, 5 and 7 days of storage at 20, 10 and 6 °C, respectively. On the other hand, plant pigment content did not respond to changes in temperature. In the case of samples stored at 20 °C, the end of shelf-life was already observed after 3 days of storage, while no significant changes were yet observed in plant pigment content. This suggests that these changes are too slow to be consistently used as indicators of shelf-life, as visual quality loss clearly precedes changes in leaf biochemistry. Although, a significant difference was found in AsA content between samples stored at 1 and 10 °C, changes in this parameter were not sensitive enough when spinach leaves were stored at 1 and 6 °C. Furthermore, inconsistent changes at different storage temperatures were observed for DHA content.

It is clear that from commercial perspective, leaf textural and visual qualities (sensory quality) are of key importance as people buy with their eyes. If fresh produce does not look good, no one will buy it even if the nutritional content is improved.

Chapter 4 Effect of light conditions on quality changes of baby leaf spinach

4.1 Introduction

Fresh produce is exposed to various light conditions during its displayed shelf-life. Leafy vegetables are an interesting crop group as the marketed product is composed of leaves that can maintain photosynthetic activity during postharvest storage. It is not surprising that in recent years, there has been an increasing interest in studying the effects of light on quality changes during the storage of leafy vegetables such as chard (Sanz *et al.*, 2008), Chinese kale (Noichinda *et al.*, 2007), lettuce (Martinez-Sanchez *et al.*, 2011, Zhan *et al.*, 2012, Zhan *et al.*, 2013) and spinach (Lester *et al.*, 2010b). The response of a number of different quality characteristics has been studied and a number of authors have reported the effects of light exposure on leaf texture (Sanz *et al.*, 2008, Martinez-Sanchez *et al.*, 2011, Medina *et al.*, 2012), visual quality (Sanz *et al.*, 2008, Kobori *et al.*, 2011, Martinez-Sanchez *et al.*, 2011, Medina *et al.*, 2012) and nutritional quality (Noichinda *et al.*, 2007, Lester *et al.*, 2010b, Martinez-Sanchez *et al.*, 2011, Zhan *et al.*, 2013).

Light exposure has been reported to decrease respiration rate during the storage of lettuce (Martinez-Sanchez *et al.*, 2011), which means that carbohydrates already present in the leaves at harvest, do not have to be broken down to be used as an energy supply to prevent the leaves from reaching senescence stage. Continuous light exposure during storage, has also been reported to support maintenance of photosynthetic activity of Chinese kale (Noichinda *et al.*, 2007), lettuce (Zhan *et al.*, 2013) and spinach leaves (Toledo *et al.*, 2003a). Increased sugar level, however, may be responsible for inducing leaf senescence, most likely via hexokinase function (Yoshida, 2003). According to these authors, if sugar content in the leaves is too high and reaches saturation point, photosynthetic activity will be repressed. Thus, to avoid greater senescence in light-stored leaves, light intensity should be relatively low, so that leaves can photosynthesize and senescence is not induced.

Light exposure during storage has been reported to reduce the solute leakage from lettuce (Martinez-Sanchez *et al.*, 2011) and spinach leaves (Kar and Choudhuri, 1986) when compared with their dark-stored counterparts. Martinez-Sanchez *et al*. (2011) suggested that this may be related to changes in gas composition inside the bags with fresh produce. Gas composition inside the bags with leafy vegetables, however, is only an indicator of the balance between photosynthesis and respiration. Photosynthetic activity is maintained in light-stored leaves (Toledo *et al*., 2003a), while it is not the case in dark-stored counterparts. Thus, lower level of CO_2 would be expected to be observed in the bags kept in the light. Furthermore, low respiration rate in light-stored leaves (Martinez-Sanchez *et al*., 2011) would not result in CO_2 development inside the bags. Leaves that can maintain their photosynthetic activity during storage and have sufficient energy supply (*e.g*. carbohydrates) to maintain the stability of cell walls are expected to better maintain their texture (Clarkson *et al*., 2003; Wagstaff *et al*., 2010).

Several authors have found slower AsA loss during light-storage of leafy vegetables, including Chinese kale (Noichinda *et al.*, 2007), lettuce (Zhan *et al.*, 2012, Zhan *et al.*, 2013) and spinach leaves (Toledo *et al.*, 2003b, Lester *et al.*, 2010b) when compared with dark-stored counterparts. Toledo *et al*. (2003b) suggested that this could be due to higher availability of carbohydrates – precursors of AsA. Lester *et al*. (2010b), however, observed that the effect is cultivar specific with higher AsA content in light-stored spinach in cultivar Lazio but not in cultivar Samish, where no difference in AsA content between two storage conditions was found after 6 days of storage. The level of DHA can also be influenced by light levels and significantly greater DHA content was found in dark-stored lettuce (Zhan *et al.*, 2012, Zhan *et al.*, 2013) and rocket (Barbieri *et al.*, 2011). However, inconsistent changes in DHA content were reported in two cultivars of spinach (Lester *et al.*, 2010b), while Toledo *et al*. (2003b) did not observe any difference in DHA content between dark and light-stored spinach leaves until day 16. Total AsA is a sum of its reduced (AsA) and oxidized (DHA) form. Thus, the increase in DHA may compensate the loss in AsA and result in similar total AsA content between dark and light-stored samples. No difference in total AsA between dark and light-stored lettuce has been

reported by Martinez-Sanchez *et al*. (2011). It is not clear from the literature, how light exposure during storage affects the balance between AsA and DHA. The response of the leaves during storage may be affected by pre-harvest conditions, *i.e*. the amount of light the crop received before being harvested, which will differ between growing seasons.

Experiment 3 was conducted to investigate the effect of light conditions during storage on quality changes of cold-stored spinach. Bagged spinach was stored under three different light intensities (in the dark or under continuous low (30-35 µmol m^{-2} s^{-1}) or high (130-140 µmol m^{-2} s^{-1}) intensity light) at 1 °C. In Experiment 4, the range of light intensities was narrowed (only low and high intensity light were used). In Experiment 4, to ensure that all the leaves received similar amount of light, the time of exposure to high intensity light (130-140 µmol m^{-2} s^{-1}) was reduced to 6 h, while for the remaining time (18 h) spinach leaves were kept in the dark. These treatments were chosen to (i) determine the effect of light exposure on quality changes during the storage of spinach and (ii) to investigate whether the observed response was due to light intensity or the amount of light received by the leaves.

This Chapter reports findings from two independent experiments conducted with field-grown spinach. The aim was to determine the effect of light conditions during storage on changes in the quality characteristics of commercially bagged spinach.

The following null hypothesis was tested: light conditions (intensity + photoperiod) during storage do not affect the maintenance of nutritional, textural and/or visual quality characteristics of baby leaf spinach.

4.2 Materials and Methods

4.2.1 Plant material and handling

Spinach used in Experiment 3 was harvested on 3rd and 28th of October 2011. Spinach was bagged at PDM Produce Ltd and transported to the laboratory (~15 minutes) in insulated opaque containers as described in section 2.1.

In Experiment 3, bags with baby leaf spinach were kept under three different light levels (in the dark or under continuous low (30-35 µmol m^{-2} s^{-1}) or high (130-140 µmol m^{-2} s^{-1}) intensity light) at 1±1 °C for 7 days. Light intensities in the range 20-35 µmol m^{-2} s^{-1} have been used by others (Toledo *et al*., 2003b; Noichinda *et al*., 2007; Lester *et al*. 2010b; Zhan *et al*., 2012, 2013), while Martinez-Sanchez *et al*. (2011) stored lettuce under the light intensity of 6±1 µmol m^{-2} s^{-1}. On the other hand, Ferrante *et al*. (2004) stored rocket, chicory and Swiss chard leaves under high intensity light of 150 µmol m^{-2} s^{-1}. The actual observed average temperature was 1.2 °C as recorded with Tinytag™ temperature loggers (Gemini Data Loggers Ltd, UK). All measurements (gas composition, solute leakage, total ascorbic acid, and total carotenoids and chlorophylls content, leaf colour) were taken on the harvesting day and then when samples were collected from storage.

Based on the results from Experiment 3, the decision was made to investigate whether the effect of light exposure during storage was due to light intensity or the amount of light received by the leaf. Spinach used in Experiment 4 was harvested on 23rd and 27th of July 2012. Spinach was bagged at PDM Produce Ltd and transported to the laboratory (~15 minutes) in insulated opaque containers as described in section 2.1. Bags with baby leaf spinach were kept at 1±1 °C under continuous (24 hours) low intensity light (30-35 µmol m^{-2} s^{-1}) or photoperiod of 6 h high intensity light (130-140 µmol m^{-2} s^{-1}) with 18 h dark per 24 hours for 10 days. The total amount of light received by the leaves was similar over a 24 hour period. The effect of short day (SD) on quality changes in lettuce has previously been studied by Martinez-Sanchez *et al*. (2011). The actual observed average temperature was 1.4 °C as recorded with Tinytag™ temperature loggers (Gemini Data Loggers Ltd, UK). All measurements (gas composition, solute leakage, total ascorbic acid,

and total carotenoids and chlorophylls content, leaf colour) were taken on the harvesting day and then when samples were collected from storage.

4.2.2 Measurements

All measurements were taken following methods described in Chapter 2.

4.2.3 Statistical analyses

Each experiment was repeated twice with very similar results (verified by the Bartlett's homogeneity test and CV (%) values). Data are presented as mean values from two experiments that were used as blocks. Results were analysed using one-way ANOVA to identify the factors/treatments that had significant effect on quality changes during the storage of baby leaf spinach. Tukey's test was used to allow the comparisons between individual treatments. All statistical analyses were performed using GenStat 14th Edition software (Payne *et al*., 2010).

4.3 Results

4.3 a) Experiment 3

4.3.1 Leaf dry matter

Leaf dry matter at harvest was 6.1%. It did not change significantly throughout the storage period in spinach samples stored in the dark (6.2-6.4%) and under low intensity light (6.1-6.3%). A significant (P<0.001) increase in dry matter (7.1-7.2%), however, was observed already after 3 days of storage under high intensity light conditions. This was associated with enhanced water loss from these samples.

4.3.2 Gas composition

Light conditions during storage had a significant (P<0.001) effect on gas composition inside the bags with spinach leaves. In the case of spinach leaves stored at 1 °C under light (low intensity light, high intensity light) conditions, oxygen level remained high and carbon dioxide was not detected during 7 day storage period, suggesting that under both conditions respiration was compensated by photosynthetic activity of the leaves (Table 4.1). Decrease in oxygen level with simultaneous increase in carbon dioxide throughout the storage period was found in the bags stored at 1 °C in the dark. The concentration of CO_2 in those bags increased from 0.0% to 2.5% and 3.1% after 3 and 7 days of storage, respectively.

Table 4.1 Changes in the gas composition inside the bags with spinach stored for 7 days at 1 °C under different light conditions (dark (DK), low intensity light (LL) and high intensity light (HL)). Data represent mean values from 6 replicates.

Time of storage	Light conditions	O_2	CO_2
Day 0		20.9%	0.0%
Day 3	dark (DK)	17.7% b	2.5% a
	low intensity light (LL)	20.9% a	0.0% b
	high intensity (HL)	20.5% a	0.0% b
Day 7	dark (DK)	17.3% b	3.1% a
	low intensity light (LL)	20.9% a	0.0% b
	high intensity (HL)	20.6% a	0.0% b

within columns, for each day, different letters indicate that values are significantly different (P<0.05). O_2: 3 d (P<0.001; SEM=0.347, CV=2.2%), 7 d (P<0.001; SEM=0.423, CV=1.7%). CO_2: 3 d (P<0.001; SEM=0.244, CV=4.0%), 7 d (P<0.001; SEM=0.473, CV=5.2%).

4.3.3 Solute leakage

After 3 days of storage, solute leakage decreased significantly (P<0.05) from 4.6% (initial value) to 3.5% in samples stored under low intensity light at 1 °C (Figure 4.1), whereas no significant change was observed in samples stored at 1 °C either in the dark (4.3%) or under high intensity light (4.4%) conditions. After 7 days, a small increase in solute leakage was observed in the samples stored in the dark (4.8%) or under high intensity light (4.9%) conditions, while a small non-significant decrease occurred under low intensity light (3.2%).

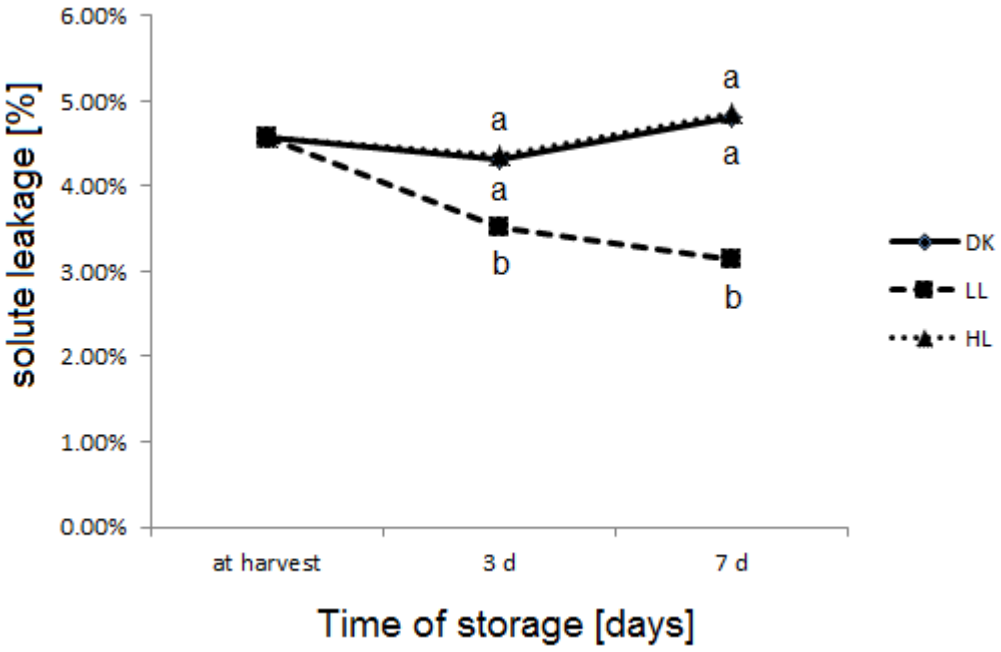

Figure 4.1 Solute leakage from spinach leaves stored for 7 days (d) at 1 °C under different light conditions (dark (DK), low intensity light (LL) and high intensity light (HL)). Solute leakage: 3 d (P=0.010; SEM=0.170, CV=4.1%), 7 d (P<0.001; SEM=0.232, CV=5.3%). Different letters indicate that values are significantly different (P<0.05) at each time point. Each data point is the mean of 6 replicates.

4.3.4 Total ascorbic acid (ascorbic acid (AsA) + dehydroascorbic acid (DHA))

The content of AsA in spinach leaves decreased during the 7 day storage period. A significant (P<0.001) decrease in AsA content from 4.12 mg g^{-1} DW (initial value) to 3.26 mg g^{-1} DW was already observed after 3 days in samples stored under high intensity light conditions, while no change took place in those stored in the dark or under low intensity light conditions, where AsA content was 4.14 and 4.16 mg g^{-1} DW, respectively (Table 4.2). After 7 days, AsA content decreased in all samples, the loss being more pronounced with increasing light intensity.

Table 4.2 The content of ascorbic acid (AsA) and dehydroascorbic acid (DHA) on a dry weight (DW) basis in spinach leaves stored for 7 days at 1 °C under three different light conditions (dark (DK), low intensity light (LL) and high intensity light (HL)). Data represent mean values from 6 replicates.

Light conditions	**AsA [mg/g DW]**			**DHA [mg/g DW]**		
	storage time [days]					
	0	3	7	0	3	7
DK	4.12	4.14 a	3.75 a	0.05	0.25 a	0.68 ab
LL		4.16 a	3.18 ab		0.31 a	1.04 a
HL		3.26 b	3.04 b		0.08 b	0.25 b
Significance:						
Treatment		<0.001	0.040		0.003	0.040
SEM		0.156	0.298		0.055	0.254
CV		3.0%	6.2%		11.5%	66.3%

within columns, for each day, different letters indicate that values are significantly different (P<0.05).

DHA content in spinach increased during the 7 day storage period. The lowest DHA content was observed in spinach leaves stored at 1 °C under high intensity light conditions (Table 4.2). After 3 days of storage, DHA content in these samples was 0.08 mg g^{-1} DW and was significantly (P=0.003) lower than in the counterparts stored in the dark (0.25 mg g^{-1} DW) or under low intensity light conditions (0.31 mg g^{-1} DW). After 7 days of storage DHA content increased in all samples (Table 4.2). The highest DHA content of 1.04 mg g^{-1} DW was found in the samples stored under low intensity light conditions.

In the case of total AsA content (AsA + DHA), the pattern was similar to the one observed for AsA (Figure 4.2). No difference in total AsA content was observed between spinach leaves stored in the dark and under low intensity light conditions. Total AsA content in those samples, however, was significantly (P<0.001) higher than in counterparts stored under high intensity light conditions (Figure 4.2).

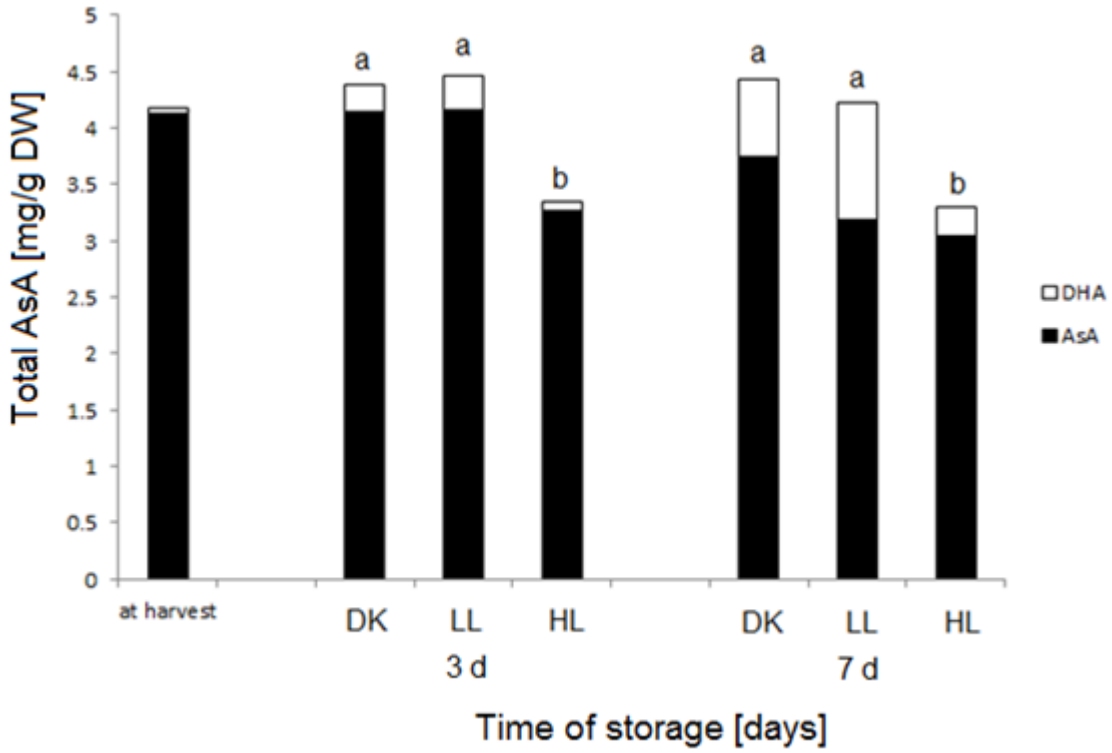

Figure 4.2 Changes in total AsA (AsA + DHA) content on a dry weight (DW) basis in spinach leaves stored for 7 days (d) at 1 °C under different light conditions (dark (DK), low intensity light (LL) and high intensity light (HL)). Total AsA: 3 d (P<0.001; SEM=0.132 CV=3.2%), 7 d (P<0.001; SEM=0.455, CV=11.1%). Different letters indicate that values are significantly different (P<0.05) at each time point. Each data point is the mean of 6 replicates.

4.3.5 Total carotenoid and chlorophyll content

After 3 days of storage total carotenoid content significantly (P=0.008) increased in spinach leaves stored at 1 °C under high intensity light conditions, while it remained relatively stable in the samples stored in the dark or under low intensity light conditions (Table 4.3). After 7 days, however, total carotenoid content increased in spinach leaves stored in the dark and under low intensity light conditions and was no longer significantly lower when compared with the samples stored under high intensity light conditions (Table 4.3).

Chlorophyll *a* content increased after 3 days of storage under high intensity light conditions, and was then significantly (P<0.001) higher when compared with the samples stored in the dark or under low intensity light conditions (Table 4.3). After 7 days, however, chlorophyll *a* content decreased in the samples stored in the light (both low

intensity light and high intensity light), while its content did not change in those stored in the dark. Thus, after 7 days the highest chlorophyll *a* content was observed in dark-stored samples. In the case of chlorophyll *b*, an increase was observed in all samples after 3 days of storage (Table 4.3). The highest increase being observed in spinach leaves stored under high intensity light conditions. After 7 days, however, chlorophyll *b* content decreased in the samples stored in the light, while its content did not change in those stored in the dark. Thus, after 7 days the highest chlorophyll *b* content was observed in dark-stored samples.

Table 4.3 Chlorophyll *a*, chlorophyll *b*, *a*: *b* ratio and total carotenoid content on a dry weight (DW) basis in spinach leaves stored for 7 days at 1 °C under different light conditions (dark (DK), low intensity light (LL) and high intensity light (HL)). Data represent mean values from 6 replicates.

Light conditions	**chlorophyll *a* [mg/g DW]**			**chlorophyll *b* [mg/g DW]**		
	storage time [days]					
	0	3	7	0	3	7
DK	17.75	19.85 b	20.20 a	4.63	5.28 b	5.27 a
LL		18.04 b	15.95 b		5.03 b	4.39 b
HL		23.10 a	18.54 ab		6.02 a	4.79 ab
Significance:						
Treatment		<0.001	0.038		0.001	0.007
SEM		0.868	1.523		0.203	0.237
CV		1.6%	6.2%		1.8%	6.3%
Light conditions	**chlorophyll *a: b* ratio**			**total carotenoids [mg/g DW]**		
	storage time [days]					
	0	3	7	0	3	7
DK	3.83	3.76 a	3.83 a	5.09	4.95 b	5.75 a
LL		3.59 a	3.63 a		4.90 b	5.32 a
HL		3.84 a	3.87 a		5.60 a	5.38 a
Significance:						
Treatment		0.317	0.324		0.008	0.076
SEM		0.164	0.313		0.200	0.202
CV		3.0%	6.2%		1.8%	6.3%

within columns, for each day, different letters indicate that values are significantly different ($P<0.05$).

4.3.6 Leaf colour changes

There was no significant difference (between light conditions) in leaf lightness (L^*) value after 3 days of storage. After 7 days, however, spinach leaves stored under light

conditions (low intensity light, high intensity light) were significantly lighter when compared with their counterparts stored in the dark (Table 4.4). There was no difference in greenness (*a**) value throughout the storage period (Table 4.4).

Table 4.4 Leaf colour changes during the storage of spinach leaves for 7 days at 1 °C under different light conditions (dark (DK), low intensity light (LL) and high intensity light (HL)). Data represent mean values from 6 replicates.

Light conditions	***L*(lightness)***		
	storage time [days]		
	0	3	7
DK	42.17	42.24 a	41.72 b
LL		41.93 a	43.23 a
HL		43.03 a	43.62 a
Significance:			
Treatment		0.189	0.003
SEM		0.541	0.603
CV		1.1%	0.9%
Light conditions	***a* (greenness)***		
	0	3	7
DK	-15.23	-15.15 a	-15.42 a
LL		-15.53 a	-16.01 a
HL		-15.74 a	-16.03 a
Significance:			
Treatment		0.101	0.097
SEM		0.288	0.336
CV		1.5%	1.7%
Light conditions	***b* (yellowness)***		
	0	3	7
DK	26.48	26.56 b	26.84 b
LL		26.95 ab	28.36 ab
HL		28.16 a	29.52 a
Significance:			
Treatment		0.051	0.001
SEM		0.647	0.764
CV		2.0%	2.0%

within columns, for each day, different letters indicate that values are significantly different (P<0.05).

Leaf yellowness (*b**) increased with increasing intensity of light during storage (Table 4.4). No significant difference in leaf yellowness was observed between spinach

leaves stored in the dark and under low intensity light conditions; however leaves stored under high intensity light conditions were significantly more yellow than dark-stored counterparts already after 3 days of storage (Figure 4.3).

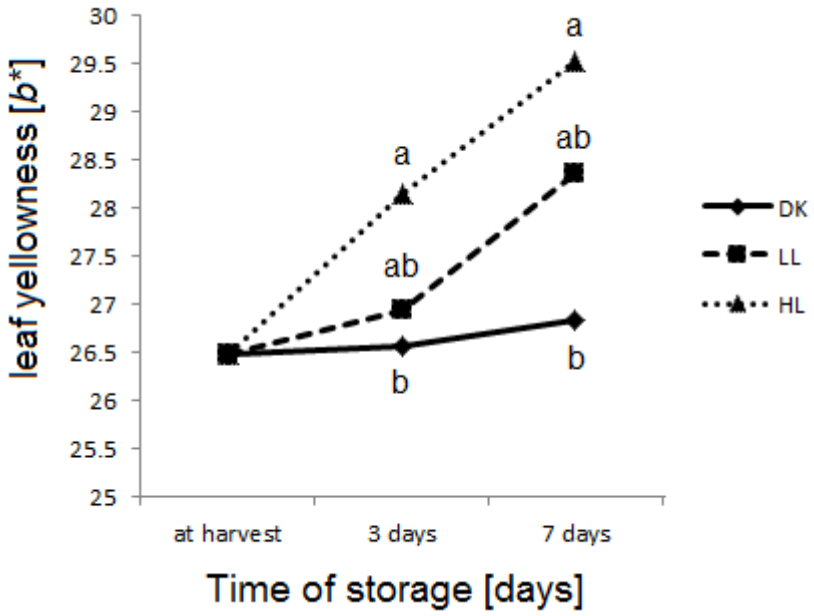

Figure 4.3 Changes in leaf yellowness value (*b**) during the storage of spinach leaves for 7 days at 1 °C under different light conditions (dark (DK), low intensity light (LL) and high intensity light (HL)). *b**: 3 d (*P*=0.051; SEM=0.647 CV=2.0%), 7 d (*P*=0.001; SEM=0.764, CV=2.0%). Different letters indicate that values are significantly different (*P*<0.05) at each time point. Each data point is the mean of 6 replicates.

4.3 b) Experiment 4

4.3.7 Leaf dry matter

Leaf dry matter at harvest was 6.9%. It did not change significantly throughout the storage period in spinach samples stored under continuous (24 hours) low intensity light conditions (6.7-6.9%) and those stored under SD (6 h of high intensity light followed by 18 h in the dark) conditions (6.7-7.3%).

4.3.8 Gas composition

No significant difference was found in gas composition inside the bags with spinach stored under continuous (24 hours) low intensity light and SD conditions (6 hours high intensity light/ 18 hours dark) during 10 days of storage at 1 °C, suggesting that

under both conditions respiration was compensated by photosynthetic activity of the leaves, thus no significant increase in CO_2 concentration was observed (Table 4.5).

Table 4.5 Changes in the gas composition inside the bags with spinach stored for 10 days at 1 °C under different light conditions (low intensity light (24 h) (LL (24 h)) and high intensity light/dark (6/18 h) (HL/DK (6/18 h)). Data represent mean values from 12 replicates.

Time of storage	Light conditions	O_2	CO_2
Day 0		20.6%	0.0%
Day 5	LL (24 h)	20.0% a	0.0% a
	HL / DK (6/18 h)	19.2% a	0.2% a
Day 10	LL (24 h)	20.4% a	0.0% a
	HL / DK (6/18 h)	20.5% a	0.0% a

within columns, for each day, different letters indicate that values are significantly different (P<0.05). O_2: 5 d (P=0.832; SEM=0.292, CV=3.7%), 10 d (P=0.846; SEM=0.195, CV=2.1%). CO_2: 5 d (P=0.827; SEM=0.027, CV=2.0%), 10 d (P=0.852; SEM=0.021, CV=1.4%).

4.3.9 Solute leakage

No significant difference was observed in solute leakage from spinach leaves stored under continuous (24 hours) low intensity light and SD (6 hours high intensity light/ 18 hours dark) during 10 days of storage at 1 °C. Only a slight, non-significant decrease from 0.6% (initial value) to 0.5% was reported after 5 days of storage for both treatments. After 10 days, solute leakage returned to initial level of 0.6% under both storage conditions.

4.3.10 Total ascorbic acid (ascorbic acid (AsA) + dehydroascorbic acid (DHA))

The content of AsA in the samples stored under continuous low intensity light increased significantly from 3.54 mg g^{-1} DW to 4.15 mg g^{-1} DW after 10 days of storage suggesting increased synthesis of AsA. On the other hand, AsA content did not change in samples stored under SD conditions. Thus, after 10 days of storage, AsA content in the samples stored under continuous low intensity light was significantly (P=0.003) higher than in those stored under SD conditions (Table 4.6). In contrast to AsA, the content of DHA did not change significantly over the storage period (Table 4.6).

Table 4.6 The content of ascorbic acid (AsA) and dehydroascorbic acid (DHA) on a dry weight (DW) basis in spinach leaves stored for 10 days at 1 °C under two different light conditions (low intensity light (24 h) (LL (24 h)) and high intensity light/dark (6/18 h) (HL/DK (6/18 h)). Data represent mean values from 12 replicates.

Light conditions	**AsA [mg/g DW]**			**DHA [mg/g DW]**		
	storage time [days]					
	0	5	10	0	5	10
LL (24 h)	3.54	3.94 a	4.15 a	0.41	0.41 a	0.46 a
HL / DK (6/18 h)		3.63 a	3.66 b		0.34 a	0.41 a
Significance:						
Treatment		0.130	0.003		0.386	0.164
SEM		0.138	0.113		0.054	0.023
CV		15.5%	17.3%		32.8%	25.7%

within columns, for each day, different letters indicate that values are significantly different (P<0.05).

No significant difference in either AsA or DHA was observed after 5 days of storage, however, due to the fact that the content of both compounds was slightly higher in the samples stored under continuous low intensity light, total AsA content was significantly (P=0.049) higher in these samples (Figure 4.4). After 10 days, due to an increase in AsA in the samples stored under continuous low intensity light, total AsA content in those samples was significantly (P=0.002) higher than in their counterparts stored under SD conditions.

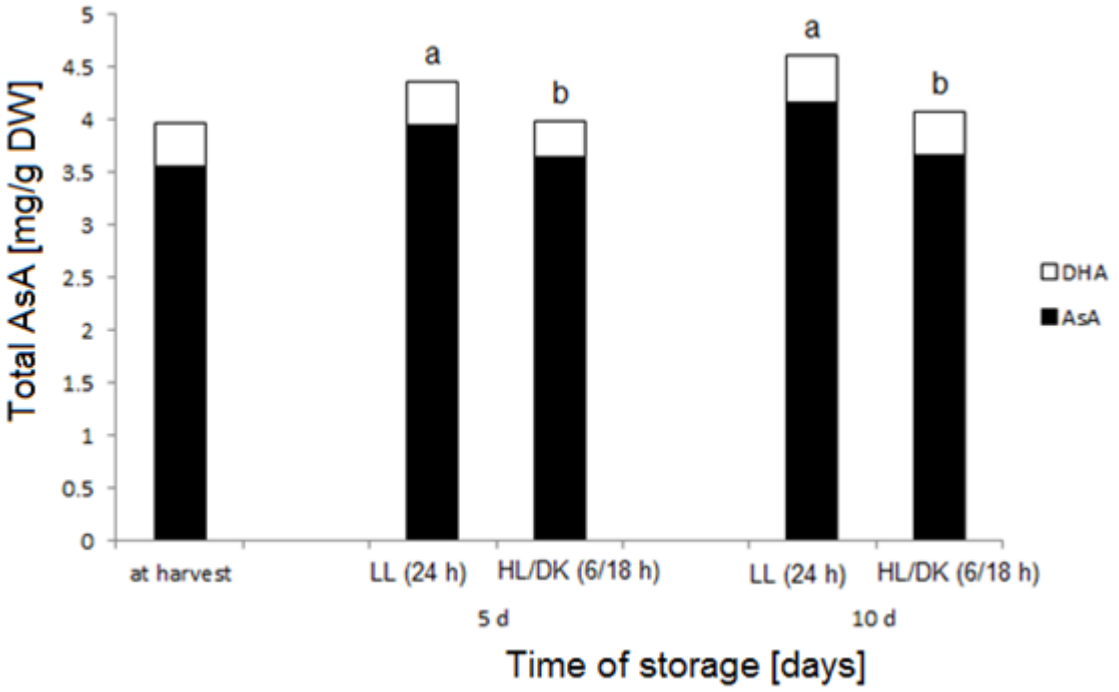

Figure 4.4 Changes in total AsA (AsA + DHA) content on a dry weight (DW) basis in spinach leaves stored for 10 days (d) at 1 °C under different light conditions (low intensity light (24 h) (LL (24 h)) and high intensity light/dark (6/18 h) (HL/DK (6/18 h)). Total AsA: 5 d (P<0.049; SEM=0.128 CV=13.1%), 10 d (P=0.002; SEM=0.117, CV=16.2%). Different letters indicate that values are significantly different (P<0.05) at each time point. Each data point is the mean of 12 replicates.

4.3.11 Total carotenoid and chlorophyll content

There was no significant difference in the content of total carotenoids, chlorophyll *a* and *b* between spinach samples stored under continuous low intensity light and those stored under SD conditions (Table 4.7).

Table 4.7 Chlorophyll *a*, chlorophyll *b*, ratio and total carotenoid content on a dry weight (DW) basis of spinach leaves stored for 10 days at 1 °C under different light conditions (low intensity light (24 h) (LL (24 h)) and high intensity light/dark (6/18 h) (HL/DK (6/18 h)). Data represent mean values from 12 replicates.

Light conditions	**chlorophyll *a* [mg/g DW]**			**chlorophyll *b* [mg/g DW]**		
	storage time [days]					
	0	5	10	0	5	10
LL (24 h)	9.61	8.95 a	9.02 a	2.72	2.70 a	2.76 a
HL / DK (6/18 h)		9.25 a	9.19 a		2.80 a	2.87 a
Significance:						
Treatment		0.173	0.344		0.195	0.077
SEM		0.575	0.169		0.383	0.147
CV		4.2%	8.7%		16.2%	2.9%
Light conditions	**chlorophyll *a: b* ratio**			**total carotenoids [mg/g DW]**		
	storage time [days]					
	0	5	10	0	5	10
LL (24 h)	3.53	3.32 a	3.27 a	3.11	3.17 a	3.32 a
HL / DK (6/18 h)		3.30 a	3.20 a		3.27 a	3.31 a
Significance:						
Treatment		0.312	0.746		0.308	0.860
SEM		0.104	0.146		0.093	0.230
CV		7.2%	9.3%		5.5%	9.8%

within columns, for each day, different letters indicate that values are significantly different (P<0.05).

Spinach leaves stored under continuous low intensity light were significantly lighter (L^*) than those stored under SD conditions after 5 days of storage (Table 4.8). This difference, however, was no longer present after 10 days of storage. No difference between two storage conditions was observed for leaf greenness (a^*) and leaf yellowness (b^*) values over the 10 day storage period (Table 4.8).

Table 4.8 Leaf colour changes during the storage of spinach leaves for 10 days at 1 °C under different light conditions (low intensity light (24 h) (LL (24 h)) and high intensity light/dark (6/18 h) (HL/DK (6/18 h)). Different letters indicate that values are significantly different (P<0.05). Data represent mean values from 12 replicates.

Light conditions	***L* (lightness)***		
	storage time [days]		
	0	5	10
LL (24 h)	46.84	48.36 a	47.97 a
HL / DK (6/18 h)		47.45 b	47.68 a
Significance:			
Treatment		<0.001	0.494
SEM		0.36	0.268
CV		5.5%	9.8%
Light conditions	***a* (greenness)***		
	0	5	10
LL (24 h)	-15.92	-16.34 a	-16.78 a
HL / DK (6/18 h)		-16.53 a	-16.71 a
Significance:			
Treatment		0.724	0.361
SEM		0.186	0.263
CV		11.6%	12.5%
Light conditions	***b* (yellowness)***		
	0	5	10
LL (24 h)	26.93	29.17 a	30.48 a
HL / DK (6/18 h)		29.43 a	30.47 a
Significance:			
Treatment		0.859	0.894
SEM		0.494	0.122
CV		4.6%	10.5%

within columns, for each day, different letters indicate that values are significantly different (P<0.05).

4.4 Discussion

Two experiments were conducted to determine the effect of light exposure on quality changes during the storage of spinach and to investigate whether the observed response was due to light intensity or the amount of light received by the leaves. Experiment 3 was conducted in October 2011, while Experiment 4 in July 2012. This could account for the differences in the quality of the leaves. Leaf dry matter content was significantly higher in Experiment 4. The quality of the leaves used in Experiment 3 was lower when compared with those used in Experiment 4. Texture of the leaves used in Experiment 3 (indicated by higher solute leakage at harvest) was worse than those used in Experiment 4. Plant pigment content was higher in spinach leaves used in Experiment 3; leaves used in Experiment 4 were significantly lighter and more yellow (at harvest) when compared with those used in Experiment 3. Finally, nutritional quality did not differ between experiments.

Gas composition

Previous studies have found a decrease in O_2 concentration with simultaneous increase in CO_2 concentration already after 3 days of storage of spinach at 5 °C (Allende *et al.*, 2004b; Conte *et al*., 2008) and 7 °C (Tudela *et al*., 2013) in the dark. In agreement with those studies CO_2 development was also observed in our study, in bags with spinach stored in the dark. In the light-stored counterparts, however, no change in the gas composition was observed, suggesting that respiration was compensated by photosynthetic activity of the leaves (Toledo *et al.*, 2003a). Similarly, the amount of CO_2 produced, as a result of respiration, was used in the photosynthesis by spinach leaves stored under SD conditions (6 h light/18 h dark). Monitoring the changes in gas composition inside the bags with spinach is a useful indicator of the balance between photosynthesis and respiration. Increase in respiration, as indicated by higher CO_2 development would indicate tissue deterioration.

The increase in dry matter content was only observed in the spinach samples stored under continuous high light conditions. This increase resulted from enhanced water loss which was probably associated with higher numbers of stomata that remained open

in light-stored leaves when compared with dark-stored samples as has previously been reported by others (Noichinda *et al.*, 2007, Martinez-Sanchez *et al.*, 2011).

Texture

Excess water loss leads to a loss of turgor and thus the decrease in textural quality of the leaf (Martin-Diana *et al.*, 2006, Wagstaff *et al.*, 2007, Aguero *et al.*, 2008). In agreement with other studies (Kar and Choudhuri, 1986, Martinez-Sanchez *et al.*, 2011), solute leakage from spinach leaves was reduced by exposure to low intensity light when compared with dark-stored samples. No difference in solute leakage was found between samples stored under continuous low intensity light and those stored under SD conditions. This would suggest that, it is the amount of light received by the leaf rather than the light intensity that is responsible for reducing the solute leakage. The stability of cell walls (Wagstaff *et al*., 2010) was probably better maintained in spinach leaves that received certain amount of light during storage. No benefit of light exposure during storage, however, was noticed when light level/amount was too high. Too much light probably can lead to excess oxidative stress that caused tissue damage (Foyer and Shigeoka, 2011).

Nutritional quality

In our study, there was no significant difference in the content of AsA between samples stored in the dark and under continuous low intensity light. This is in contrast with other studies (Toledo *et al.*, 2003b, Lester *et al.*, 2010b, Zhan *et al.*, 2013) that reported better retention of AsA in light-stored samples. It is important to note that here AsA content is reported on dry weight (DW) basis while others (Toledo *et al.*, 2003b, Zhan *et al.*, 2013) reported its content on fresh weight (FW) basis, meaning that loss of leaf water will have a significant influence on AsA content and changes may be due to the water loss rather than physiological changes in AsA biosynthesis. Higher FW loss was indeed observed during the storage of leafy vegetables under light conditions when compared with dark-stored counterparts (Sanz *et al.*, 2008, Martinez-Sanchez *et al.*, 2011, Zhan *et al.*, 2012, Zhan *et al.*, 2013). Thus, it could be expected that difference in AsA content between light and dark-stored samples would be smaller when expressed on DW basis (Bergquist *et al.*, 2006). Furthermore, seasonal (Barbieri *et al.*, 2011) and cultivar (Lester

et al., 2010b) differences have been reported. Lester *et al*. (2010b) observed higher AsA content in light-stored spinach in cultivar Lazio, but not in the case of cultivar Samish, where no difference in AsA content between two storage conditions was found for top- and medium-canopy leaves after 9 days of storage. These cultivar differences may explain why no difference in AsA content between samples stored in the dark and under low intensity light was found in our study. On the other hand, significant decrease in AsA content was found under high intensity light conditions (Experiment 3), suggesting that if the level of oxidative stress is too high, nutritional quality of spinach leaves will be reduced. Exposure of spinach leaves to high intensity light probably leads to increase in AOS, which then need to be scavenged by AsA. Thus, the more AOS is produced, more AsA needs to be oxidised. This was further confirmed in Experiment 4, where only 6 h exposure to high intensity light resulted in lower AsA content after 10 days of storage at 1 °C when compared with samples stored under continuous low intensity light conditions.

It has been reported by several authors (Lester *et al.*, 2010b, Martinez-Sanchez *et al.*, 2011, Zhan *et al.*, 2013) that the loss in AsA may be compensated by the increase in DHA. This was indeed the case in spinach leaves stored in the dark or under continuous low intensity light (Experiment 3), where decline in AsA content was associated with an increase in DHA. No difference in total AsA was observed in our study between spinach leaves stored in the dark and those stored under low intensity light conditions. This is in agreement with others who also did not report differences in total AsA during the storage of spinach (Lester *et al.*, 2010b) and lettuce (Martinez-Sanchez *et al.*, 2011), respectively. Decline in total AsA was observed in spinach leaves stored under continuous high intensity light conditions, which suggests that a certain amount of total AsA was lost, probably due to DHA being degraded further *e.g*. to 2, 3-diketogulonic acid. Interestingly, total AsA content remained relatively stable during 10 day storage under SD conditions, while an increase was observed under continuous low intensity light. This increase was associated with biosynthesis of AsA, as DHA content in those samples was retained. The different response of spinach leaves stored under continuous low intensity light between Experiment 3 and 4 would suggest that changes in nutritional quality during the storage of

spinach leaves are influenced by pre-harvest conditions, *e.g*. the amount of light received during the growing season. It could be possible that leaves used in Experiment 3 (harvested in October) were more sensitive to the light during storage probably because during their growth they received far less light than leaves used in Experiment 4 (harvested in July).

Total carotenoid content was found to be relatively stable under all storage conditions tested. This is in agreement with Bergquist *et al*. (2006) and Lester *et al*. (2010b) who reported carotenoids to be relatively stable or even increase during the storage of spinach. Kopas-Lane and Warthesen (1995), however, have reported enhanced carotenoids degradation in light-stored spinach. It is important to note that total carotenoid content reported in this study and those of Bergquist *et al*. (2006) and Lester *et al*. (2010b) was on DW basis while Kopas-Lane and Warthesen (1995) reported them on FW basis, whereas the water loss during storage is significantly higher in light-stored samples.

Visual quality

Chlorophyll content decreased in light-stored samples when compared with dark-stored counterparts. This is in agreement with Kopas-Lane and Warthesen (1995) and Ferrante *et al*. (2004) who demonstrated that chlorophyll degradation was enhanced by light exposure during the storage of spinach and rocket leaves, respectively.

Conte *et al*. (2008) did not observe any changes in colour of spinach leaves stored in the dark at 5 °C for 13 days. Similar to their study; no changes in leaf colour was observed, in this work in dark stored spinach leaves. With increasing light intensity, however, spinach leaves became significantly lighter and more yellow. Similar observations have previously been reported by Toledo *et al*. (2003b) who found that yellowing of spinach leaves stored at 8 °C was faster in the case of light-stored leaves when compared with their dark-stored counterparts. According to these authors leaves may become more yellow due to chlorophyll loss with simultaneous retention of carotenoids - change in the chlorophyll: carotenoids ratio. Stronger colour alteration has

also been observed in light-stored lettuce (Martinez-Sanchez *et al.*, 2011) when compared with dark-stored counterparts.

Effect of photoperiod

No differences in the quality of lettuce stored under continuous (24 hours) low intensity light and photoperiod (low intensity light/ dark (12/12 h)) were reported by Martinez-Sanchez *et al*. (2011) with respect to gas composition, visual quality, leaf colour changes, solute leakage, AsA, DHA and total AsA. Similar to their study, no differences were observed in our study in all these parameters, with an exception of AsA and total AsA. The concentration of AsA and total AsA was lower in spinach samples stored under SD conditions (high intensity light/ dark (6/18 h)). This suggests that even relatively short exposure to high intensity light may lead to an excess oxidative stress that accelerates AsA degradation, thus reducing nutritional quality of spinach leaves.

The findings from Experiment 3 and 4 suggest that exposure to continuous low intensity light may improve texture maintenance of spinach leaves. This could be associated with photosynthesis that still occurs during postharvest storage. Light intensity, however, has to be low enough not to cause excess oxidative stress, which would lead to accelerated senescence. As has been shown in Experiment 4, no significant difference in textural and visual quality was observed between samples stored under continuous low intensity light and those stored under photoperiod conditions. Nutritional quality (AsA and total AsA content), however, was better if samples were stored under continuous low intensity light.

Nutritional quality was the only parameter affected by photoperiod, whereas light intensity was found to affect texture, plant pigment content, leaf colour and nutritional quality of spinach. Thus, light intensity seems to be more important than the total amount of light received by the leaf. It is clear, that light intensity should be adjusted accordingly during spinach displayed shelf-life. Where possible, retailers should keep spinach at refrigerated temperature under continuous low intensity light.

The null hypothesis tested in this research has to be rejected as it has been found

that all parameters that were measured were affected by changes in light conditions during storage.

4.5 Conclusions

Experiment 3 has shown that nutritional content of the leaves (AsA, DHA, total AsA, and total carotenoids) was preserved during the storage of spinach under continuous low intensity light conditions. Low intensity light exposure during storage also prevented development of CO_2 in the bags; this would reduce the development of off-odours. Furthermore, solute leakage, which indicates membrane damage/texture loss, was reduced in the samples stored under continuous low intensity light. On the other hand, decrease in chlorophyll content in light-stored spinach leaves resulted in leaves being lighter and more yellow when compared with their dark-stored counterparts. Although this difference was detected using the chroma meter, it would be almost impossible to notice from the consumer point of view.

Experiment 4 has investigated the effect of photoperiod (6 h of high intensity light/ 18 h in the dark) in comparison with continuous (24 h) low intensity light on quality changes of spinach. The total amount of light received by the leaf in both treatments was very similar. Nutritional quality of the leaves stored under SD conditions was reduced, while no other differences were found between the treatments. This decrease was due to oxidative stress induced by high intensity light. This finding suggests that it is light intensity rather than total amount of light received by the leaf that will affect its quality during postharvest storage.

Overall, storage of spinach leaves under continuous low intensity light conditions seems to be beneficial for spinach quality by improving its texture without any loss in the nutritional quality of the product. The main focus for future research should be on optimizing light intensity/level during displayed shelf-life of spinach.

Chapter 5 Influence of temperature and light on quality of baby leaf spinach

5.1 Introduction

Leafy vegetables are exposed to various temperature and light conditions during their displayed shelf-life. The shelf-life of spinach is relatively short (7-10 days) and, similar to other leafy vegetables, is influenced by initial quality at harvest (Wagstaff *et al.*, 2010) and subsequent storage conditions (Piagentini *et al.*, 2005). Thus, it is not surprising that, in recent years, there has been an increasing interest in studying the effects of temperature (Pandrangi and LaBorde, 2004, Bergquist *et al.*, 2006) and light (Lester *et al.*, 2010b) on changes in the quality of spinach during storage.

It has already been demonstrated that the quality of spinach is affected by both temperature (Chapter 3) and light conditions (Chapter 4) during storage. Quality of spinach was found to decline with increasing temperature of storage and light intensity. In both studies only one of the factors varied, *i.e*. in Chapter 3 a range of temperatures (1, 6, 10 and 20 °C) was used, while light conditions (low intensity (30-35 μmol m^{-2} s^{-1}) light) were the same; in Chapter 4 a range of light conditions (dark, low intensity (30-35 μmol m^{-2} s^{-1}) and high intensity (130-140 μmol m^{-2} s^{-1}) light) was used at one storage temperature (1 °C).

It is not known if changes in the quality of spinach leaves would respond in the same way over the range of temperatures (1 and 10 °C) if stored under different light conditions (low intensity (30-35 μmol m^{-2} s^{-1}) and high intensity (130-140 μmol m^{-2} s^{-1}) light). A key question is whether the effect is additive or is there an interaction between storage temperature and light conditions?

Experiment 5 was conducted to investigate the effect of temperature and light conditions during storage on quality changes of spinach. In contrast to Chapter 3 and 4 both factors varied (2 x 2 factorial experiment). Bagged spinach was stored under two different light levels (under continuous low (30-35 μmol m^{-2} s^{-1}) or high (130-140 μmol m^{-2} s^{-1}) intensity light) at either 1 or 10 °C. These treatments were chosen to (i) determine the effect of light intensity on quality changes during the storage of spinach at two different

temperatures, (ii) to investigate how the quality of spinach stored under two light intensities changes with increasing temperature of storage, (iii) to determine whether there is an interaction between both factors, and (iv) to identify which of these two factors has larger effect on quality maintenance of spinach.

The following null hypothesis was tested: temperature and light conditions affect the maintenance of nutritional, textural and/or visual quality characteristics of baby leaf spinach independent of each other.

5.2 Materials and Methods

5.2.1 Plant material and handling

Spinach used in Experiment 5 was harvested on 12th of August 2011 and 10th of August 2012. Spinach was bagged at PDM Produce Ltd and transported to the laboratory (~15 minutes) in insulated opaque containers as described in section 2.1.

In Experiment 5, bags with baby leaf spinach were kept under two different light conditions – continuous (24 hours) low intensity (30-35 μmol m^{-2} s^{-1}) or high intensity (130-140 μmol m^{-2} s^{-1}) light at 1±1 °C or 10±1 °C for 7 days. These treatments have already been used in Chapter 3 and 4. The actual observed average temperatures were 1.1-1.3 and 9.7-10.0 °C as recorded with Tinytag™ temperature loggers (Gemini Data Loggers Ltd, UK). The photosynthetically active radiation (PAR) was measured with quantum sensor (Skye Instruments Ltd, UK). Spinach leaves were prepared for further analyses (gas composition analyses, solute leakage, total ascorbic acid, and total carotenoids and chlorophyll content, leaf colour) on the harvesting day and after 3 and 7 days of storage, respectively.

5.2.2 Measurements

All measurements were taken following methods described in Chapter 2.

5.2.3 Statistical analyses

Experiment was repeated twice with very similar results (verified by the Bartlett's homogeneity test and CV (%) values). Data are presented as mean values from two experiments that were used as blocks. Results were analysed using one-way ANOVA and two-way ANOVA to identify the treatments/factors that had significant effect on quality of baby leaf spinach. Tukey's test was used to allow the comparisons between individual treatments. All statistical analyses were performed using GenStat 14th Edition software (Payne *et al*., 2010).

5.3 Results

5.3 Experiment 5

5.3.1 Leaf dry matter

Leaf dry matter at harvest was 6.7%. After 3 days of storage, dry matter remained in the range 6.9-7.2% for all the treatments. A significant (P<0.001) difference in dry matter between treatments was observed after 7 days of storage. In spinach stored at 1 °C under low intensity and high intensity light dry matter was in the range 6.8-7.1% and 6.9-7.3%, respectively. In samples stored at 10 °C under low intensity light dry matter was in the range 7.0-7.4% and was slightly but non-significantly higher, whereas significantly higher dry matter of 7.4-8.1% was found in samples stored at 10 °C under high intensity light. Changes in dry matter are associated with water loss.

5.3.2 Gas composition

The gas composition for all the treatments did not change during the 7 day storage period (Table 5.1). In the case of spinach leaves stored at either 1 or 10 °C under light (low intensity light, high intensity light) conditions, oxygen level remained high and carbon dioxide was not detected.

Table 5.1 Changes in the gas composition inside the bags with spinach stored for 7 days at 1 or 10 °C under different light conditions (low intensity light (LL) and high intensity light (HL)). Data represent mean values from 6 replicates.

Time of storage	Light conditions	Temperature	O_2	CO_2
Day 0			20.9%	0.0%
Day 3	low intensity light (LL)	1 °C	20.7% a	0.0% a
	low intensity light (LL)	10 °C	20.9% a	0.0% a
	high intensity light (HL)	1 °C	20.5% a	0.0% a
	high intensity light (HL)	10 °C	20.7% a	0.0% a
Day 7	low intensity light (LL)	1 °C	20.9% a	0.0% a
	low intensity light (LL)	10 °C	20.6% a	0.0% a
	high intensity light (HL)	1 °C	20.7% a	0.0% a
	high intensity light (HL)	10 °C	20.9% a	0.0% a

within columns, for each day, different letters indicate that values are significantly different (P<0.05). O_2: 3 d (P=0.802; SEM=0.301, CV=3.9%), 7 d (P=0.852; SEM=0.295, CV=2.5%).

5.3.3 Solute leakage

After 3 days of storage, there was no significant difference in solute leakage between the treatments (Figure 5.1). For all the treatments (LL at 1 °C, LL at 10 °C, HL at 1 °C and HL at 10 °C) solute leakage on average was in the range of 1.2 – 1.3%. After 7 days of storage, solute leakage significantly increased in all treatments, with the exception of samples stored at 1 °C under low intensity light conditions. Solute leakage increased with increasing temperature of storage and increasing light intensity (Figure 5.1). Thus, the highest solute leakage of 4.1% was observed in samples stored at 10 °C under high intensity light, while the lowest (1.5%) at 1 °C under low intensity light.

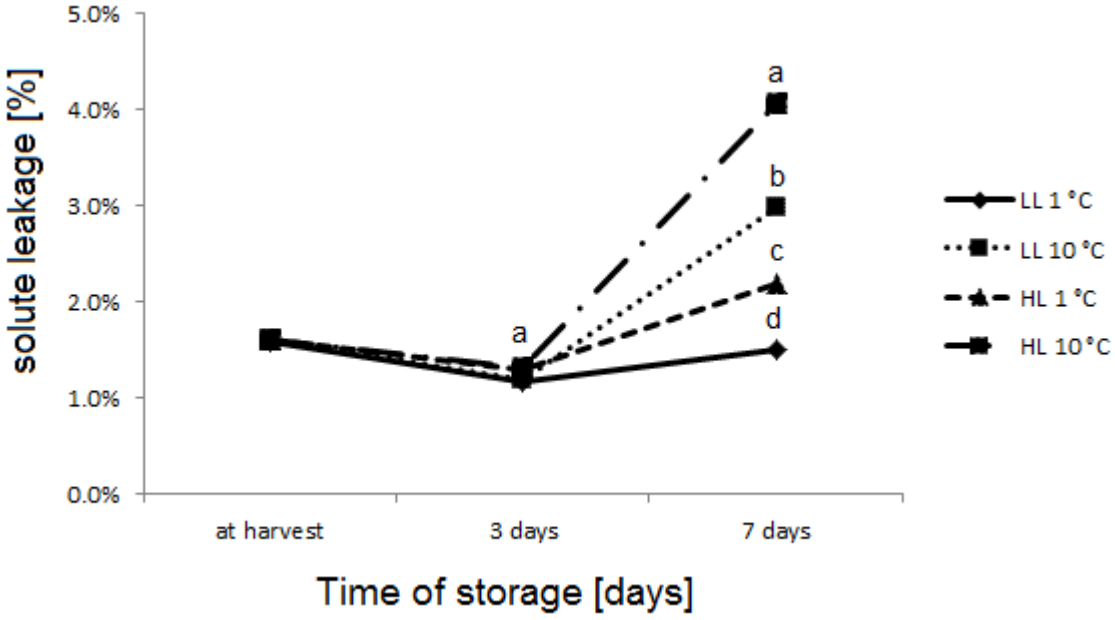

Figure 5.1 Solute leakage from spinach leaves stored for 7 days (d) at 1 or 10 °C under different light conditions (low intensity light (LL) and high intensity light (HL)). Solute leakage: 3 d (P=0.362; SEM=0.370, CV=9.7%), 7 d (P<0.001; SEM=0.169, CV=5.3%). Different letters indicate that values are significantly different (P<0.05) at each time point. Each data point is the mean of 6 replicates.

5.3.4 Total ascorbic acid (ascorbic acid (AsA) + dehydroascorbic acid (DHA))

Temperature had a main effect on AsA content after 3 days of storage, and the content of AsA significantly (P<0.001) decreased in samples stored at 10 °C when compared with their counterparts stored at 1 °C (Table 5.2), while no effect of light intensity was observed. After 7 days, however, both temperature (P<0.001) and light (P=0.022) had a significant effect on AsA content in spinach. The highest AsA content of 2.16 mg g^{-1} DW was observed in the samples stored at 1 °C under low intensity light. The content of AsA significantly decreased (1.63 mg g^{-1} DW) when samples were stored at the same temperature under high intensity light conditions. Even greater AsA loss was found in the samples stored at 10 °C (Table 5.2). A significant interaction (P=0.035) was found between temperature and light conditions after 7 days of storage. This was due to AsA content being higher at 1 °C under low intensity light when compared with their counterparts stored at 1 °C under high intensity light, while no difference between the light conditions was observed at 10 °C (Table 5.2), where AsA content was 0.65 and 0.67 mg g^{-1} DW in the samples stored under low intensity and high intensity light, respectively.

Table 5.2 The content of ascorbic acid (AsA) and dehydroascorbic acid (DHA) on a dry weight (DW) basis in spinach leaves stored for 7 days at 1 or 10 °C under different light conditions (low intensity light (LL) and high intensity light (HL)). Data represent mean values from 6 replicates.

Light conditions	**Temperature**	**AsA [mg/g DW]**			**DHA [mg/g DW]**		
		storage time [days]					
		0	3	7	0	3	7
LL	1 °C	2.81	2.35 a	2.16 a	0.19	0.16 a	0.47 a
LL	10 °C		1.39 b	0.65 c		0.14 a	0.39 ab
HL	1 °C		2.40 a	1.63 b		0.21 a	0.19 b
HL	10 °C		1.53 b	0.67 c		0.24 a	0.46 a

Storage time [days]	**Factor**		Mean	SEM	*P*	Mean	SEM	*P*
3	Light conditions	LL	1.87	0.062	0.317	0.15	0.018	0.009
		HL	1.96			0.23		
	Temperature	1 °C	2.37		<0.001	0.19		0.888
		10 °C	1.46			0.19		
Significance of interaction:								
Light conditions x Temperature				0.087	0.634		0.026	0.324
			CV		13.7%	CV		41.4%
Storage time [days]	**Factor**		Mean	SEM	*P*	Mean	SEM	*P*
7	Light conditions	LL	1.41	0.082	0.022	0.43	0.060	0.080
		HL	1.15			0.33		
	Temperature	1 °C	1.90		<0.001	0.33		0.139
		10 °C	0.66			0.42		
Significance of interaction:								
Light conditions x Temperature				0.116	0.035		0.085	0.008
			CV		27.3%	CV		19.7%

within columns, for each day, different letters indicate that values are significantly different (P<0.05).

After 3 days of storage, there was no significant difference in DHA content between the treatments. After 7 days, a significant interaction (P=0.008) was found between temperature and light conditions. The lowest DHA content of 0.19 mg g^{-1} DW was found in the samples stored at 1 °C under high intensity light (Table 5.2). DHA content in these samples was lower than in other samples, where DHA content increased.

In the case of total AsA content (AsA + DHA), the pattern was similar to the one observed for AsA (Figure 5.2). No difference in total AsA was observed after 3 d of storage between spinach leaves stored under two light conditions. The content of total AsA significantly (P<0.001) decreased in samples stored at 10 °C when compared with their counterparts stored at 1 °C (Figure 5.2). After 7 days, however, both temperature (P<0.001) and light (P=0.049) had a significant effect on total AsA content in spinach. The highest total AsA content of 2.63 mg g^{-1} DW was observed in the samples stored at 1 °C under low intensity light. The content of total AsA significantly decreased (1.82 mg g^{-1} DW) when samples were stored at the same temperature under high intensity light conditions. Even greater total AsA loss was found in the samples stored at 10 °C (Figure 5.2).

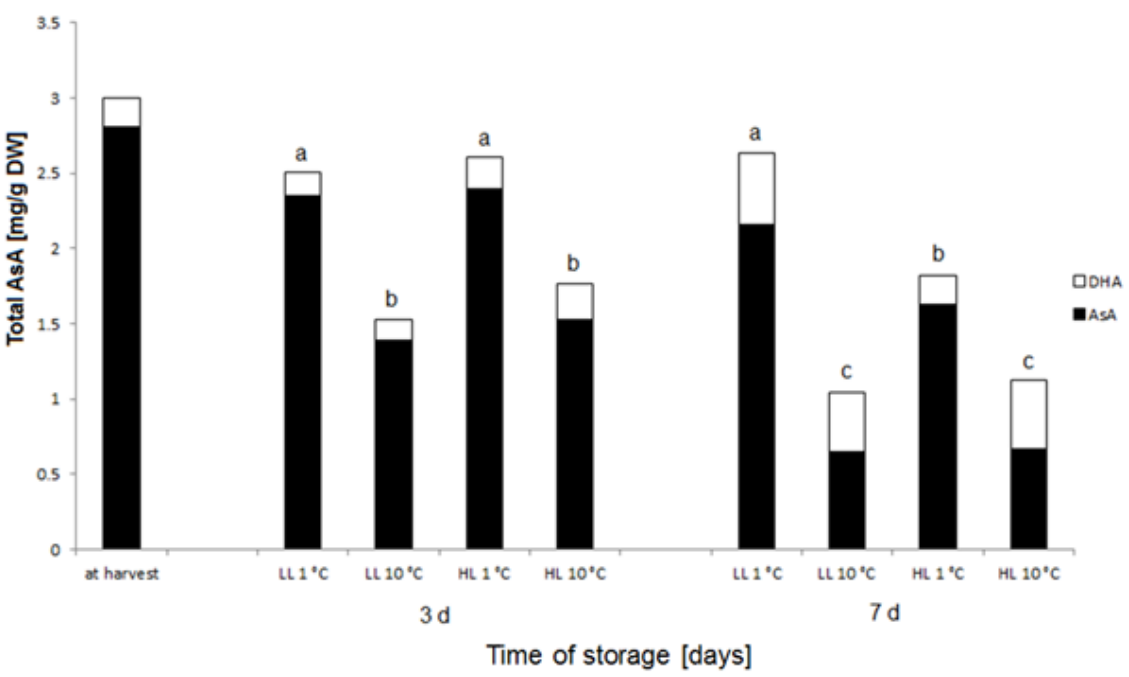

Figure 5.2 Changes in total AsA (AsA + DHA) content on a dry weight (DW) basis in spinach leaves stored for 7 days (d) at 1 or 10 °C under different light conditions (low intensity light (LL) and high intensity light (HL)). Total AsA: 3 d (P<0.001; SEM=0.199, CV=13.2%), 7 d (P<0.001; SEM=0.129, CV=11.3%). Different letters indicate that values are significantly different (P<0.05). Each data point is the mean of 6 replicates.

A significant interaction (P=0.016) was found between temperature and light conditions after 7 days of storage. This was due to total AsA content being higher at 1 °C under low intensity light when compared with their counterparts stored at 1 °C under high

intensity light, while no difference between the light conditions was observed at 10 °C (Figure 5.2), where total AsA content was 1.04 and 1.13 mg g^{-1} DW in the samples stored under low intensity and high intensity light, respectively.

5.3.5 Total carotenoid and chlorophyll content

After 3 days of storage, there was no significant difference in total carotenoid content between the treatments (Table 5.3). Total carotenoid content in all treatments was in the range of 2.86 – 3.21 mg g^{-1} DW. After 7 days of storage, both temperature (P=0.023) and light (P=0.012) had a significant effect on total carotenoid content (Table 5.3). Total carotenoid content remained relatively stable in all treatments, with an exception of spinach leaves stored at 10 °C under high intensity light condition, where total carotenoid content decreased to 1.89 mg g^{-1} DW.

Table 5.3 Total carotenoid content on a dry weight (DW) basis in spinach leaves stored for 7 days at 1 or 10 °C under different light conditions (low intensity light (LL) and high intensity light (HL)). Data represent mean values from 6 replicates.

Light conditions	**Temperature**		**total carotenoids [mg/g DW]**		
			storage time [days]		
			0	3	7
LL	1 °C		3.16	3.21 a	2.70 a
LL	10 °C			2.87 a	2.49 a
HL	1 °C			2.93 a	2.69 a
HL	10 °C			2.86 a	1.89 b
Storage time [days]	**Factor**		Mean	SEM	*P*
3	Light conditions	LL	3.04	0.131	0.981
		HL	2.90		
	Temperature	1 °C	3.07		0.210
		10 °C	2.87		
Significance of interaction:					
Light conditions x Temperature				0.227	0.213
			CV		10.4%
Storage time [days]	**Factor**		Mean	SEM	*P*
7	Light conditions	LL	2.60	0.093	0.012
		HL	2.29		
	Temperature	1 °C	2.70		0.023
		10 °C	2.19		
Significance of interaction:					
Light conditions x Temperature				0.247	0.185
			CV		9.0%

within columns, for each day, different letters indicate that values are significantly different (P<0.05).

After 3 days of storage, chlorophyll *a* content declined in all samples except the one stored at 1 °C under low intensity light (Table 5.4). Thus, chlorophyll *a* content was found to decrease significantly with increasing temperature (P<0.001) and light intensity (P=0.006). After 7 days, no significant difference in chlorophyll *a* content was found between samples stored at 1 °C under low intensity and high intensity light conditions. The content of chlorophyll *a* in the samples stored at 10 °C under low intensity light was significantly lower than in those mentioned above. A much greater decline in chlorophyll *a*

content was observed in the samples stored at 10 °C under high intensity light conditions (Table 5.4).

After 3 days of storage, chlorophyll *b* content declined in all samples except those stored at 1 °C under low intensity light (Table 5.4). Thus, chlorophyll *b* content was found to decrease significantly with increasing temperature (P=0.001) and light intensity (P=0.004). After 7 days, no significant difference in chlorophyll *b* content was found between samples stored at 1 °C under low intensity and high intensity light and those stored at 10 °C under low intensity light conditions. Decline in chlorophyll *b* content, however, was observed in the samples stored at 10 °C under high intensity light conditions (Table 5.4).

Table 5.4 Chlorophyll *a* and chlorophyll *b* content on a dry weight (DW) basis in spinach leaves stored for 7 days at 1 or 10 °C under different light conditions (low intensity light (LL) and high intensity light (HL)). Different letters indicate that values are significantly different (P<0.05). Data represent mean values from 6 replicates.

Light conditions	Temperature		chlorophyll *a* [mg/g DW]			chlorophyll *b* [mg/g DW]			chl *a*: *b* ratio		
			storage time [days]			storage time [days]			storage time [days]		
			0	3	7	0	3	7	0	3	7
LL	1 °C		11.61	11.31 a	8.28 a	2.98	3.1 a	2.28 a	3.90	3.65 a	3.63 a
LL	10 °C			9.22 b	7.48 b		2.60 b	2.25 a		3.55 a	3.32 a
HL	1 °C			9.91 b	8.22 a		2.58 b	2.26 a		3.84 a	3.64 a
HL	10 °C			9.04 b	5.39 c		2.52 b	1.60 b		3.59 a	3.37 a
Storage time [days]	**Factor**		Mean	SEM	*P*	Mean	SEM	*P*	Mean	SEM	*P*
3	Light conditions	LL	10.27	0.240	0.006	2.85	0.071	0.001	3.60	0.210	0.711
		HL	9.48			2.55			3.72		
	Temperature	1 °C	10.61		<0.001	2.84		0.004	3.75		0.581
		10 °C	9.13			2.56			3.57		
Significance of interaction:											
Light conditions x Temperature				0.35	0.958		0.143	0.756		0.33	0.832
			CV		10.3%	CV		10.6%	CV		8.3%
Storage time [days]	**Factor**		Mean	SEM	*P*	Mean	SEM	*P*	Mean	SEM	*P*
7	Light conditions	LL	7.88	0.350	<0.001	2.27	0.101	<0.001	3.48	0.190	0.892
		HL	6.81			1.93			3.51		
	Temperature	1 °C	8.25		<0.001	2.27		0.02	3.64		0.481
		10 °C	6.44			1.93			3.35		
Significance of interaction:											
Light conditions x Temperature				0.480	0.666		0.203	0.712		0.310	0.715
			CV		8.5%	CV		8.2%	CV		5.6%

5.3.6 Leaf colour changes

After 3 days of storage, leaf lightness (L^*) value increased significantly (P=0.002) with increasing temperature of storage (Table 5.5). On the other hand, there was no significant difference in leaf lightness between the light conditions. After 7 days of storage, both temperature (P<0.001) and light intensity (P=0.026) had a significant effect on leaf lightness. Spinach leaves became lighter with increasing temperature of storage and light intensity. A significant interaction (P=0.014) was found between temperature and light conditions after 7 days of storage. This was due to leaf lightness being higher at 10 °C under high intensity light when compared with their counterparts stored at 10 °C under low intensity light, while no difference between the light conditions was observed at 1 °C (Table 5.5).

After 3 days of storage, there was no significant difference in leaf greenness (a^*) value between all treatments stored at 1 or 10 °C under low intensity or high intensity light (Table 5.5). After 7 days, the greenness value was significantly ($P<0.001$) lower in the samples stored at 10 °C when compared with their counterparts stored at 1 °C. A significant interaction ($P=0.029$) was found between temperature and light conditions after 7 days of storage. This was due to leaf greenness being higher at 10 °C under high intensity light when compared with their counterparts stored at 10 °C under low intensity light, while no difference between the light conditions was observed at 1 °C (Table 5.5).

Table 5.5 Leaf colour changes during the storage of spinach at 1 or 10 °C under different light conditions (low intensity (LL) and high intensity (HL) light). Data represent mean values from 6 replicates.

Temperature	Light conditions		*L** (lightness)			*a** (greenness)			*b** (yellowness)		
			storage time [days]								
			0	3	7	0	3	7	0	3	7
1 °C	LL		44.30	44.28 b	45.15 c	-14.33	-14.78 a	-14.63 a	22.58	23.63 c	24.80 c
10 °C	LL			45.54 a	48.70 b		-15.07 a	-15.21 ab		24.93 b	29.97 b
1 °C	HL			44.41 b	45.02 c		-14.84 a	-14.83 a		24.09 c	24.18 c
10 °C	HL			45.35 a	51.23 a		-15.43 a	-15.72 b		25.85 a	34.94 a
Storage time [days]	**Factor**		Mean	SEM	*P*	Mean	SEM	*P*	Mean	SEM	*P*
3	Light conditions	LL	44.91	0.246	0.923	-14.93	0.188	0.433	24.28	0.408	0.232
		HL	44.88			-15.14			24.97		
	Temperature	1 °C	44.35		0.002	-14.81		0.105	23.86		0.009
		10 °C	45.45			-15.25			25.39		
Significance of interaction:											
Light conditions x Temperature				0.348	0.655		0.266	0.575		0.577	0.692
			CV		0.5%	CV		0.9%	CV		1.4%
Storage time [days]	**Factor**		Mean	SEM	*P*	Mean	SEM	*P*	Mean	SEM	*P*
7	Light conditions	LL	46.93	0.376	0.026	-14.92	0.166	0.606	27.39	0.467	0.001
		HL	48.13			-15.28			29.56		
	Temperature	1 °C	45.09		<0.001	-14.73		<0.001	24.49		<0.001
		10 °C	49.97			-15.47			32.46		
Significance of interaction:											
Light conditions x Temperature				0.531	0.014		0.235	0.029		0.660	<0.001
			CV		1.1%	CV		1.3%	CV		2.0%

[1]within columns, for each day, different letters indicate that values are significantly different ($P<0.05$).

After 3 days of storage, there was no significant difference in leaf yellowness (*b**) value between samples stored at 1 °C under low intensity or high intensity light (Table 5.5), while spinach leaves stored at 10 °C were significantly (*P*=0.009) more yellow than those stored at 1 °C. The highest increase in leaf yellowness value was observed at 10 °C under high intensity light. After 7 days, still no difference was observed between samples stored at 1 °C under low intensity or high intensity light (Table 5.5). Spinach leaves stored at 10 °C were significantly (*P*<0.001) more yellow when compared with those stored at 1 °C (Table 5). Leaf yellowness also increased with increasing light intensity (*P*=0.001) due to severe yellowing of the leaves when stored at 10 °C under high light conditions. This led to significant interaction (*P*<0.001) being observed between temperature and light conditions.

5.4 Discussion

Experiment 5 was conducted in August 2011 and August 2012. No significant difference in the quality of the leaves was observed between two growing seasons. It was possible to analyse the data together using two-way ANOVA, where experiments were used as blocks. The treatments used in this Experiment 5 have previously been used in Chapter 3 and 4.

Gas composition

The increase in temperature of storage from 1 to 10 °C has previously been reported (Chapter 3) to affect gas composition inside the bags with spinach, while no change in the gas composition was observed in light-stored samples (Chapter 4). In agreement with the data reported in Chapter 4 no CO_2 development was reported in this study inside the bags with spinach, suggesting that respiration was fairly low and was compensated by photosynthetic activity of the leaves (Toledo *et al.*, 2003a). This is however in contrast with Chapter 3, where with increasing storage temperature, respiration increased. This different response may be partly explained by the fact that the number of bags that were used in both experiments differed. The number of bags per treatment/storage conditions in Experiment 1 (Chapter 3) and Experiment 5 were 42 and

12, respectively and this could be accounted for small changes in the gas composition inside the bags and within the growing cabinets. The difference between experiments could also be due to differences in film thickness, which has not been measured. Furthermore, the difference between two experiments was in the sampling times. In Experiment 1, samples were collected daily, while in Experiment 5 samples were analysed only on day 3 and 7.

Increase in dry matter has previously been reported with increasing temperature (Chapter 3) and light intensity (Chapter 4). In agreement with these findings, the highest dry matter was found in the samples stored at 10 °C under high intensity light conditions probably due to high transpiration. This treatment was then followed by spinach leaves stored at 10 °C under low intensity light conditions. This finding suggests that storage temperature affects dry matter more than light intensity.

Texture

A significant (P=0.039) increase in solute leakage has previously been reported (Chapter 3) after 5 days of storage when comparing samples stored at 1 and 10 °C. In agreement with these findings no difference in solute leakage between spinach leaves stored at 1 and 10 °C was observed until day 7 of storage, when solute leakage indeed increased in samples stored at 10 °C, regardless of light intensity. Low intensity light has previously been shown (Chapter 4) to reduce solute leakage from the leaves compared with the samples stored under high intensity light. The same observation was reported in this study for spinach leaves stored either at 1 and 10 °C. This observation suggests that increase in both storage temperature and light intensity reduces textural quality of the leaves, which is in agreement with Luo *et al*. (2009) who observed an increase in solute leakage with increasing temperature of storage from 1 to 12 °C. Our findings are further supported by Babic *et al*. (1996) who observed marked decrease in textural quality of spinach leaves when leaves were stored at 10 °C. The possible mechanism relates to tissue dehydration (Aguero *et al.*, 2008), which has been demonstrated to increase with increasing storage temperature (Pandrangi and LaBorde, 2004) and light intensity (Lester

et al., 2010b). In agreement with these studies, dry matter significantly increased in spinach leaves stored at 10 °C under high intensity light.

Nutritional quality

A decline in AsA content has previously been reported with storage temperature increasing from 1 to 10 °C (Chapter 3) and light intensity from 30-35 µmol m^{-2} s^{-1} to 130-140 µmol m^{-2} s^{-1} (Chapter 4). A similar response was observed in this study, however, storage temperature had a significant (P<0.001) effect on AsA content already after 3 days of storage, while significant (P=0.022) differences between two light conditions were observed after 7 days of storage. High intensity light led to AsA loss, and this could be explained by high oxidative stress that will cause tissue damage (Foyer and Shigeoka, 2011). This suggests that changes in AsA are more sensitive to changes in the temperature of storage than to intensity of light. Interestingly, a significant (P=0.035) interaction was found between storage temperature and light. This was due to a stronger decline in AsA content under high intensity light when spinach was stored at 1 °C, whereas no difference between light conditions was observed at 10 °C. Changes in the content of DHA in spinach leaves were inconsistent, as has previously been reported by Bergquist *et al*. (2006). Total AsA content has previously been reported to decline with storage temperature increasing from 1 to 10 °C (Chapter 3) and light intensity from 30-35 µmol m^{-2} s^{-1} to 130-140 µmol m^{-2} s^{-1} (Chapter 4). In agreement with these findings, total AsA declined with increasing temperature already after 3 days of storage, while the difference between light conditions was not observed until day 7.

A decline in total carotenoid content has previously been reported with storage temperature increasing from 1 to 10 °C (Chapter 3). On the other hand, light intensity had no effect on total carotenoid content (Chapter 4). This is in agreement with others (Bergquist *et al*., 2006, Lester *et al*., 2010b) who reported carotenoids to be relatively stable or even increase during the storage of spinach. In this study, both temperature (P=0.023) and light intensity (P=0.012) had a significant effect on total carotenoid content after 7 days of storage. This was, however, mainly due to a significant decline in total carotenoid content in spinach leaves stored at 10 °C under high intensity light, while light

intensity had no effect on total carotenoid content in spinach stored at 1 °C. On the other hand, total carotenoid content declined with increasing storage temperature in the samples stored under high intensity light conditions, while non-significant decline was observed under low intensity light conditions.

Visual quality

The content of chlorophyll *a* and *b* has previously been reported to decline with storage temperature increasing from 1 to 10 °C (Chapter 3), while no difference was observed between spinach leaves stored under low and high intensity light (Chapter 4). In agreement with these findings the content of both chlorophylls declined with increasing temperature from 1 to 10 °C and was not affected by light intensity in samples stored at 1 °C in this study. Storage of spinach leaves under high intensity light at 10 °C led to a significant decline in the content of both chlorophylls.

Leaf lightness and yellowness have previously been reported to increase with increasing temperature of storage (Chapter 3), while light intensity had no effect on leaf colour (Chapter 4). These parameters increased with increasing temperature. Leaf lightness and yellowness also increased with increasing light intensity, however, the increase was only observed in the case of spinach leaves stored at 10 °C, while no effect of light was found when spinach was stored at 1 °C, which supports the findings from Chapter 4. Significant interactions between temperature and light conditions during storage were found for all leaf colour parameters – lightness (P=0.014), greenness (P=0.029) and yellowness (P<0.001). These were mainly due to changes observed at 10 °C between two light conditions, while no difference between light conditions was found at 1 °C.

Similar effect of increasing storage temperature and light intensity after 7 days of storage was found in terms of solute leakage, total carotenoids, chlorophyll *a* and *b*, and leaf colour. This led to cumulative effect of both factors, *i.e*. out of all treatments the quality of spinach stored at 10 °C under high intensity light was the lowest. Nutritional quality of spinach was affected by storage temperature already after 3 days of storage

while light had no effect until day 7. Interaction between storage temperature and light intensity was found for nutritional (AsA, DHA) and visual (leaf colour) quality after 7 days of storage, which means that both factors should be considered during displayed shelf-life of spinach.

5.5 Conclusions

The results obtained in this study clearly support the findings of Chapter 3 (Effect of storage temperature on quality changes of baby leaf spinach) and 4 (Effect of light conditions on quality changes of baby leaf spinach). In the case of spinach leaves quality loss (*i.e.* membrane integrity, total AsA content and leaf colour) is accelerated with increasing temperature (Chapter 3) and intensity of light (Chapter 4). The rise in temperature caused more severe damage to the leaves than increase in the intensity of the light. Increase in storage temperature from 1 to 10 °C significantly reduced the quality of spinach leaves, as indicated by increased solute leakage, decline in the content of AsA, total AsA, total carotenoids and chlorophylls and reduced visual quality - leaf colour. Increase in light intensity from 30-35 μmol m^{-2} s^{-1} to 130-140 μmol m^{-2} s^{-1} resulted in decline of chlorophylls and total carotenoids, and reduced visual quality of spinach leaves stored for 7 days at 10 °C, while no effect of light was observed in samples stored at 1 °C. On the other hand, the increase in light intensity reduced nutritional quality (AsA and total AsA content) of the leaves when stored at 1 °C, while no difference was found between spinach leaves stored at 10 °C. This could be explained by the strong decline in the content of this bioactive compound already in response to the temperature rise. This means that spinach leaves are less responsive to changes in the light intensity when stored at 1 °C. Nonetheless, high intensity light may reduce nutritional quality of the leaves, even when they are stored at low temperature. Low storage temperature and low intensity light should thus be used to maintain the quality of spinach leaves during their displayed shelf-life. Both excessive temperature and intensive light lead to oxidative stress in spinach leaves, in this way inducing their senescence.

Chapter 6 Effect of hot water treatments on quality of baby leaf spinach

6.1 Introduction

There is potential to impose a postharvest period of heat to manipulate leaf biochemistry. A number of workers have reported benefits of periods of hot water treatment immediately after harvest to either visual quality or biochemical properties of leafy vegetables. These benefits have included reduced enzymatic browning of fresh-cut lettuce by repressing induction of phenylalanine ammonia-lyase (PAL) activity (Murata *et al.*, 2004); maintained leaf colour in rocket (Koukounaras *et al.*, 2009) and reduced tissue breakdown and chlorophyll loss in spinach (Gomez *et al.*, 2008). Whilst Gomez *et al*. (2008) reported that hot water treatment at 37-43 °C for 3.5 min extended the shelf-life of spinach leaves, their results were obtained following storage at 23 °C. Commercial growers, however, routinely store spinach leaves at refrigerated temperatures (0-5 °C) and it has been demonstrated by Zhang *et al*. (2009) with broccoli that the response to hot water treatment may vary between the samples subsequently stored under different temperature regimes.

Before hot water treatments can be recommended as a viable technique for improving postharvest quality of spinach, it is necessary to determine whether they could potentially be used for spinach leaves stored at commercially realistic refrigerated temperatures. The level of heat stress (*i.e.* temperature and duration) imparted by hot water treatment therefore needs to be great enough to elicit an antioxidant response but not so high as to lead to irreversible tissue damage (Delaquis *et al.*, 1999).

This Chapter reports findings from 2 experiments conducted with field-grown spinach. The aim was to determine the effect of hot water treatment prior to storage on changes in the quality characteristics of baby leaf spinach during storage. The hypothesis tested was: hot water treatment prior to storage does not affect the maintenance of texture, nutritional and/or visual quality characteristics of baby leaf spinach.

6.2 Materials and Methods

6.2.1 Plant material and handling

Spinach (*Spinacia oleracea* L.) cultivar Toucan was commercially grown at PDM Produce Ltd, Shropshire, TF10 9BN, UK. For each experiment, approximately 3 kg of leaves were collected at harvest (April 2012 – Experiment 6; 18th and 21st of May 2012 – Experiment 7) and transported to the laboratory (~15 minutes) in insulated opaque containers and treated immediately on arrival.

In Experiment 6, spinach was subjected to hot water (40, 45 or 50 °C) treatment for 0, 30, 60 or 120 s. Leaves were subsequently washed with cold water (4 °C) for 120 s and then carefully blotted with absorbent paper, before storage at 4 °C in the dark for 10 days.

In Experiment 7, spinach was subjected to hot water treatment at 45 °C for 0 or 60 s and was then subsequently washed in cold (4 °C) distilled water containing 100 µL L^{-1} active chlorine (Cl) (Koukounaras *et al.*, 2009) for 120 s. Leaves were carefully blotted with absorbent paper, before storage at 4 °C in the dark for 10 days.

6.2.2 Measurements

All measurements were taken following methods described in Chapter 2.

6.2.3 Statistical analyses

Each experiment was repeated twice with very similar results (verified by the Bartlett's homogeneity test and CV (%) values). Data are presented as mean values from two experiments that were used as blocks. Results were analysed using two-way ANOVA to identify the factors/treatments that had significant effect on quality changes during the storage of baby leaf spinach. Tukey's test was used to allow the comparisons between individual treatments. Results were analysed using one-way ANOVA to identify whether the treatment had significant effect on quality changes of baby leaf spinach. All statistical analyses were performed using GenStat 14th Edition software (Payne *et al.*, 2010).

6.3 Results

6.3 a) Experiment 6

6.3.1 Gas composition

Hot water treatments at 40 and 45 °C had no effect on changes in CO_2 concentration inside the packages with spinach leaves when compared with unheated samples (Table 6.1); the CO_2 concentration in these samples on average did not exceed 0.2%. In contrast, hot water treatment at 50 °C significantly (P<0.001) enhanced average CO_2 concentration in the packages up to 0.67 and 0.85% in samples treated for 60 and 120 s, respectively. The increase being more pronounced (P=0.020) with increasing treatment time (Table 6.1), however, this trend was only observed in case of samples treated at 50 °C. A significant (P=0.014) interaction between temperature of treatment and treatment time was observed. This was accounted for the samples treated at 50 °C, as no difference in the gas composition inside the packages with spinach was found between unheated samples and those treated with hot water at 40 and 45 °C for 30, 60 and 120 s, respectively.

Table 6.1 Changes in the concentration of O_2 [%] and CO_2 [%] in response to hot water treatments at 40, 45 or 50 °C for 0, 30, 60 or 120 s, respectively. O_2 and CO_2 concentrations were determined after 10 days of storage at 4 °C.

Temperature [°C]	Treatment Time [s]	O_2	CO_2
	0	20.8% a	0.1% c
40	30	20.8% a	0.1% c
40	60	20.8% a	0.1% c
40	120	20.9% a	0.0% c
45	30	20.9% a	0.0% c
45	60	20.7% a	0.2% c
45	120	20.7% a	0.2% c
50	30	20.6% ab	0.3% bc
50	60	20.2% b	0.7% ab
50	120	20.1% b	0.9% a

Different letters indicate that values are significantly different (P<0.05). Data represent mean values from 6 replicates.O_2 (P<0.001; SEM=0.191, CV=3.4%), CO_2 (P<0.001; SEM=0.210, CV=2.6%).

6.3.2 Solute leakage

After 10 days of storage, significant (P=0.007) differences in solute leakage were observed between spinach leaves treated with various hot water treatments prior to storage (Figure 6.1). Hot water treatments at 40 and 45 °C, however, had no significant effect on solute leakage when compared with unheated samples (Figure 6.1). Solute leakage values for samples treated with hot water at 40 °C were in the range from 4.18 to 4.35%. In the case of spinach leaves treated at 45 °C for 30 or 60 s and at 50 °C for 30 s solute leakages were 3.20, 3.82 and 3.97%, respectively. Solute leakage increased in samples treated with hot water at 45 °C for 120 s (10.62%), at 50 °C for 60 s (10.23%). Hot water treatment at 50 °C for 120 s resulted in the greatest membrane damage (19.79%). Solute leakage increased significantly (P=0.004) with increasing treatment time. This trend, however, was only observed in samples treated at 45 and 50 °C. A significant (P=0.023) interaction was found between temperature of the treatment and treatment time, which informs that the response to increase in treatment time (from 30 to 120 s) was different over three temperatures (40, 45 and 50 °C) tested.

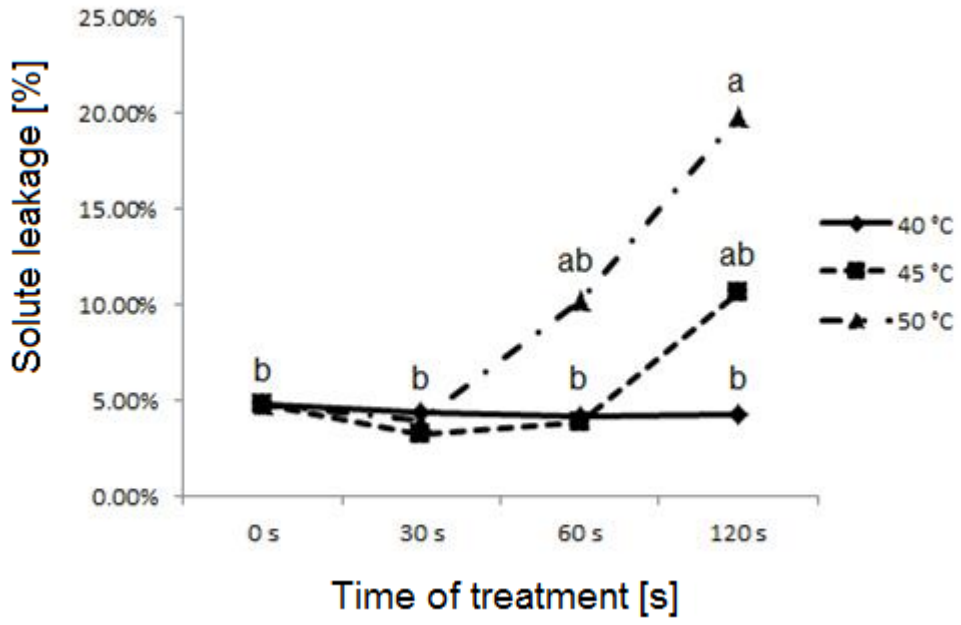

Figure 6.1 Solute leakages [%] from spinach leaves treated with hot water at 40, 45 or 50 °C for 0, 30, 60 or 120 s, respectively. Solute leakage was determined after 10 days of storage at 4 °C. Solute leakage (P=0.007; SEM=2.506, CV=40.0%). Different letters indicate that values are significantly different (P<0.05). Where data points are close together one letter refers to those points. Each data point is the mean of 6 replicates.

6.3.3 Leaf colour changes

Among leaf colour characteristics measured, only leaf yellowness was affected by hot water treatments (Table 6.2). There was no significant difference observed between unheated leaves and those treated either with hot water at 40 °C for 30, 60 or 120 s or those treated with hot water at 45 °C for 30 or 60 s, respectively. Spinach leaves treated with hot water at 45 °C for 120 s and those treated with hot water at 50 °C for 30 or 60 s were significantly more yellow than unheated control.

The maximum treatment (temperature and duration) that did not result in a significant reduction in the quality of spinach leaves stored for 10 days at 4 °C was a hot water treatment at 45 °C applied for 60 s. This treatment was selected for further study to determine its effect on the nutritional quality of cold-stored spinach leaves.

Table 6.2 Effect of hot water treatments prior to storage applied at three different temperatures (40, 45 and 50 °C) for 0, 30, 60 or 120 s on leaf colour changes during the storage of spinach leaves for 10 days at 4 °C. Different letters indicate that values are significantly different (P<0.05).

Temperature [°C]	**Treatment Time [s]**	***L* (lightness)***			***a* (greenness)***			***b*(yellowness)***		
unheated	0	40.98 ab			-13.38 a			23.14 d		
40	30	40.04 b			-13.69 ab			23.21 d		
40	60	40.64 ab			-13.79 abc			23.77 cd		
40	120	40.90 ab			-13.44 a			23.40 cd		
45	30	41.67 ab			-13.90 abc			24.31 cd		
45	60	41.12 ab			-14.02 abc			24.33 cd		
45	120	41.43 ab			-14.29 bc			24.85 bc		
50	30	41.67 ab			-14.46 c			26.35 a		
50	60	42.13 a			-13.92 abc			25.94 ab		
50	120	40.16 b			-13.80 abc			24.38 cd		
Factor		Mean	SEM	*P*	Mean	SEM	*P*	Mean	SEM	*P*
Temperature [°C]	unheated	40.98	0.379	0.023	-13.38	0.149	<0.001	23.14	0.327	<0.001
	40	40.53			-13.64			23.46		
	45	41.41			-14.07			24.50		
	50	41.32			-14.06			25.56		
Treatment Time [s]	0	40.98		0.521	-13.38		0.580	23.14		0.309
	30	41.12			-13.96			24.49		
	60	41.29			-13.86			24.55		
	120	40.82			-13.79			24.07		
Significance of interaction:										
Temperature x Treatment Time			0.544	0.001		0.214	0.002		0.469	<0.001
		CV		6.3%	CV		7.3%	CV		9.1%

6.3 b) Experiment 7

6.3.4 Gas composition

No significant difference between heated (hot water treatment at 45 °C applied for 60 s) spinach and unheated control was found in the gas composition inside the packages after 5 and 10 days of storage. The concentration of CO_2 increased over time with a simultaneous decrease in the concentration of O_2 (Table 6.3).

Table 6.3 Changes in O_2 and CO_2 concentrations [%] in response to hot water treatments at 45 °C for 0 s (unheated) or 60 s (heated), respectively. Gas composition inside the packages was determined after 5 and 10 days of storage at 4 °C, respectively.

Time of storage	Treatment	O_2	CO_2
Day 5	Unheated	17.98% a	2.87% a
	Heated	17.94% a	2.91% a
Day 10	Unheated	17.58% a	3.27% a
	Heated	17.28% a	3.57% a

Different letters indicate that values are significantly different (P<0.05). Data represent mean values from 12 replicates.O_2: 5 d (P=0.721; SEM=0.128, CV=3.0%), 10 d (P=0.678; SEM=0.255, CV=3.6%). CO_2: 5 d (P=0.757; SEM=0.180, CV=2.5%), (P=0.699; SEM=0.251, CV=3.2%).

6.3.5 Solute leakage

Solute leakage from unheated and heated spinach leaves decreased to 0.77 % (unheated) and 0.58 % (heated) after 5 days of storage and remained lower until 10 days of storage – 1.00 % (unheated) and 0.87 % (heated) when compared with initial value of 1.37 % (Figure 6.2). No significant difference was observed between unheated and heated samples throughout the storage period.

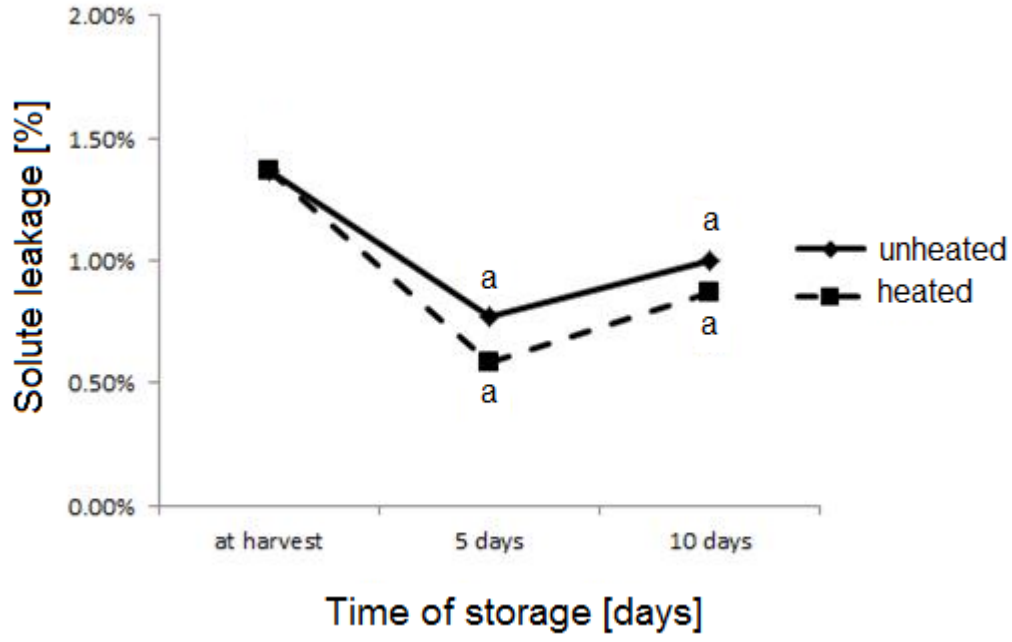

Figure 6.2 Solute leakage from spinach leaves subjected to hot water treatment applied at 45 °C for 0 s (unheated) or 60 s (heated) prior to storage. Solute leakage was determined after 5 and 10 days of storage at 4 °C, respectively. Solute leakage: 5 d (P=0.084; SEM=0.214, CV=31.2%), 10 d (P=0.362; SEM=0.084, CV=9.7%). Different letters indicate that values are significantly different (P<0.05). Each data point is the mean of 12 replicates.

6.3.6 Total ascorbic acid (ascorbic acid (AsA) + dehydroascorbic acid (DHA))

No significant difference was found in AsA content between heated and unheated samples throughout the storage period (Table 6.4). DHA content decreased from 0.36 to 0.23 mg g^{-1} DW in heated samples after 5 days of storage, and was significantly (P<0.001) lower than in unheated samples. After 10 days, however, DHA content in these samples increased to 0.41 mg g^{-1} DW and was no longer significantly different when compared with unheated samples (Table 6.4).

Table 6.4 Effect of hot water treatment applied at 45 °C for 0 s (unheated) or 60 s (heated) prior to storage on ascorbic acid (AsA) and dehydroascorbic acid (DHA)) concentration on a dry weight (DW) basis in spinach leaves after 5 and 10 days of storage at 4 °C, respectively. Different letters indicate that values are significantly different (P<0.05). Data represent mean values from 12 replicates.

Treatment	**AsA [mg/g DW]**			**DHA [mg/g DW]**		
	storage time [days]					
	0	5	10	0	5	10
unheated	6.78	6.62 a	6.13 a	0.36	0.38 a	0.35 a
heated		6.43 a	5.89 a		0.23 b	0.41 a
Significance:						
Treatment		0.500	0.438		<0.001	0.385
SEM		0.197	0.180		0.024	0.044
CV		13.7%	11.8%		48.5%	63.4%

Total AsA content decreased after 10 d of storage from 7.14 mg g^{-1} DW at day 0 to 6.48 and 6.30 mg g^{-1} DW in unheated and heated samples, respectively (Figure 6.3). No difference in total AsA was observed between unheated and heated leaves throughout the storage period.

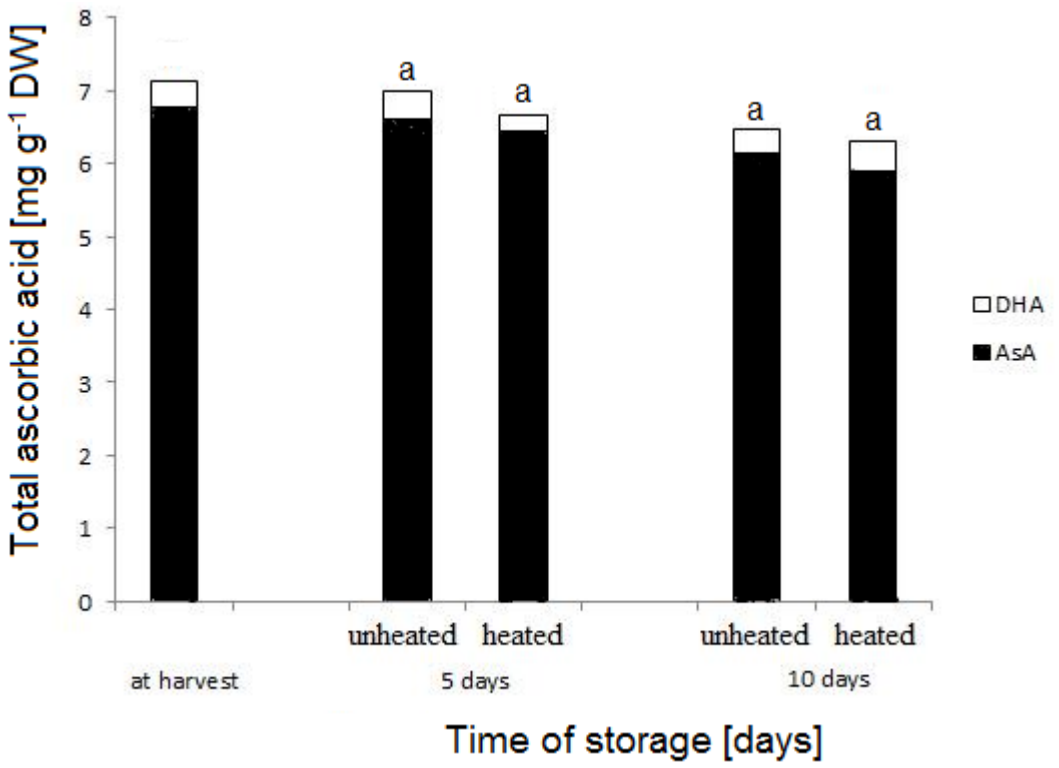

Figure 6.3 Effect of hot water treatment applied at 45 °C for 0 s (unheated) or 60 s (heated) prior to storage on total ascorbic acid (ascorbic acid (AsA) + dehydroascorbic acid (DHA)) concentration [mg g^{-1} DW] in spinach leaves after 5 and 10 days of storage at 4 °C, respectively. Total AsA: 5 d (*P*=0.229; SEM=0.282, CV=13.2%), 10 d (*P*=0.642; SEM=0.129, CV=11.3%). Different letters indicate that values are significantly different (*P*<0.05). Each data point is the mean of 12 replicates.

6.3.7 Total carotenoid and chlorophyll content

No difference was found in chlorophyll *a* and *b* content between the samples during storage (Table 6.5). No significant difference was found for chlorophyll *a: b* ratio. Change was observed in total carotenoid content after 10 days of storage where it decreased significantly in unheated spinach leaves from 2.83 mg g^{-1} DW at day 0 to 2.56 mg g^{-1} DW, while it did not change significantly in heated leaves. This resulted in significantly (*P*=0.049) higher total carotenoid content being observed in heated samples (Table 6.5). No correlation was found between leaf colour changes and plant pigment content of spinach leaves.

Table 6.5 Chlorophyll *a* and chlorophyll *b*, ratio and total carotenoid content on a dry weight (DW) basis in spinach leaves subjected to hot water treatment at 45 °C for either 0 s (unheated) or 60 s (heated). Spinach leaves were subsequently stored for 10 days at 4 °C. Different letters indicate that values are significantly different (P<0.05). Data represent mean values from 12 replicates.

Treatment	**chlorophyll *a* [mg/g DW]**			**chlorophyll *b* [mg/g DW]**		
	storage time [days]					
	0	5	10	0	5	10
unheated	8.32	8.04 a	7.31 a	1.73	1.77 a	1.55 a
heated		7.83 a	7.87 a		1.76 a	1.65 a
Significance:						
Treatment		0.474	0.138		0.852	0.229
SEM		0.201	0.256		0.049	0.058
CV		11.4%	11.2%		15.6%	12.0%
Treatment	**chlorophyll *a: b* ratio**			**total carotenoids [mg/g DW]**		
	storage time [days]					
	0	5	10	0	5	10
unheated	4.81	4.54 a	4.72 a	2.83	2.79 a	2.56 b
heated		4.45 a	4.77 a		2.75 a	2.85 a
Significance:						
Treatment		0.314	0.184		0.644	0.049
SEM		0.140	0.082		0.062	0.099
CV		4.5%	6.1%		7.8%	12.2%

6.3.8 Leaf colour changes

At harvest, all leaves had a dark green colour. Leaf colour changed during 10 days of storage at 4 °C. Leaf lightness and yellowness values increased, while the greenness value decreased (Table 6.6). No significant difference in leaf colour between unheated and heated samples was found after 5 days of storage. After 10 days heated leaves were significantly lighter, greener and more yellow than unheated counterparts.

Table 6.6 Effect of hot water treatments at 45 °C for 0 s (unheated) or 60 s (heated) on leaf colour changes during the storage of spinach leaves for 10 days at 4 °C. Different letters indicate that values are significantly different (P<0.05). Data represent mean values from 12 replicates.

Treatment	***L* (lightness)***		
	storage time [days]		
	0	5	10
unheated	41.99	42.73 a	43.27 b
heated		42.72 a	44.15 a
Significance:			
Treatment		0.983	0.018
SEM		0.354	0.366
CV		4.5%	6.5%
Treatment	***a* (greenness)***		
	0	5	10
unheated	14.99	-15.84 a	-15.79 a
heated		-15.89 a	-16.11 b
Significance:			
Treatment		0.743	0.037
SEM		0.177	0.152
CV		6.1%	7.4%
Treatment	***b* (yellowness)***		
	0	5	10
unheated	25.81	27.35 a	28.16 b
heated		27.78 a	30.36 a
Significance:			
Treatment		0.392	<0.001
SEM		0.503	0.495
CV		10.0%	13.1%

6.4 Discussion

A number of authors have reported benefits of hot water treatment on the shelf-life of spinach (Gomez *et al.*, 2008) and rocket (Koukounaras *et al.*, 2009). In the work reported here, spinach was treated with hot water at 40, 45 and 50 °C covering the optimum temperatures reported by both Gomez *et al*. (2008) and Koukounaras *et al*. (2009). Data obtained in Experiment 6 suggest that the maximum 'safe' hot water treatment that did not result in a significant deterioration in the quality of spinach leaves was hot water treatment at 45 °C applied for 60 s prior to storage. Treatment for a longer time, or at a higher temperature, proved counterproductive as the damage observed would result in a decreased shelf-life even if increased antioxidant content was observed. For instance, the level of solute leakage markedly increased in samples treated with hot water at 50 °C suggesting that cellular integrity was adversely affected. This is in agreement with work on tissue integrity in lettuce that reported solute leakage to be enhanced with increasing temperature and treatment time (Delaquis *et al.*, 1999, Delaquis *et al.*, 2004). Solute leakage was unaffected by hot water treatment at 45 °C for 60 s when compared with the control but Gomez *et al*. (2008) reported a reduction in solute leakage following hot water treatment at 40 °C for 3.5 min. This discrepancy might be due to the fact that these authors used a markedly higher storage temperature (23 °C) than the one used in this study. The storage temperature used in the current study (4 °C) was chosen to be representative of typical commercial storage conditions.

The maximum hot water treatment of 45 °C applied for 60 s was hotter and of a shorter duration than the 40 °C for 3.5 min suggested by Gomez *et al*. (2008) for spinach. However, Gomez *et al*. (2008) only studied one duration of hot water treatment (3.5 min) at moderate temperatures (37, 40 and 43 °C) making direct comparison difficult. In contrast, Koukounaras *et al*. (2009) recommended a maximum hot water treatment for rocket at 50 °C for 30 s but did not study lower temperatures. In the study reported here, heat treatments at 50 °C applied for 30 s or longer damaged spinach leaves and led to significant changes in colour, solute leakage and respiration after 10 days.

Using the maximum acceptable treatment (45 °C for 60 s), the respiration rate was not affected when spinach leaves were subsequently stored at 4 °C. A similar observation has previously been reported (Martinez-Sanchez *et al.*, 2008a) for mizuna and watercress leaves treated with hot water at 50 °C for 60 s and then subsequently stored at 4 °C. The same treatment did not increase the respiration rate of rocket leaves when subsequently stored at 8 °C (Koukounaras *et al.*, 2009). This work identified that hot water treatment at 45 °C for 60 s did not increase tissue respiration in spinach, which is associated with decreased shelf-life (Masih *et al.*, 2002, Martinez-Sanchez *et al.*, 2008a).

Solute leakage remained low in all samples, regardless of whether tissue received the hot water treatment or not, further indicating that this treatment does not cause significant loss to tissue integrity. The values observed were in the same range as those reported for spinach by Allende *et al*. (2004b) and others (Medina *et al.*, 2012, Tudela *et al.*, 2013), but lower than reported by Gomez *et al*. (2008). This is not surprising as Gomez *et al*. (2008) stored spinach leaves at 23 °C which is a higher temperature than 4 °C used here and 5 °C used by Allende *et al*. (2004b). Furthermore, a marked decrease in textural quality of spinach leaves has already been observed when leaves were stored for 5 days at 10 °C (Babic *et al.*, 1996), suggesting that higher storage temperature may cause damage to the leaves.

A significant decrease in total AsA content was observed after 10 days of storage, an observation that has previously been reported by other authors (Gil *et al.*, 1999, Bergquist *et al.*, 2006, Bergquist *et al.*, 2007, Bottino *et al.*, 2009). No significant difference in AsA content was observed between heated and unheated spinach leaves. In support, others have found that hot water treatments had no effect on AsA content in spinach (Gomez *et al.*, 2008) and rocket leaves (Koukounaras *et al.*, 2009), respectively. The content of DHA in spinach leaves remained at a similar level during the storage period. This is in contrast with other studies that reported an increase in DHA content during the storage of spinach (Gil *et al.*, 1999, Bottino *et al.*, 2009), while inconsistent changes in DHA content have also been reported by Bergquist *et al*. (2006).

Leaf lightness increased during the storage of unheated spinach leaves, a different response to that reported by Conte et al. (2008) who did not observe any changes in leaf lightness, greenness and yellowness when spinach leaves were stored for 13 d at 5 °C. On the other hand, in this work, after 10 days of storage at 4 °C, spinach leaves were significantly more yellow than at day 0. Spinach leaves treated at 45 °C for 60 s were significantly lighter, greener and more yellow after 10 days when compared with unheated leaves. These results are in contrast with the response of rocket leaves (Koukounaras *et al.*, 2009) which were significantly less green, less yellow and darker when treated with hot water at 50 °C for 30 s. Care must be taken when using leaf colour as a measure of postharvest quality; in darker leaves green colour may be masked by the presence of other pigments.

Leaf colour changes may be related to chlorophyll degradation (Pandrangi and LaBorde, 2004) and it was observed that chlorophyll content decreased during the storage of unheated spinach leaves. This was in agreement with Pandrangi and LaBorde (2004), but in contrast to those reporting chlorophyll concentrations to be relatively stable during the storage of spinach leaves (Bergquist *et al.*, 2006, Conte *et al.*, 2008). The difference in chlorophyll retention may also be a consequence of studying a different cultivar to other workers, or else seasonal differences previously reported (Bergquist *et al.*, 2006, Conte *et al.*, 2008). In this study only chlorophyll *a* concentration decreased during storage, while chlorophyll *b* remained relatively stable. This is in contrast to Kopas-Lane and Warthesen (1995) and Noichinda *et al*. (2007) who reported chlorophyll *b* degradation to occur faster in spinach and Chinese kale, respectively. On the other hand, Pandrangi and LaBorde (2004) did not find any difference in chlorophyll *a* and *b* degradation rate, while Bergquist *et al*. (2006) observed a decrease in chlorophyll *a*: *b* ratio. In contrast to reported observations with rocket (Koukounaras *et al.*, 2009) and spinach (Gomez *et al.*, 2008), chlorophyll retention was not affected by hot water treatment at 45 °C for 60 s in this study. The difference may be due to several factors; Gomez *et al*. (2008) kept spinach at 23 °C, while spinach was kept at 4 °C in this study; rocket leaves have a different texture to spinach leaves which may affect heat transfer properties. Furthermore, different

methods were used to quantify chlorophyll content of the leaves. Gomez *et al*. (2008) used chlorophyll meter (SPAD-502, Minolta) while a spectrophotometer was used in this study.

The effect of hot water treatment at 45 °C on carotenoid content of spinach has not been previously reported but it is known that cooking temperatures, *i.e.* 98 °C, can degrade carotenoids (Aman *et al.*, 2005). Compared with untreated leaves, hot water treatment at 45 °C for 60 s gave leaves with significantly higher total carotenoid content after 10 days of storage. The difference in carotenoid concentration after 10 days in this work was either associated with increased biosynthesis and/or with reduced carotenoid breakdown in the heated leaves, but further work will be needed to establish the underlying mechanism. Total carotenoid concentration in unheated spinach leaves declined throughout the storage period. This is in agreement with previous findings (Pandrangi and LaBorde, 2004, Bunea *et al.*, 2008) but in contrast to those reporting carotenoids to be relatively stable during the storage of spinach leaves (Bergquist *et al.*, 2006, Bergquist *et al.*, 2007, Lester *et al.*, 2010b). No correlation was found between chlorophyll and total carotenoid content and leaf colour changes during the storage of spinach leaves. Similar observation has previously been reported (Pandrangi and LaBorde, 2004, Bergquist *et al.*, 2006). Bergquist *et al*. (2006) suggested that it was due to the loss of colour being unevenly distributed.

Although there were some benefits to the maintenance of carotenoid content in the leaves, overall, data presented in this Chapter suggest that hot water treatment at 45 °C for 60 s (the maximum ‘safe’ treatment) does not increase the shelf-life of spinach leaves. This is in contrast to the findings of Gomez *et al*., (2008), and suggests that the maximum hot water treatment for spinach (45 °C for 60 s) does not offer a practical method for producers for shelf-life extension or antioxidant concentration increase. Treatment for a longer time or at a higher temperature would result in unacceptable tissue damage and is not commercially viable.

6.5 Conclusions

The postharvest hot water treatment at 45 °C for 60 s applied immediately after harvest was identified as the maximum stress treatment that could be applied before subsequent damage was observed in spinach leaves. Subsequent studies showed that this hot water treatment did not prolong the shelf-life of spinach; increase tissue quality or either maintain or increase total AsA content. The treatment did appear to protect total carotenoid concentration compared to untreated samples. Results presented here indicate that hot water treatments have limited commercial potential for quality improvement of spinach leaves.

Chapter 7 General discussion, conclusions and recommendations for future study

The aim of this research was to investigate whether postharvest quality of baby leaf spinach can be improved or maintained either through optimising storage conditions (temperature and light) or by imposing a pre-storage stress (hot water treatment).

The current knowledge on the role of temperature and light exposure during storage and the effects of pre-storage hot water treatments on quality of leafy vegetables have been reviewed in Chapter 1. Seven duplicated experiments were conducted with field-grown spinach to improve our understanding of the role of temperature and light exposure during storage and whether pre-storage hot water treatments could be used by industry to improve or maintain the quality of baby leaf spinach.

In this chapter, the experiments will be summarized and compared with the published data. Broader questions will be addressed using the combined data, recommendations will be made for optimising spinach leaf quality and areas for future work will be identified.

7.1 Effect of storage temperature on quality changes of spinach leaves

The first objective of this project was to determine the effect of storage temperature on quality changes of baby leaf spinach. Furthermore, there was a need to identify which of the quality measures (from those used in this study) can potentially be used as good indicators of shelf-life.

All quality measures studied, with an exception of leaf greenness (*a** value), responded to storage temperature with rapid deterioration in quality at 20 °C. This deterioration slowed as the temperature was reduced and based on the results obtained in Experiment 1, 2 and 5, it can be concluded that storage of spinach leaves at 1 °C maintained spinach leaf quality. This could be observed in the development of CO_2 inside the bags, solute leakage, changes in AsA, total AsA and plant pigment content (chlorophyll *a* and *b*, and total carotenoids), and leaf colour changes.

The most responsive quality measures to temperature were AsA and total AsA content, development of CO_2, solute leakage, leaf yellowness and lightness. In contrast, changes in plant pigment content were inconsistent and are not well suited to being used as indicators of shelf-life.

The content of AsA and total AsA declined with temperature increasing from 1 to 10 or 20 °C already after 1 day of storage. This rapid change in AsA content is not surprising as it is a key antioxidant in leaf tissue (Conklin, 2001; Mittler, 2002). Its role is to scavenge AOS that are produced in excess under stress conditions, *e.g.* increase in temperature. Plant cells have a capability to reduce the damage caused by AOS using antioxidant enzymes (SOD, APX, GR, CAT) and metabolites, including AsA and GSH, to transform AOS to less toxic compounds, *e.g.* water, using AsA as an electron donor (Mittler, 2002; Foyer and Noctor, 2009). In the reaction catalysed by APX, AsA is changed into DHA. Thus, changes in antioxidant content are a good indication of oxidative stress (Mittler, 2002). On the other hand, AsA content was found to be relatively stable during the storage of spinach leaves at 1 °C. Better retention of AsA in spinach leaves stored at lower temperature could be explained by either its biosynthesis or reduced turnover due to

higher activity of AsA-GSH cycle that is responsible for regeneration of AsA (Mittler, 2002; Blokhina *et al*., 2003). This finding suggests that storage at low temperature helps in maintaining nutritional quality of baby leaf spinach during its shelf-life.

Rapid decline in AsA observed in spinach leaves stored either at 10 or 20 °C clearly suggests that cells were under strong oxidative stress and probably were not able to scavenge all AOS, which usually results in damage to cellular components, *e.g.* membrane lipids and proteins (Mittler, 2002). Peroxidation of membrane lipids occurs when AOS react with unsaturated fatty acids which lead to increased solute leakage. Lipid peroxidation of membranes is another reflection of stress-induced damage at the cellular level.

Severe tissue damage in spinach leaves stored at 20 °C was also reflected in high respiration rate as indicated by strong development of CO_2 inside the bags reported already after 1 day of storage. Spinach leaves have relatively high respiration rate and it has been reported by others (Martinez-Sanchez *et al*., 2008a) that respiration rate of leafy vegetables increases with increasing temperature of storage from 1 to 12 °C. Higher respiration rate is associated with reduced shelf-life. Proteins (Masih *et al*., 2002), lipids and carbohydrates (Buchanan-Wollaston, 1997) are often used as substrates for respiration. No development of CO_2 was observed in bags with spinach stored at 1 °C, which suggests that the respiration was balanced by photosynthesis (Toledo *et al*., 2003a) and/or that film permeability to O_2 and CO_2 met physiological requirements of baby leaf spinach (Martinez *et al*., 2005).

As already mentioned above, oxidative stress leads to increased solute leakage. Thus, it is not surprising that in the study reported here (Experiment 1, 2 and 5), solute leakage increased significantly with increasing temperature of storage from 1 to 6, 10 and 20 °C after 7, 5 and 3 days of storage, respectively. This finding is in agreement with the findings of Luo *et al*. (2009) who observed increased solute leakage from spinach leaves when comparing the samples stored at 1 and 12 °C. The values observed in our study were in the same range as those reported by others (Allende *et al.*, 2004b, Luo *et al.*,

2009, Medina *et al.*, 2012, Tudela *et al.*, 2013) but lower than reported by Gomez *et al*. (2008). This is not surprising as Gomez *et al*. (2008) stored spinach leaves at 23 °C, which is a higher temperature than the one used by us and others (Allende *et al.*, 2004b, Luo *et al.*, 2009, Medina *et al.*, 2012, Tudela *et al.*, 2013). A marked decrease in textural quality of spinach leaves has already been observed when leaves were stored at 10 °C (Babic *et al.*, 1996), explaining why higher storage temperature would cause damage to the leaves. In contrast to Luo *et al*. (2009), who did not observe any difference in solute leakage between spinach leaves stored at 1, 5 and 8 °C, in our study (Experiment 2), even a small difference in storage temperature (1 vs. 6 °C) significantly reduced textural quality of spinach leaves.

Increase in solute leakage indicates the severity of the damage caused by AOS. The loss of texture may also be related to solubilisation of cell wall pectin and loss of carbohydrates as a result of enhanced respiration. These modifications reduce cell wall strength and cell to cell adhesion, leading to leaf softening (Clarkson *et al*., 2003). To reduce the damage from AOS, plants evolved the ability to regulate membrane fluidity in response to changes in temperature (Murata and Los, 1997); this can be achieved through saturation/unsaturation of membrane fatty acids. Membrane fluidity decreases with decreasing temperature of storage. The fact that solute leakage differed significantly between spinach leaves stored at 1, 6, 10 and 20 °C suggests that plants can show remarkable responses even to such small changes in temperature.

Leaf lightness (*L** value) and leaf yellowness (*b** value) increased with increasing temperature of storage (Experiment 1, 2 and 5), while leaf greenness (*a** value) did not respond to changes in temperature. Leaf yellowness (*b** value) increased significantly with storage temperature increasing from 1 to 6, 10 and 20 °C after 7, 5 and 3 days of storage, respectively. Leaf lightness (*L** value) on the other hand, increased with temperature increase from 1 to 10 °C, whereas no difference in this parameter was observed between spinach leaves stored at 1 and 6 °C. Our findings are in agreement with Luo *et al*. (2009) who have also demonstrated that in spinach, the loss of visual quality is accelerated with increasing temperature of storage. It is not surprising that from all leaf colour

characteristics (*L**, *a** and *b**) the strongest changes were reported for leaf yellowness, as the process of leaf yellowing is the main issue associated with the loss of visual quality during the storage of spinach leaves. Leaf yellowing is often associated with changes in plant pigment content, *e.g.* changes in chlorophyll content or chlorophyll: carotenoid ratio (Toivonen and Brummell, 2008), however, as the changes in chlorophylls and carotenoids were inconsistent in our study, it is difficult to find a clear correlation between plant pigment content and leaf colour changes. Other groups (Pandrangi and LaBorde, 2004; Bergquist *et al*., 2006) also could not find a correlation between pigment content and visual quality of the leaves. This suggests that leaf colour, as measured with a chroma meter, may be influenced not only by pigment content but also by changes in texture and water content because plant pigments are often determined on DW basis, while leaf colour is always assessed on fresh tissue. Furthermore, plant pigments are not homogenously distributed within the leaf, so there might be a mismatch between the readings.

Overall, the loss of chlorophyll was accelerated with increasing temperature of storage (Experiment 1, 2 and 5) which is probably related to higher activity of enzymes involved in chlorophyll degradation (Hortensteiner, 2006) and enhanced membrane disruption (Ferrante *et al*., 2004) as indicated by increased solute leakage, because chlorophyllase, enzyme involved in chlorophyll degradation, is separated from its substrate and does not come into contact with it until membrane integrity is reduced (Hortensteiner, 2006). This may explain, why no change in chlorophyll content was observed during 3 days of storage at 20 °C, suggesting that changes in plant pigment content are relatively slow. Changes in chlorophyll content were inconsistent between experiments. Inconsistent changes were observed in samples stored at 1 °C; where chlorophyll content either declined (Experiment 2 and 5) or remained relatively stable (Experiment 1). Good retention of chlorophyll has previously been reported (Bergquist *et al.*, 2006, Conte *et al.*, 2008), while Pandrangi and Laborde (2004) observed a decline in chlorophyll content during the storage of spinach. An increase in chlorophyll degradation rate with increasing storage temperature from 4 to 20 °C has previously been observed by

others (Gnanasekharan *et al.*, 1992, Pandrangi and LaBorde, 2004). On the other hand, Bergquist *et al*. (2006) have not reported significant differences between spinach leaves stored at 2 and 10 °C.

Total carotenoid content was found to be relatively stable during 3 days of storage at 1, 6, 10 and 20 °C (Experiment 1, 2 and 5). After 7 days of storage, however, changes in total carotenoid content were inconsistent between experiments. In Experiment 1, total carotenoid content decreased with increase in storage temperature from 1 to 10 °C, while no difference between these storage temperatures was observed in Experiment 5. Inconsistent changes in carotenoid content have previously been reported. A decrease in carotenoid content with increasing temperature of storage has been reported by Pandrangi and LaBorde (2004), while Bergquist *et al*. (2006) suggested that synthesis of carotenoids may occur and that carotenoids are quite well preserved during storage.

All these data helped us to identify leaf texture and colour (leaf yellowness) as the best indicators of shelf-life as both parameters clearly indicated quality loss after 3, 5 and 7 days of storage at 20, 10 and 6 °C, respectively. Although, a significant difference was found in AsA content between samples stored at 1 and 10 °C, changes in this parameter were not sensitive enough as no difference was found between spinach leaves stored at 1 and 6 °C. Bergquist *et al*. (2006) investigated the effect of storage temperature on AsA retention in baby leaf spinach. Similar to our findings, they found better retention of AsA in spinach leaves stored at 2 °C when compared with their counterparts stored at 10 °C and suggested that AsA content may be a good predictor of shelf-life. The finding from our study (Experiment 2), however, suggests that AsA may not be the best parameter for determining the shelf-life of spinach. On the other hand, changes in plant pigment content were inconsistent and are not suitable as indicators of shelf-life.

7.2 Effect of light exposure during storage on quality changes of spinach leaves

Another objective of this project was to determine the effect of light exposure during storage on quality changes of baby leaf spinach and to investigate whether the observed responses were due to the light intensity or the total amount of light received by the leaves.

Not all quality measures studied responded to light environment. In general, high light led to deterioration in postharvest quality and this effect was most marked in leaf membrane integrity, antioxidant content and leaf colour. On the other hand, membrane integrity maintenance was improved by exposure of spinach leaves to low intensity light, whereas chlorophyll *a* and *b* content decreased. There was little response to photoperiod suggesting that the key factor in postharvest response was light intensity, with high light intensity leading to oxidative stress and quality loss.

Based on the results obtained in Experiment 3, 4 and 5 it can be concluded that during the storage of spinach leaves at 1 and 10 °C under light conditions (30-35 and 130-140 μmol m^{-2} s^{-1}) respiration was compensated by the photosynthetic activity of the leaves (Toledo *et al.*, 2003a) as no CO_2 development was detected inside the bags. No photosynthesis could occur in the dark, thus development of CO_2, as observed, was expected to take place in dark-stored samples (Experiments 3, 6 and 7).

In agreement with other studies (Kar and Choudhuri, 1986, Martinez-Sanchez *et al.*, 2011) solute leakage from spinach leaves was reduced by exposure to low intensity light (Experiment 3) when compared with their dark-stored counterparts. No benefit of light exposure during storage, however, was observed when light intensity was too high as solute leakage increased with increasing light intensity (Experiment 3 and 5). High intensity light may lead to excess oxidative stress that will cause tissue damage (Foyer and Shigeoka, 2011), which is often associated with increased lipid peroxidation (Hodges and Forney, 2003). The possible mechanism may also relate to tissue dehydration (Aguero *et al.*, 2008) which has been demonstrated to increase with increasing storage temperature (Pandrangi and LaBorde, 2004) and light intensity (Lester *et al.*, 2010b).

Furthermore, due to photosynthetic activity of spinach leaves stored under light conditions, the pool of carbohydrates may be increased (Toledo *et al*., 2003b), thus supplying substrates for respiration, whereas in the dark photosynthesis cannot proceed. This may result in leaf membrane lipids being used in respiration (Buchanan-Wollaston, 1997), which would lead to texture loss and could possibly explain why solute leakage from spinach leaves stored under low intensity light condition was improved when compared with their dark-stored counterparts.

Ascorbic acid content was found to be relatively stable during the storage of spinach leaves under low intensity light at 1 °C (Experiment 1, 2, 3, 4 and 5) or in the dark at 1 °C (Experiment 3) or 4 °C (Experiment 7). No significant difference in the content of AsA was found between samples stored in the dark and under continuous low intensity light. This is in contrast with other studies (Toledo *et al.*, 2003b, Lester *et al.*, 2010b) that reported better retention of AsA in light-stored samples. It is important to note that in our study AsA content is reported on dry weight (DW) basis while Toledo *et al*. (2003b) reported it on fresh weight (FW) basis. Furthermore cultivar differences have been reported by Lester *et al*. (2010b) who observed higher AsA content in light-stored spinach in cultivar Lazio, but not in the case of cultivar Samish, where no difference in AsA content between two storage conditions was found for top- and medium-canopy leaves after 9 days of storage. This may explain why no difference in AsA content between samples stored in the dark and under low intensity light was found in our study.

The loss of AsA was enhanced with increasing light intensity (Experiments 3, 4 and 5), which could possibly be explained by the increased water loss (Aguero *et al*., 2008). Decline in AsA content in spinach leaves stored under high light intensity may also be due to oxidative stress, where the level of AOS produced in response to high light exceeds the capacity of cells to scavenge them, thus leading to photooxidative damage (Asada, 2006). AsA acts not only as an antioxidant itself but plays a role in photoprotection, being a cofactor for violaxanthin deepoxidase in the xanthophyll cycle (Eskling *et al*., 1997). The xanthophyll cycle, where under high intensity light violaxanthin is transformed to zeaxanthin, is responsible for non-photochemical quenching of AOS,

which are mainly generated in the reaction centres of photosystem I and II. The xanthophyll cycle is induced by changes in pH across thylakoid membranes, which result from exposure to high intensity light (Asada, 2006).

Changes in the content of DHA in spinach leaves were inconsistent between experiments, as previously reported by Bergquist *et al*. (2006). The content of DHA is often used as an indication of stress in the leaf; however, care is needed as DHA can undergo further conversion, *e.g*. an irreversible hydrolysis to 2, 3-diketogulonic acid (Yang and Loewus, 1975).

Spinach leaves stored under light conditions (low intensity and high intensity light) were significantly lighter than their dark-stored counterparts (Experiment 3) but in terms of leaf yellowness, leaves stored under low intensity light were not significantly different from those stored in the dark. Increase in leaf lightness could be explained by a decrease in chlorophyll: carotenoids ratio. Leaf greenness (*a** value) was not affected by light conditions during storage at 1 °C (Experiment 3 and 5).

Kopas-Lane and Warthesen (1995) suggested that chlorophyll degradation is enhanced by light exposure during storage. Chlorophyll content decreased in samples stored under low intensity light when compared with their dark-stored counterparts, whereas no difference in chlorophyll content was observed between leaves stored under low and high intensity light conditions at 1 °C (Experiment 3 and 5).

Light exposure had no effect on total carotenoid content. This may be explained by their important role in photosynthesis and protection of chlorophylls and chloroplasts from photooxidative damage (Demmig-Adams *et al*., 1996). Furthermore, as already mentioned above, carotenoids may be transformed from one to another (xanthophyll cycle) in response to changes in light intensity.

Overall, increase in light intensity from 30-35 and 130-140 μmol m^{-2} s^{-1} resulted in a decline in AsA, DHA and total AsA, and increased solute leakage from spinach leaves. In contrast, change in light intensity had no effect on plant pigment content and leaf colour characteristics after 7 days of storage at 1 °C (Experiment 3 and 5).

To answer the question whether these observed responses were due to the light intensity or total amount of light received by the leaves, an experiment was conducted where spinach leaves received similar amounts of light by being exposed either to continuous (24 hours) low intensity light (30-35 μmol m^{-2} s^{-1}) or a short day (SD) photoperiod of 6 hours high intensity light (130-140 μmol m^{-2} s^{-1}) with 18 hours in the dark.

No differences in the quality of spinach stored under continuous (24 hours) low intensity light and SD (high intensity light/ dark (6/18 hours)) were observed (Experiment 4) with respect to gas composition, texture and visual quality (plant pigment content and leaf colour), however, the concentration of AsA and total AsA was lower in spinach leaves stored under SD conditions (high intensity light/ dark (6/18 hours)). This suggests that even relatively short exposure to high intensity light (130-140 μmol m^{-2} s^{-1}) may lead to an excess oxidative stress that accelerates AsA degradation, thus reducing nutritional quality of spinach leaves. This observation suggests, that exposure of spinach leaves to high intensity light (130-140 μmol m^{-2} s^{-1}) for only 6 hours, still induces stress response and production of AOS, which need to be scavenged within AsA-GSH cycle with concomitant decline in AsA. Further investigation should be carried out to identify the threshold in light intensity that does not compromise spinach quality, so that recommendations can be made to retailers and the supply chain.

7.3 Effect of temperature and light on quality of baby leaf spinach

It was clear from the previous experiments that both temperature and light conditions during storage are important in terms of quality maintenance during the storage of spinach. There were questions that still needed to be answered: how the changes in the quality of spinach leaves would respond over the range of temperatures (1 and 10 °C) if stored under different light conditions (low intensity (30-35 µmol m^{-2} s^{-1}) and high intensity (130-140 µmol m^{-2} s^{-1}) light)? Would the effect be additive or is there an interaction between two factors? Finally, which of two factors – temperature or light – has a greater effect on the quality of spinach?

In Experiment 5, solute leakage increased with increasing temperature of storage from 1 to 10 °C and light intensity from 30-35 to 130-140 µmol m^{-2} s^{-1}. This finding was in agreement with findings from Experiment 1 and 3. The effect of temperature and light conditions during storage on leaf texture was additive as the lowest solute leakage was observed in spinach leaves stored at 1 °C under low intensity light followed by samples stored under high intensity light at the same temperature. The highest solute leakage was reported for spinach leaves stored at 10 °C under high intensity light condition. It also gives clear evidence that storage temperature has a greater effect on leaf texture than intensity of light.

In Experiment 5, AsA and total AsA content declined with increasing temperature, while increase in light intensity significantly reduced AsA and total AsA content only in case of samples stored at 1 °C, but not those stored at 10 °C. This finding is in agreement with findings from Experiment 1 and 3. It does suggest that storage temperature has a much greater effect on nutritional quality of spinach leaves, whereas the effect of light on AsA content at higher temperature seems to be minor.

No differences in plant pigment content (chlorophyll *a*, *b* and total carotenoids) were observed in Experiment 5 between spinach leaves stored at 1 and 10 °C under low intensity light. This finding is in contrast with Experiment 1, where plant pigment content decreased with increasing temperature of storage. No difference was also observed

between spinach leaves stored at 1 °C under low and high intensity light conditions, which was in agreement with findings from Experiment 3, where light had no effect on plant pigment content in spinach. This lack of consistency further supports the statement that plant pigments are not good indicators of shelf-life. Interestingly, the greatest decline in all plant pigments was reported in spinach leaves stored at 10 °C under high intensity light conditions, which suggests that these compounds are very sensitive to changes in light intensity when stored at higher temperature. This may be due to chemical reactions (*e.g.* degradation of chlorophyll) that are faster at 10 than at 1 °C being further accelerated (additive effect) by oxidative stress induced by excessive light.

In experiment 5, leaf lightness (*L** value) and yellowness (*b** value) increased with increasing temperature from 1 to 10 °C, while these parameters were not affected by light intensity when spinach was stored at 1 °C. These findings are in agreement with Experiment 1 and 3. Interestingly, as a result of interaction between temperature and light conditions during storage, the greatest decline in visual quality of the leaves was reported in spinach leaves stored at 10 °C under high intensity light conditions, which suggests that these parameters are very sensitive to changes in both temperature and light intensity.

Production of AOS in plant cells increases with increasing temperature from 1 to 10 °C and is further enhanced by exposure to high intensity light which causes additional damage at cellular level. These two stresses combined together may cause damage to photosynthetic apparatus, which would probably induce leaf senescence as physiological processes in the cell could not be maintained. Enhanced quality deterioration in spinach leaves stored at 10 °C under high intensity light conditions, gives clear evidence that the response of leaves subjected to a combination of two different stresses is different from the response to each of them applied independently (Experiment 1 and 3). Thus, studying plant responses to multiple stresses is a serious challenge; it requires good understanding of different signalling pathways and the interactions (cross-talk) between them (Fujita *et al*., 2006). These complex signalling networks include several components, *e.g.* AOS, plant hormones (abscisic acid, salicylic acid, jasmonic acid and ethylene) and kinases.

The mechanisms how these pathways are coordinated in the cell, however, are still not yet well understood.

Hewezi *et al*. (2008) have conducted a study, where they investigated gene expression patterns in sunflower (*Helianthus annuus* L.) plants exposed to high temperature and high light stresses applied individually and in combination. They found that the expression of number of genes was induced in response to a particular stress. Based on their observations, suggestion can be made that the response of a plant to two different stresses cannot be deduced from results obtained with these stresses applied independently from each other.

There is a clear message to the industry sector that even though the temperature of storage is a key factor affecting the quality maintenance of baby leaf spinach, having greater effect on spinach quality than light, the role of the latter one cannot be underestimated. Light has been reported to affect textural and nutritional quality of spinach leaves when bags were stored at 1 °C (Experiment 3), and the important role of light has been demonstrated with increasing temperature of storage (Experiment 5).

The reasons why temperature may have a greater effect on quality of spinach leaves than light exposure have been discussed in the sections 7.1 and 7.2. Briefly, both stresses induce production of AOS which is enhanced with increasing dose (temperature or light intensity). Based on the results obtained in this research it can be postulated that increase in temperature within the range studied, enhances the respiration rate and alters activity of the enzymes involved in metabolism, including those responsible for scavenging of AOS, cell wall/membrane degradation and biosynthesis/turnover of phytochemicals. On the other hand, with increasing light intensity within the range studied, membrane integrity and antioxidant content were the only parameters that were affected. Care must also be taken when interpreting these observations because the range of temperature studied was bigger than the range of light intensities, *i.e.* the highest temperature of 20 °C caused severe damage to the leaf, where the end of shelf-life was reached after 3 days of storage, while the effect of highest light intensity was less pronounced. The combined

study gives clear indication about the important role of temperature and light exposure during the storage of baby leaf spinach and highlights the necessity to consider both factors in the future studies.

7.4 Effect of pre-storage hot water treatment on quality changes of spinach leaves

Another question to be answered was whether pre-storage hot water treatments can enhance or maintain postharvest quality of spinach leaves? The pre-storage hot water treatment at 45 °C for 60 s applied immediately after harvest was identified as the maximum heat shock treatment that could be used before subsequent damage was observed in spinach leaves. A second more detailed study showed that this hot water treatment did not prolong the shelf-life of spinach or increase tissue quality. The treatment did appear to protect total carotenoid concentration compared to untreated samples. Results indicate that hot water treatments have limited commercial potential for quality improvement of spinach leaves. This finding is in contrast with findings of Gomez *et al*. (2008) and Koukounaras *et al*. (2009) who reported improved quality of spinach and rocket leaves in response to pre-storage hot water treatments at 40 °C for 3.5 min and 50 °C for 30 s, respectively. This different response may be explained by different temperature conditions during subsequent storage (Gomez *et al*., 2008) or sensitivity of different commodities. This finding gives valuable information to the industry, as in contrast to number of papers reporting benefits of pre-storage hot water treatment on quality maintenance during subsequent storage of lettuce (Murata *et al*., 2004; Martin-Diana *et al*., 2006), rocket (Koukounaras *et al*., 2009) and spinach (Gomez *et al*., 2008), this is not the case, when spinach is stored under commercial, refrigerated conditions.

7.5 Broader questions

Does antioxidant content correlate with shelf-life?

There was a need to answer the question whether the content of particular compounds, *i.e*. antioxidants (AsA or carotenoids), is important in terms of shelf-life improvement in spinach as suggested by Bergquist *et al*. (2006). To answer this question, correlation analyses were conducted (Appendix 1). Neither AsA nor total carotenoid content correlated with shelf-life related characteristics (*e.g.* solute leakage, leaf colour), thus it can be concluded that shelf-life in spinach is not associated with the content of these particular antioxidants.

How does chlorophyll content relate to leaf texture?

Another intriguing question was why there was a difference in solute leakage values between experiments. A moderate correlation (r=0.535) was found between total chlorophyll content and solute leakage (Figure 7.1). To confirm, whether the correlation is correct the data for all experiments was checked. High total chlorophyll content (Experiment 1 and 3) was indeed associated with higher solute leakage. This finding is supported by others (Cuppett *et al*., 1999) who found a correlation between total chlorophyll content and the level of nitrogen applied in the fertilizer. Increasing the nitrogen level leads to darker green leaves. These leaves, however, were found to be softer (Cuppett *et al*., 1999), which is likely to result in shorter shelf-life (Clarkson *et al.*, 2003) during postharvest storage. Leaf strength was also found to be reduced in response to applied nitrogen in iceberg lettuce (Newman *et al*., 2005), however, these authors observed that this loss in texture can be reduced if plants are grown with addition of calcium. This means that the level of nitrogen in the fertilizer should be carefully adjusted bearing in mind its effect on postharvest quality of the product. This might be a challenge for breeders and growers as there is a high consumer demand for dark green leaves, however, if the quality of the product could be maintained and its shelf-life extended this might be a feasible solution.

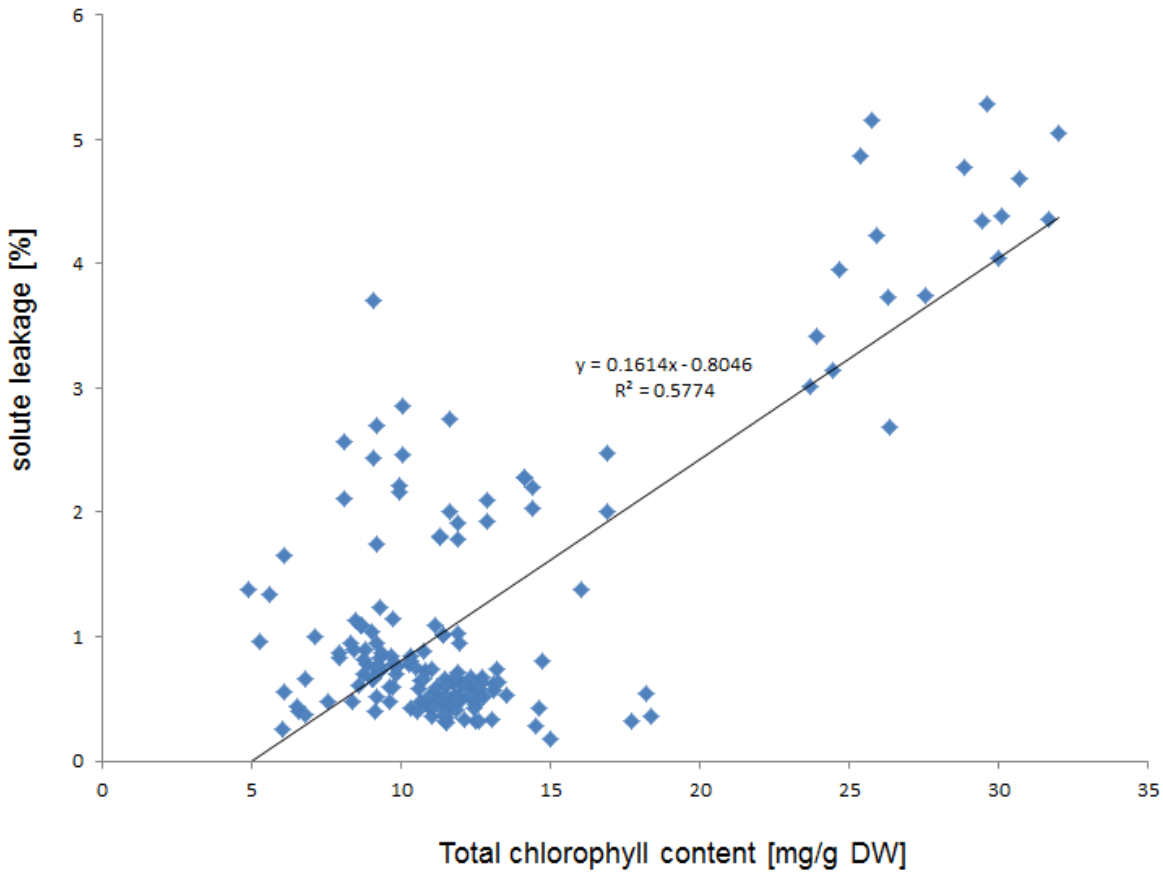

Figure 7.1 Correlation between total chlorophyll content [mg/g DW] and solute leakage from spinach leaves during storage. Data points include readings from experiments 1, 2, 3, 4, 5 and 7, excluding the values from high (10 and 20 °C) temperature of storage.

Are leaf colour parameters correlated with each other?

Care must be taken when comparing different studies as seasonal differences in colour parameters of spinach leaves have been reported by Conte *et al*. (2008). Furthermore, using leaf greenness (*a** value) as a measure of postharvest quality might be a bit misleading as dark green leaves could often be seen (by Minolta) as less green when compared with bright green leaves. To support the latter statement, correlation analyses were conducted. The outcome from these analyses is presented on Figure 7.2. It can be seen (Figure 7.2 A) that leaf lightness (*L** value) correlates (r=0.601) well with leaf yellowness (*b** value). An even stronger correlation (r=-0.746) was found between leaf greenness (*a** value) and leaf yellowness (Figure 7.2 B). This means, that with increasing yellowness spinach leaves become lighter and greener. Thus, as mentioned before bright green leaves are recognised as being greener than their dark green counterparts.

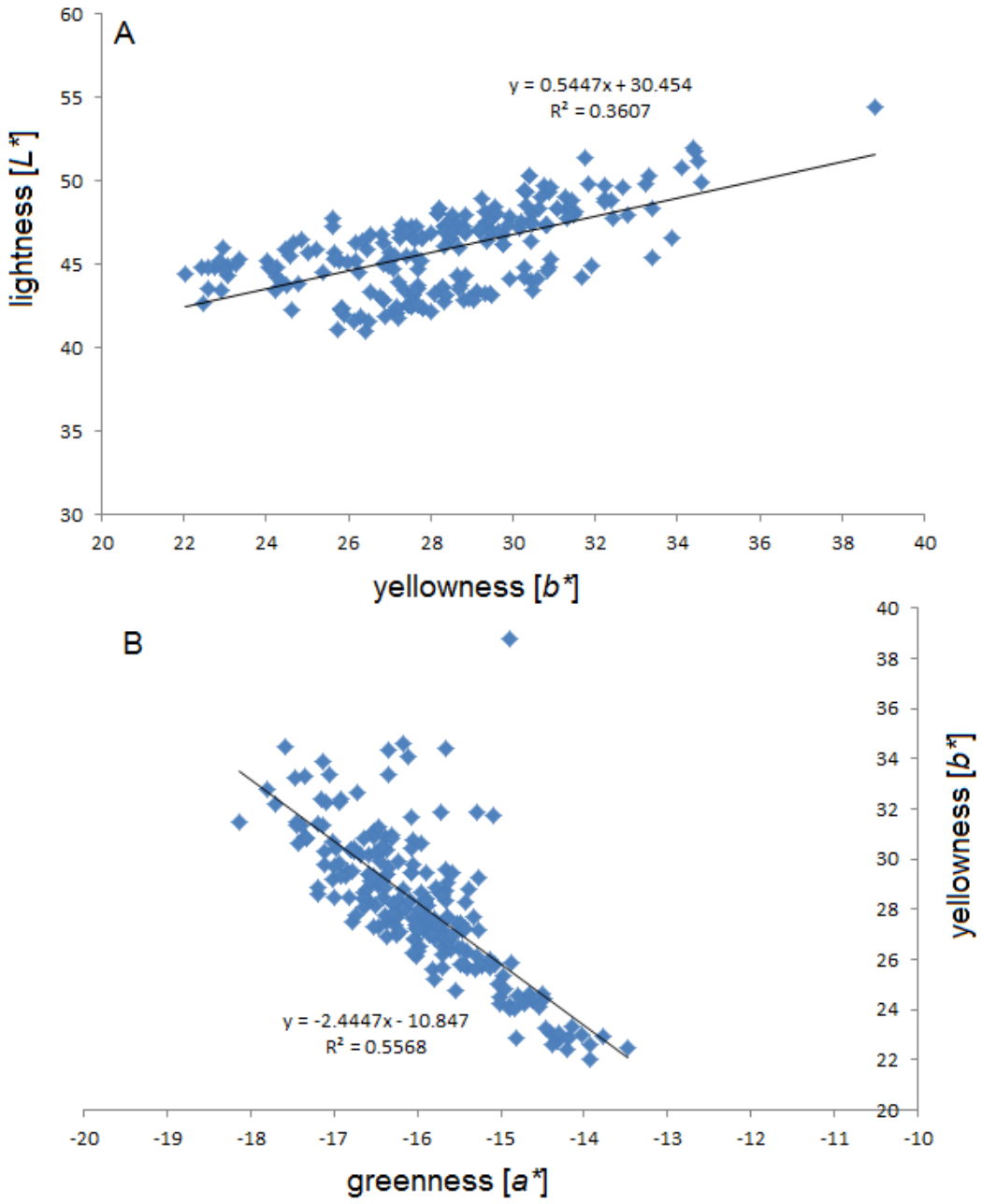

Figure 7.2 Correlations between leaf colour characteristics: (A) between leaf yellowness (*b**) and lightness (*L**), (B) between leaf greenness (*a**) and yellowness (*b**). Data points include readings from all experiments.

Do changes in chlorophyll a: b ratio correlate with leaf colour changes?

In addition to correlations mentioned above, a weak, nonetheless significant (P<0.05) correlation (r=-0.260) was found between chl *a*: chl *b* ratio and leaf lightness (L^* value). This correlation suggests that with decreasing chl *a*: chl *b* ratio leaf lightness increases, which could be explained by the fact that chl *b* is brighter than chl *a* (Hortensteiner, 2006). Interestingly, the ratio did not correlate with other colour characteristics – leaf greenness (a^* value) and yellowness (b^* value), which suggests that chl *a*: chl *b* ratio is not a good indicator for assessing the quality of baby leaf spinach.

Are pre-harvest conditions important in terms of postharvest shelf-life?

Finally, a number of authors (Bergquist *et al*., 2006; Conte *et al*., 2008) reported seasonal differences in the quality of spinach leaves. Even though Boese and Huner (1990) suggested that spinach was not stressed when grown at the temperature of 5 °C, they did observe an increase in the leaf thickness and strength compared with the leaves grown at 16 °C. Antioxidant content, however, was not determined in their study. Interestingly, in our research, the highest AsA content was found in Experiment 7, conducted on spinach grown at the lowest average temperature of 8.7 °C, while the lowest content was reported in Experiment 5, when the temperature (14.9 °C) was highest. This may suggest that growth at low temperature may actually induce some stress related responses leading to increased nutritional value of baby leaf spinach, thus requiring further investigation.

7.4 Overall conclusions

A. Storage temperature affected nutritional quality of baby leaf spinach. Quality loss was accelerated with increasing temperature of storage;

B. Storage temperature affected visual and textural quality of baby leaf spinach. Visual quality declined (spinach leaves were more yellow) with increasing temperature of storage;

C. Light conditions during storage affected nutritional quality of baby leaf spinach. High intensity light led to oxidative stress and loss of nutritional value of spinach;

D. Light conditions during storage affected visual quality of baby leaf spinach. With increasing light intensity, spinach leaves became lighter and more yellow;

E. Light conditions during storage affected texture of baby leaf spinach. Texture was well maintained under low intensity light conditions, whereas high intensity light led to tissue damage;

F. Both temperature and light conditions during storage are important with respect to quality maintenance in baby leaf spinach;

G. Pre-storage hot water treatment affected nutritional quality of baby leaf spinach (higher total carotenoid content) but had no effect on leaf texture;

H. Pre-storage hot water treatment affected visual quality of baby spinach. Heated spinach leaves were lighter and more yellow than their unheated counterparts;

I. Shelf-life in spinach is not associated with the content of antioxidants;

J. Leaf greenness (*a** value) should not be used as a measure of quality of spinach.

7.5 Recommendation for future studies

Effect of hot water treatments on individual carotenoids and flavonoids in spinach

As mentioned in the literature review (Chapter 1) information on changes in carotenoids and flavonoids in response to hot water treatments is not available. To our knowledge, this is the first time (Chapter 6) when the effect of hot water treatment at 45 °C for 60 s on total carotenoid content of spinach has been reported. More detailed analysis – changes in the concentrations of individual compounds, *e.g.* lutein and *β*-carotene – however, is still missing. It is important to understand changes in the carotenoid biochemistry of spinach in response to hot water treatment because different carotenoids play a different role in human health. For the same reason it would be interesting to investigate changes in individual flavonoids – a group of compounds that possess antioxidant capacity and have also been reported to be important to humans.

Effect of light exposure during storage on membrane integrity

Maintaining membrane integrity seems to be one of the key factors in terms of extending the shelf-life of spinach. The role of membrane in postharvest physiology has been reviewed (Marangoni *et al*., 1996). More recently, Wagstaff *et al*. (2010) demonstrated that lettuce with reduced membrane permeability and modified cell wall properties exhibited improved shelf-life. Light exposure during storage has been reported to reduce solute leakage from lettuce (Martinez-Sanchez *et al.*, 2011) and spinach leaves (Kar and Choudhuri, 1986) when compared with dark-stored counterparts. A similar observation was reported in our study (Experiment 3). The mechanism behind this response, however, remains unclear. Thus, it would be useful to conduct more detailed analyses (similar to those of Wagstaff *et al*. (2010)) of cell wall properties of spinach leaves stored under low intensity light and in the dark. Better understanding of this mechanism could be beneficial for plant breeders, so that the new cultivars, possibly with extended shelf-life would appear on the market.

Manipulating light quality during storage

Information on the effects of light quality manipulation during the storage of spinach is scarce. Only studies that compared light- and dark-stored leaves have been conducted (Kopas-Lane and Warthesen, 1995; Toledo *et al*., 2003b; Lester *et al*., 2010b). Thus, to consider potential application of photobiology in postharvest storage of spinach, a better understanding of the effects of different light wavelengths on plant's biochemistry is necessary.

Plants have specific photoreceptors that sense different wavelengths of light. These are phytochromes (red and far red light), cryptochromes (blue light) and phototropins (Gyula *et al.*, 2003, Karpinski *et al.*, 2003). The signal transduction pathways that are regulated by phytochrome (Quail, 2002, Schafer and Bowler, 2002, Gyula *et al.*, 2003), cryptochrome and phototropins (Quail, 2002, Gyula *et al.*, 2003, Liscum *et al.*, 2003) have been reviewed and the expression of large numbers of genes is induced in response to light.

Due to the different wavelengths being absorbed by different pigments in plant leaves (Lefsrud *et al.*, 2008) it may be expected that the change in light spectrum may influence the concentration of these compounds. Recently, light emitting diodes (LEDs) have been found to be a useful and promising tool to investigate the effect of light wavelength manipulation on plant biochemistry (Ohashi-Kaneko *et al.*, 2007, Lefsrud *et al.*, 2008, Li and Kubota, 2009).

Light manipulation can be used to improve nutritional quality of spinach by increasing the concentration of bioactive compounds (Ohashi-Kaneko *et al*., 2007; Lefsrud *et al*., 2008; Li and Kubota, 2009), *e.g*. neither blue nor red light applied individually had a significant effect on AsA content in spinach, whereas when both blue and red light were applied simultaneously AsA increased (Ohashi-Kaneko *et al*., 2007). Blue light enhanced carotenoid and chlorophyll concentrations in spinach (Matsuda *et al.*, 2007, Ohashi-Kaneko *et al.*, 2007). LEDs can either be used as the only source of light during storage or in addition (light supplementation) to white light.

Use of metabolomics in postharvest studies

It seems that in the near future, postharvest studies should consider the use of metabolomics (Cevallos-Cevallos *et al*., 2009; Kim *et al*., 2011). ^{1}H NMR metabolite profiling might be a solution, as it is a fast and simple method for determining the presence of specific compounds within the leaf tissue (Kim *et al*., 2011). Furthermore, being able to screen multiple compounds at a time, means that biochemical pathways could be constructed and this would improve our understanding of leaf biochemistry.

Recommendation for growers and retailers

In agreement with Nunes *et al*. (2009), who suggested that temperature maintenance in the supply chain is the most important factor affecting the quality and shelf-life of fresh produce; it has been found in this research that storage temperature has a greater effect on quality of baby leaf spinach than intensity of light during storage. The role of light exposure, however, cannot be underestimated as it has been reported that it may also affect the quality of spinach. Based on the obtained results, recommendation can be made to keep spinach at low refrigerated temperature, ideally close to 1 °C, under low intensity light conditions.

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Appendix 1

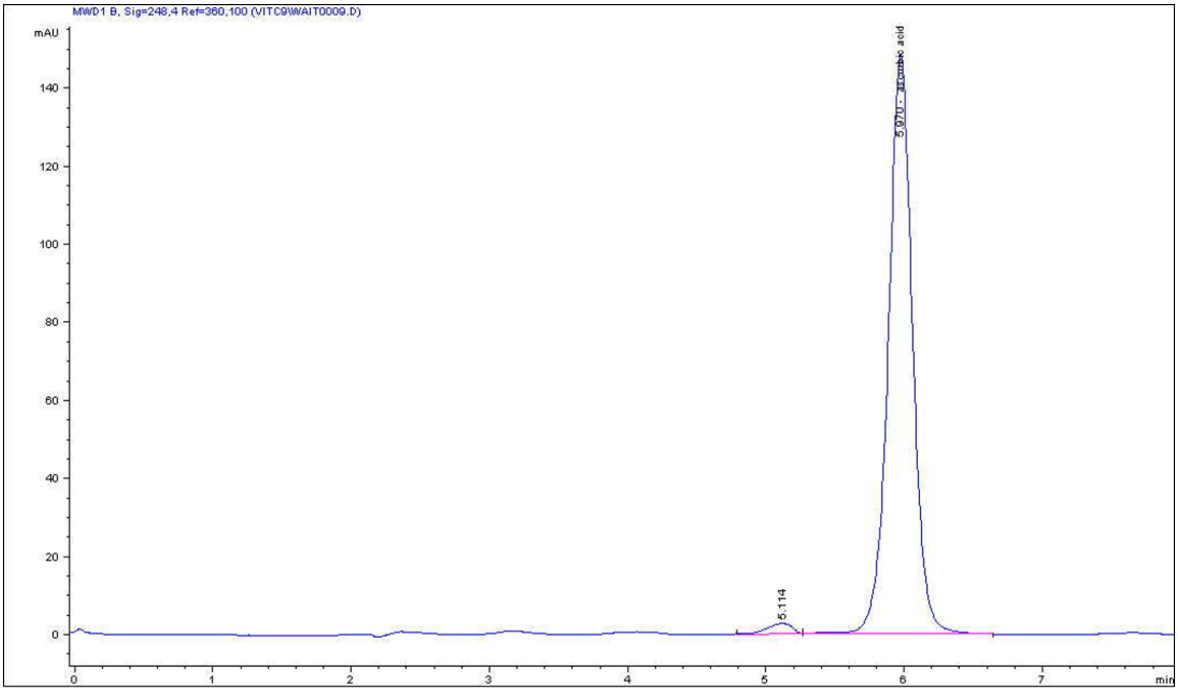
Correlation between different measures used for quality assessment of baby leaf spinach (analysis of combined data from Experiments 1-5 and Experiment 7).

	Solute leakage	AsA	total AsA	chl *a*	chl *b*	chl *a*: chl *b* ratio	total chl	total carotenoids	chl: carotenoids ratio	*L**	*a**	*b**
solute leakage	1											
AsA	-0.211	1										
total AsA	-0.188	0.986*	1									
chl *a*	0.520*	-0.200	-0.220	1								
chl *b*	0.560*	-0.170	-0.150	0.960*	1							
chl *a*: chl *b* ratio	-0.290*	0.230	0.210	-0.210	-0.430*	1						
total chl	0.535*	-0.229	-0.208	1	0.980*	-0.260*	1					
total carotenoids	-0.150	0.230	0.240	0.390*	0.420*	-0.210	0.400*	1				
chl: carotenoids ratio	0.210	-0.160	-0.140	0.900*	0.860*	-0.200	0.890*	-0.020	1			
L*	0.064	-0.203	-0.201	-0.100	0.010	-0.260*	-0.076	-0.080	0.001	1		
a*	0.028	-0.078	-0.099	-0.160	-0.230	0.190	-0.169	-0.240	-0.010	-0.461*	1	
b*	0.091	-0.042	-0.009	-0.030	0.010	-0.050	-0.021	0.160	-0.090	0.601*	-0.746	1

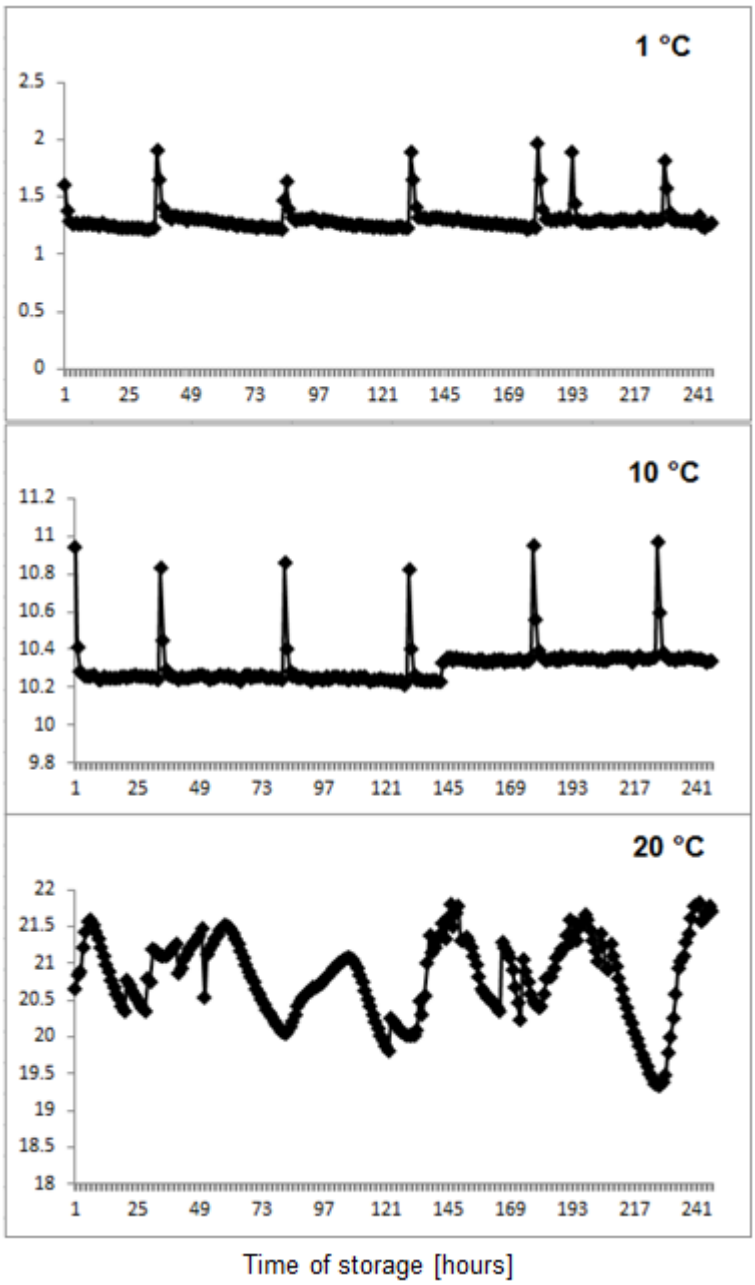
*significant at $P<0.05$.

Appendix 2

HPLC chromatogram of ascorbic acid (AsA).

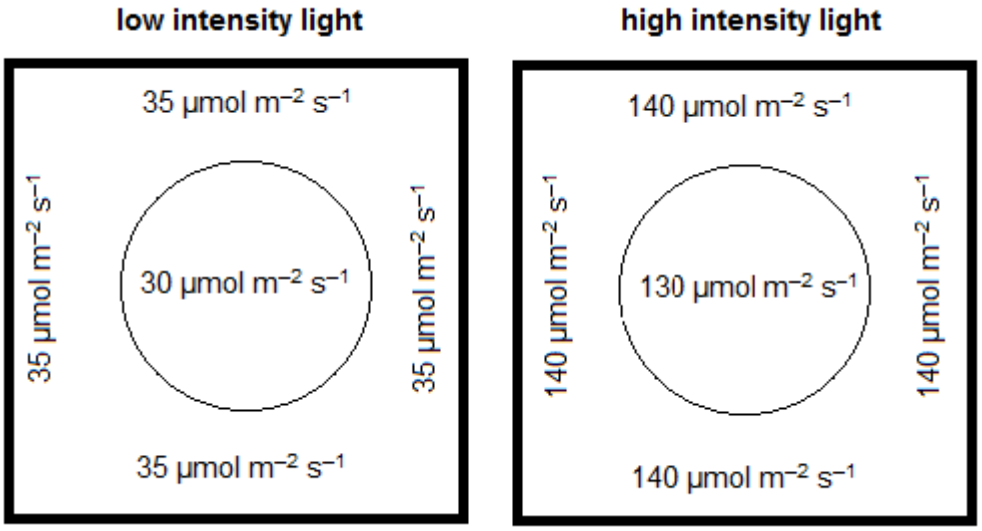


Appendix 3



Indicative data of temperature variation in the growing cabinets as recorded with Tinytag™ temperature loggers (Gemini Data Loggers Ltd, UK) during the storage of spinach samples at 1, 10 and 20 °C during the 10 days (240 hours) period.

Appendix 4



Indicative data of light intensity variation in the growing cabinets as recorded with quantum sensor (Skye Instruments Ltd, UK) during the storage of spinach.