Health-related compounds in broccoli (*Brassica oleracea* L. var. *italica*) as affected by postharvest temperature, light and UV-B irradiation

Effekt av temperatur, lys og UV-B på helserelaterte innholdsstoffer i brokkoli (*Brassica oleracea* L. var. *italica*) etter høsting

Philosophiae Doctor (PhD) Thesis

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Abstract

Broccoli (*Brassica oleracea* L. var. *italica*) belongs to *Brassica* vegetables, consumption of which has been shown to reduce the risk of several types of cancer and cardiovascular disease mortality in epidemiological studies. The health-promoting properties of broccoli have been attributed to high levels of bioactive compounds including vitamin C, flavonols and glucosinolates (GLS) in this vegetable. The contents of these compounds in broccoli at harvest are not only determined by pre-harvest factors but are further influenced by various postharvest factors on the way from producer to consumer. However, the effect of storage temperature on flavonols and GLS in particular is still not clear and little is known about how postharvest light influences vitamin C and GLS contents. Moreover, UV irradiation is considered as a tool for enhancing contents of bioactive compounds in fresh fruits and vegetables after harvest. However, little is known how temperature influences the outcome of postharvest light or UV-B radiation treatment. Therefore, the main purpose of the present study was to evaluate the effects of postharvest temperature, light and UV-B irradiation on the vitamin C, *L*-ascorbic acid (AA), dehydroascorbic acid (DHA), flavonols and GLS levels in broccoli flower buds in an experiment with multi-level design simulating mapped commercial storage conditions from harvest until consumer purchase. The contents of these compounds were examined in flower buds during pre-storage and storage of broccoli heads, representing refrigerated transport with wholesale distribution and retail, respectively. In addition, the GLS analysis was conducted for floret stalks of chosen samples and epidermal flavonols in flower buds of all broccoli heads were monitored during storage using non-destructive repeated measurements. Broccoli heads were pre-stored for 4 or 7 d at 0 or 4 °C in the dark and then stored for 3 d at 10 or 18 °C. During storage, the broccoli heads were exposed for 12 h per day

to three different levels of visible light (13, 19 or 25 μmol m^{-2} s^{-1}) or a combination of visible light (19 μmol m^{-2} s^{-1}) and UV-B irradiation (20 kJ m^{-2} d^{-1}), or they were stored in the dark.

The vitamin C, AA, DHA, flavonols and GLS levels in broccoli flower buds were not significantly changed during pre-storage at both temperatures 0 °C or 4 °C for both 4 and 7 d. Moreover, the contents of flavonols and GLS were found to be in general stable, while vitamin C levels degraded as a function of increasing pre-storage period (PP), pre-storage temperature (PT) and storage temperature (ST) during storage at higher temperatures (10 °C or 18 °C). However, the levels of epidermal flavonols, monitored with non-destructive repeated measurements of the broccoli heads during storage, were observed to increase with prolonged PP and storage period. In addition, general linear model analysis revealed that PP and ST significantly influenced almost all GLS levels, except for 4-methoxyglucobrassicin in broccoli flower buds during storage. Higher levels of these compounds were found in broccoli pre-stored for 7 d than in those pre-stored for 4 d. The 4-hydroxyglucobrassicin content was increased after storage at 18 °C, while the levels of all other GLS were higher in flower buds of broccoli after storage at 10 °C than at 18 °C. Moreover, the effect of ST on aliphatic GLS in broccoli flower buds depended on PT and it was negligible for broccoli heads pre-stored at 4 °C but significant for those pre-stored at 0 °C. For flavonols the PP × PT interaction was found significant, with higher contents after storage observed for broccoli pre-stored shorter (4 d) at 4 °C and pre-stored longer (7 d) at 0 °C. In addition, the PP × ST interaction was found significant for vitamin C and DHA and to a minor extent for AA, indicating that the difference in contents of these compounds in flower buds of broccoli stored at 10 °C and at 18 °C depended on PP and was higher for broccoli pre-stored for 4 d. The DHA level and DHA/AA ratio were stable in flower buds of broccoli pre-stored for 7 d during storage but were increased in those pre-stored for 4 d. These findings suggest a shift in the ascorbate metabolism in broccoli flower buds during storage with its higher rate observed for broccoli pre-stored for

shorter time. The radiation treatments significantly affected both quercetin content and epidermal flavonol levels in broccoli flower buds during storage, with the highest levels observed after a combination of visible light and UV-B irradiation treatment. However, the outcome of this postharvest treatment was influenced by a combination of factors including PP, PT and ST. The accumulation of epidermal flavonols upon UV-B exposure was initiated earlier in broccoli heads stored at higher temperature (18 °C), that is, already during the first day of storage. However, no effect of radiation treatments was found for vitamin C and GLS contents in broccoli flower buds during storage.

In conclusion, the present study has shown that PP and PT influence the contents of vitamin C, flavonols and GLS during storage, which emphasise the importance of pre-storage treatment for both postharvest research and commercial industry. Moreover, the potential of UV-B irradiation combined with visible light to increase flavonol contents in broccoli flower buds during storage has also been demonstrated. These findings will hopefully contribute to improved postharvest handling procedures in order to preserve high contents of health-related compounds in broccoli.

Sammendrag

Brokkoli tilhører slekten *Brassica* som representerer mange viktige grønnsaker. Disse har i epidemiologiske undersøkelservist å kunne redusere risikoen for ulike typer kreft og redusere dødeligheten ved hjertekarsykdommer hos mennesker. De positive egenskapene hos brokkoli har blitt knyttet til bioaktive forbindelser som vitamin C, flavonoler og glukosinolater (GLS). Innholdet av disse påvirkes av en rekke faktorer før høsting, under lagring og gjennom distribusjonskjeden frem til forbruker. I dag er det fortsatt uklart hvordan temperatur og lys etter høsting påvirker innholdet av flavonoler, vitamin C og glukosinolater. UV stråling har blitt sett på som et potensielt middel for å kunne øke innholdet av bioaktive forbindelser i frukt og grønt etter høsting, til tross for at lite er kjent hvordan UV-B påvirker spesifikke forbindelser og hvor trolig temperaturen er viktig.

Hensikten med denne studien var å evaluere effekten av temperatur, lys og UV-B etter høsting på innholdet av vitamin C, L-askobinsyre (AA), dehydroaskorbinsyre (DHA), flavonoler og glukosinolater i brokkoli knopper. Studien var designet som en fler-faktor studie og simulerte kommersiell distribusjon og omsetning av brokkoli fra høsting frem til forbruker. Innholdet av vitamin C, L-askobinsyre (AA), dehydroaskorbinsyre (DHA), flavonoler og glukosinolater ble analysert i knopper av brokkoli lagret under forhold tilsvarende distribusjon (pre-storage) og butikk (storage). Brokkoli ble lagret mørkt i 4 eller 7 dager ved 0 eller 4 °C (distribusjon) for deretter å bli overført til 10 eller 18 °C de påfølgende 3 dagene (butikk). I denne perioden ble grupper av brokkoli eksponert for tre ulike nivåer lys med hvite lysstoffrør (13, 19 or 25 μmol m^{-2} s^{-1}) og en kombinasjon av lys (19 μmol m^{-2} s^{-1}) og UV-B (20 kJ m^{-2} d^{-1}) i 12 timer. En kontrollgruppe ble lagret i mørke. For utvalgte behandlinger ble i tillegg brokkoli stengler analysert for innhold av glukosinolater. Innhold av flavonoler i epidermis i brokkoli knopper, ble analysert ved hjelp av en ikke-destruktivt målemetode.

Det ble ikke registrert endringer i innhold av vitamin C, AA, DHA, flavonoler og glukosinolater under distribusjon ved 0 eller 4 °C i 4 eller 7 dager. Generelt ble innholdet av flavonoler og glukosinolater funnet å være stabilt, mens vitamin C ble brutt ned som en følge av økende tid og temperatur under distribusjon, samt høyere temperatur i butikk (10 °C og 18 °C). Innholdet av flavonoler i epidermis, målt ikke-destruktivt gjennom hele perioden, økte med antall dager i distribusjonsleddet og i butikk.

Ikke destruktive målinger viste at innholdet av flavonoler i epidermis økte med økende tid i distribusjon og butikk. Statistiske beregninger viste at antall dager i distribusjon og temperatur i butikk hadde en signifikant effekt på alle glukosinolater i brokkoli knopper med unntak av 4-metoxyglukobrassicin. Høyere nivåer av flavonoler og glukosinolater ble funnet i brokkoli med 7 dager i distribusjon. Innholdet av 4-metoxyglukobrassicin var høyere etter 3 dager ved 18 °C sammenlignet med 10 °C, mens for alle andre glukosinolater var nivået høyere ved 10 °C sammenlignet med 18 °C. For alifatiske glukosinolater var effekten av temperatur i butikk avhengig av temperaturen under distribusjon med ubetydelig effekt ved 4 °C, mens 0 °C ga en betydelig effekt. For flavonoider ble det funnet en signifikant sammenheng mellom antall dager og temperatur under distribusjon og innholdet av flavonoler de påfølgende dagene i butikk, da knopper av brokkoli med kortere tid i distribusjon, 4 dager ved 4 °C hadde høyere innhold sammenlignet med knopper av brokkoli etter 7 dager ved 0°C. I tillegg ble det funnet en signifikant sammenheng mellom antall dager i distribusjon og temperatur de påfølgende dagene i butikk for vitamin C og DHA og i mindre grad for AA. Dette indikerer at de observerte forskjellene i innhold mellom knopper av brokkoli oppbevart ved 10 °C og 18 °C er påvirket av antall dager i distribusjon da innholdet var høyere etter opphold i butikk for broccoli som hadde vært i distribusjon i 4 dager. Innholdet av DHA og DHA/AA forholdet var stabilt for brokkoli som hadde vært 7 dager i distribusjon, mens det ble observert høyere verdier for brokkoli med kortere distribusjonstid. Dette indikerer en endring i metabolismen av askorbat,

der brokkoli med kortere distribusjonstid har en høyere metabolisme. Lys og UV-B påvirket innholdet av quercetin i brokkoli knopper og innholdet av flavonoler i epidermis, der de høyeste nivåene ble funnet for lys i kombinasjon med UV-B. Responsen på lys og UV-B var avhengig av antall dager og temperatur under distribusjon samt temperatur de påfølgende dagene i butikk. Akkumulering av flavonoler i epidermis i brokkoli knopper som en følge av UV-B eksponering ble initiert tidligere ved høyere temperatur (18 °C), det vil si allerede i løpet av den første dagen. Det ble ikke funnet noen effekt av lys og UV-B på innholdet av vitamin C eller glukosinolater.

Denne studien har vist at antall dager i distribusjon og temperatur i denne perioden påvirker innholdet av vitamin C, flavonoler og glukosinolater i brokkoli den påfølgende perioden i butikk. Videre viser studien at UV-B i kombinasjon med lys har potensiale til å øke innholdet av flavonoler i brokkoli knopper. Disse resultatene gir viktig informasjon når det gjelder potensielle forbedringer innen håndtering av brokkoli i distribusjon og salg med tanke på å ivareta kvaliteten.

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Ås, 27 October 2015

Anna Rybarczyk-Plonska

List of papers

Paper I

Rybarczyk-Plonska, A., Hansen, M.K., Wold, A.-B., Hagen, S.F., Borge, G.I.A., Bengtsson, G.B., 2014.

Vitamin C in broccoli (*Brassica oleracea* L. var. *italica*) flower buds as affected by postharvest light, UV-B irradiation and temperature.

Postharvest Biol. Technol. 98, 82-89.

Paper II

Rybarczyk-Plonska, A., Wold, A.-B., Bengtsson, G.B., Borge, G.I.A., Hansen, M.K., Hagen, S.F.

Flavonols in broccoli (*Brassica oleracea* L. var. *italica*) flower buds as affected by postharvest temperature and radiation treatments.

Submitted to Postharvest Biology and Technology

Paper III

Rybarczyk-Plonska, A., Hagen, S.F., Borge, G.I.A., Bengtsson, G.B., Hansen, M.K., Wold, A.-B.

Glucosinolates in broccoli (*Brassica oleracea* L. var. *italica*) as affected by postharvest temperature and radiation treatments.

Submitted to Postharvest Biology and Technology

Abbreviations

4-Me-GB	4-metoxyglucobrassicin
4-OH-GB	4-hydroxyglucobrassicin
AA	Ascorbic acid
ANOVA	Analysis of variance
A_{UV}	Relative epidermal ultraviolet A absorbance
CHI	Chalcone isomerase
ChlF	Chlorophyll fluorescence
CHS	Chalcone synthase
CoA	Coenzyme A
COP1	CONSTITUTIVELY PHOTOMORPHOGENIC 1 ubiquitin ligase
CYP	Cytochrome P
DFR	Dihydroflavonol 4-reductase
DHA	Dehydroascorbic acid
DNA	Deoxyribonucleic acid
ESP	Epithiospecifier protein
F3'5'H	Flavonoid 3'5'-hydroxylase
F3H	Flavanone 3-hydroxylase
F3'H	Flavonoid 3'-hydroxylase
FLS	Flavonol synthase
GAL	Glucoalyssin
GB	Glucobrassicin
GDP	Guanosine diphosphate
GER	Glucoerucin
GIB	Glucoiberin
GLM	General linear model
GLS	Glucosinolates
GRA	Glucoraphanin
HPLC	High performance liquid chromatography
HY5	ELONGATED HYPOCOTYL 5 transcription factor
LC-MS	Liquid chromatography – Mass spectrometry
MDHA	Monodehydroascorbic acid
MYB12	Myeloblast family transcription factor 12
NGB	Neoglucobrassicin
PAL	Phenylalanine ammonia-lyase
PCA	Principal Component analysis
PP	Pre-storage period
PT	Pre-storage temperature
R	Radiation treatments
ROS	Reactive oxygen species
SP	Storage period
ST	Storage temperature
UV-A	Ultraviolet A
UV-B	Ultraviolet B
UV-C	Ultraviolet C
UVR8	Ultraviolet B photoreceptor

1. Introduction

1.1. Broccoli

Broccoli (*Brassica oleracea* L. var. *italica*) belongs to *Brassica oleracea* species together with other commonly grown *Brassica* vegetables including kale, cabbage, Brussels sprouts, kohlrabi and cauliflower. Wild forms of *Brassica oleracea* occur along the Atlantic coast of Europe, from where they were introduced to the eastern Mediterranean, a place of broccoli origin dated already from 400 - 600 years BC (Gómez-Campo, 1999). Since then different forms of broccoli evolved, which can be divided in two main groups: sprouting broccoli and heading broccoli (calabrese type). Sprouting broccoli has multiple, almost uniform flower heads, while heading broccoli develops large, single, terminal inflorescence. Broccoli head can be distinguished from other organs early in the plant development and consists of clusters of fully differentiated flower buds arranged on a fleshy stem (Dixon, 2007). Nowadays, the broccoli of calabrese type, green head F1 hybrids, are mainly produced in many parts of the world for their edible inflorescences. One of the most extensively grown broccoli cultivar during the last decades is 'Marathon' (Prohens-Tomás and Nuez, 2007). Broccoli is a cool-season crop with an optimal growth temperature from 16 °C to 18 °C but tolerates temperatures in the range of 4 – 30 °C. Therefore it can be cultivated in both temperate regions and in some parts of tropical and sub-tropical regions (Le Strange et al., 2010; Björkman et al., 2011). Broccoli belongs to world primary vegetables and its production quantity, which was calculated as 21.3 million tonnes in 2012 (together with cauliflower), has doubled the last two decades (FAOSTAT, 2015). This vegetable, produced both for fresh and processed marked, is difficult to handle postharvest. Broccoli inflorescences are harvested when physiologically immature, hence they are exposed to stress due to sudden removal from energy, nutrients and hormones supply. This in turn leads to a rapid senescence of this vegetable during storage with

chlorophyll degradation within sepals as a visual sign (Page et al., 2001). Therefore broccoli is considered as a very perishable commodity with high respiration rate, which requires rapid cooling just after harvest and low temperature during storage to maintain quality (Dixon, 2007; Wills, 2007; Le Strange et al., 2010). The storage life of broccoli at 0 °C, the temperature recommended for this commodity, is from 21 to 28 days, at 5 °C it is reduced to 10 – 14 days, while at 20 °C it is only 2 – 3 days (Dixon, 2007; Le Strange et al., 2010).

1.2. Health-related compounds in broccoli

Epidemiological studies have shown that consumption of *Brassica* vegetables, including broccoli, reduces the risk of several types of cancers and cardiovascular disease mortality (Zhang et al., 2011; Bosetti et al., 2012; Liu et al., 2013; Liu and Lv, 2013). These health-promoting properties have been attributed to bioactive compounds, the activity of which has been extensively studied using *in vitro* and *in vivo* models (Podsędek, 2007; Jeffery and Araya, 2009; Patil et al., 2009; Latté et al., 2011). Broccoli contains several health-related compounds, namely vitamins C and E, polyphenols, carotenoids and glucosinolates. In addition, it is also a good source of micronutrients and dietary fibre (Moreno et al., 2006; Podsędek, 2007; Jeffery and Araya, 2009; Björkman et al., 2011).

1.2.1. Vitamin C

Vitamin C comprises both *L*-ascorbic acid (AA) and its oxidized form dehydroascorbic acid (DHA). AA with a structure characterized as aldono-1,4-lactone of hexonic acid (

Figure ***1.2.1***) is a main soluble antioxidant in plants due to its abundance (Davey et al., 2000; Hancock and Viola, 2005). There are four biosynthetic pathways discovered in plants, from which the *L*-galactose pathway seems to have the highest importance. In this pathway AA

is synthesised from *D*-glucose via GDP-*D*-mannose, *L*-galactose and *L*-galactono-1,4-lactone (Hancock and Viola, 2005).

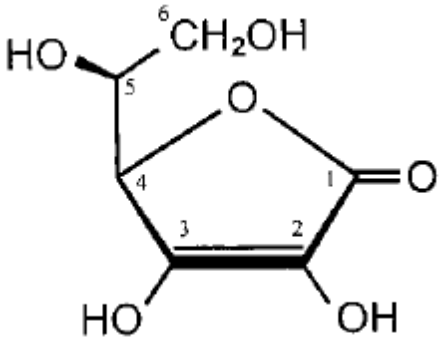


Figure 1.2.1. Chemical structure of *L*-ascorbic acid, from Davey et al. (2000).

As an antioxidant, AA can react with reactive oxygen species (ROS) that are by-products of normal oxidative metabolism and oxygenic photosynthesis in plant and animal cells. Their production is however increased by exposure to environmental stresses, which can initiate radical cascade reactions leading possibly to protein and DNA damage, lipid peroxidation and cell death. AA can scavenge ROS, including singlet oxygen ($^1O_2$), superoxide anions (O_2^-) and hydroxyl radicals (OH•), directly and indirectly being a substrate of ascorbate peroxidase, which eliminates hydrogen peroxide (H_2O_2). It can also react directly with a radical of lipophilic α-tocopherol (vitamin E) (Davey et al., 2000; Hancock and Viola, 2005).

The first product of AA oxidation, resulting from reaction with ROS, is monodehydroascorbic acid (MDHA). MDHA can be reduced back to AA by monodehydroascorbate reductase, or can disproportionate non-enzymatically to DHA and AA. In addition, dehydroascorbate reductase can reduce DHA to AA with simultaneous oxidation of glutathione, or DHA can hydrolyse to 2,3-diketogulonic acid. The recycling of AA in plants can thus be conducted using several mechanisms and seems to be crucial in responding to sudden exposure to environmental stresses (Gallie, 2013).

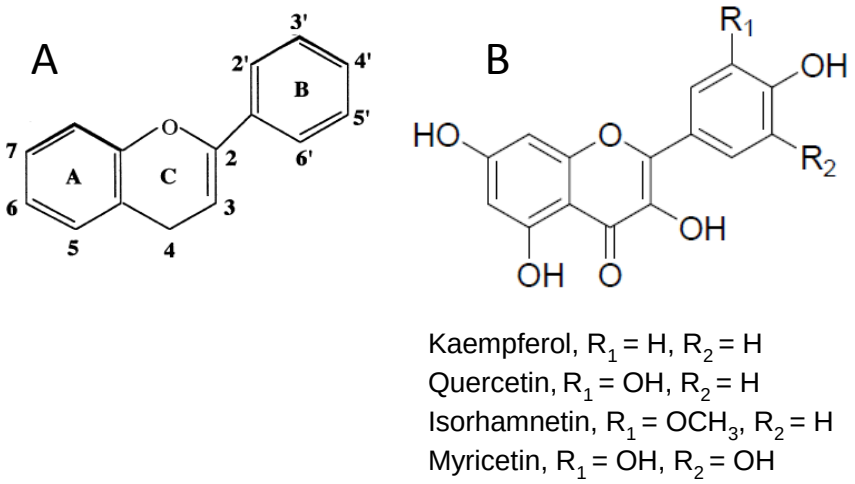
In plants, AA is indispensable for photosynthesis, not only due to its involvement in H_2O_2 elimination, but also because it serves as an electron acceptor in photosynthetic electron transport chain and as a cofactor of an enzyme involved in formation of the photoprotectant zeaxantin. Being a cofactor of other mono- or dioxygenases, AA has also implications for cell elongation and proliferation, as well as for hydrolysis of glucosinolates and biosynthesis of plant hormones such as ethylene and gibberellic acid (Davey et al., 2000). In humans, AA is involved in biosynthesis of collagen, neurotransmitters, steroid hormones and carnitine. Moreover it has been found to protect from coronary heart disease, stroke and cancer (Hancock and Viola, 2005; Patil et al., 2009).

Humans are not able to synthesise AA and are dependent on its dietary intake. Recommended daily allowance of AA was determined as 75 mg/day for woman and 90 mg/day for man to maintain optimal health and to prevent from scurvy, a vitamin C deficiency disease (Traber and Stevens, 2011). Consumption of fruits and vegetables provide more than 90% of vitamin C in human diets (Lee and Kader, 2000). *Brassica* vegetables can supply 50% of the recommended daily allowance of AA (Domínguez-Perles et al., 2014).

1.2.2. Flavonols

Flavonols are polyphenolic compounds that comprise one of the major groups of flavonoids. They are built upon a flavonoid nucleus (Figure 1.2.2.A) with a diphenylpropane structure (C6-C3-C6), which consists of the two benzene rings A and B, and one heterocyclic C ring. The C ring is condensed with the A ring and carries the B ring at 2-position. The variation in the oxidation level of the C ring is the basis for division of flavonoids into subclasses. For flavonols (Figure 1.2.2.B), the C ring is a heterocyclic pyrone having a carbonyl group in the 4-position with additional hydroxylation in the 3-position (Aherne and O'Brien, 2002). Moreover, all flavonoids are usually hydroxylated in 5- and 7-position of the A ring,

and in 4′- or 3′- and 4′- or 3′- , 4′- and 5′-position of the B ring (Ross and Kasum, 2002). Flavonols, with kaempferol, quercetin, isorhamnetin and myricetin (Figure 1.2.2.B) as the main ones, are the most abundant flavonoids in the plant kingdom. They are commonly present in plants as *O*-glycosides with conjugation occurring commonly at the 3-position of the C ring (Crozier et al., 2009). The glycosylation increases flavonol polarity and enables storage of these compounds in plant cell vacuoles (Aherne and O'Brien, 2002). Nevertheless flavonoid glycosides have been found also in chloroplast and nucleus of the plant cell (Agati et al., 2013).



Kaempferol, R_1 = H, R_2 = H
Quercetin, R_1 = OH, R_2 = H
Isorhamnetin, R_1 = OCH_3, R_2 = H
Myricetin, R_1 = OH, R_2 = OH

Figure 1.2.2. Flavonoid nucleus (A) and structures of common flavonols (B), from (Aherne and O'Brien, 2002; Es-Safi et al., 2007).

Flavonol synthesis in plants is conducted through a combination of two separate biosynthetic pathways and starts with a condensation of *p*-coumaroyl-CoA with three units of malonyl-CoA. The *p*-coumaroyl-CoA, which forms the aromatic B and C rings of the flavonol structure, is derived from phenylalanine via the shikimate pathway through its transformation by phenylalanine ammonia-lyase (PAL) to cinnamic acid, which is further hydrolysed to *p*-coumaric acid. The malonyl-CoA units, constituting the A ring of flavonol, comes from the malonic acid pathway. The condensation is catalysed by chalcone synthase (CHS) forming

naringenin-chalcone, which is then transformed by chalcone isomerase (CHI) to naringenin. The latter is converted by flavanone 3-hydroxylase (F3H) and optionally by flavonoid 3'-hydroxylase (F3'H) or flavonoid 3'5'-hydroxylase (F3'5'H) to dihydroflavonols, from which flavonols are formed by action of flavonol synthase (FLS) (Aherne and O'Brien, 2002; Winkel-Shirley, 2002; Crozier et al., 2009). Flavonol synthesis has been shown to be up-regulated by exposure to direct sunlight with increased level of quercetin and expression of genes involved in its biosynthesis, including PAL, CHS and F3H (Jaakola et al., 2004). It is suggested now, that expression of all genes involved in synthesis of dihydroxyflavonols, which occur throughout the whole-depth in sun-adapted leaves, is induced by high intensity sunlight (Agati et al., 2013).

Flavonols, due to the existence of phenol functional group in their structure, can interact with other biomolecules, proteins in particular, via hydrophobic interactions and formation of hydrogen bonds. They can also absorb UV-B irradiation (280 – 320 nm) and release a hydrogen atom to form phenoxy radicals (PhO•), and hence they show antioxidant properties. Moreover some flavonols, which have at least two adjacent hydroxyl groups on a phenyl ring, can be involved in metal chelation (Quideau et al., 2011). Due to the described properties, flavonols act in plants as antioxidants, developmental regulators and are involved in protection from UV-B irradiation (Pollastri and Tattini, 2011). The photoprotection of flavonols, which occur in plants as glycosides having absorbance maximum at ca. 350 nm, seems to be mostly attributed to their ROS reducing activity rather than UV-B screening properties (Pollastri and Tattini, 2011; Agati et al., 2013). Flavonoid glycosides located in vacuoles have been shown to play crucial role together with peroxidases and AA in reducing H_2O_2, which is produced in chloroplasts after exposure to excess light (Agati et al., 2013). Flavonols are therefore involved in plant's response to stresses, including light excess, drought, freezing temperatures and toxic metals in soil, due to their antioxidant properties but also due to the effect on auxin transport

(Winkel-Shirley, 2002; Treutter, 2005). Moreover, they play roles in plant defense against pathogens and herbivores (Treutter, 2005). In humans, dietary flavonoids, including flavonols, have been found to reduce the risk of chronic diseases and age-related disorders such as cardiovascular diseases, neurodegeneration and carcinogenesis (Quideau et al., 2011; Bondonno et al., 2015; Ivey et al., 2015). The health-promoting effects are mainly attributed to the ability of flavonoids to interact specifically with proteins such as enzymes, transcription factors and receptors (Fraga et al., 2010; Quideau et al., 2011; Del Rio et al., 2013).

Broccoli consumption provides substantial amount of flavonols in the human diet (Hollman and Katan, 1999; Ross and Kasum, 2002; Manach et al., 2004). Flavonols present in broccoli were characterized as complex 3-*O*-sophoroside/sophotrioside-7-*O*-glucoside/sophorosides of kaempferol, quercetin and isorhamnetin and less complex 3-*O*-glucoside-7-*O*-sophorosides, 3,7-*di*-*O*-glucosides, 3-*O*-sophorosides and 3-*O*-glucosides of the same aglycons. Additionally, almost all these compounds are acylated with hydroxycinnamic acids including *p*-coumaric, caffeic, ferulic and sinapic acids (Vallejo et al., 2004).

1.2.3. Glucosinolates

Glucosinolates (GLS) are sulphur- and nitrogen-containing compounds that are specific for plants from the Capparales/Brassicales order, which includes the Brassicaceae family. Their structure (Figure 1.2.3) is characterized as an *N*-hydroxyiminosulfate ester with a sulphur-linked β-D-glucopyranose and a variable side chain (R) (Halkier and Gershenzon, 2006; Clarke, 2010).

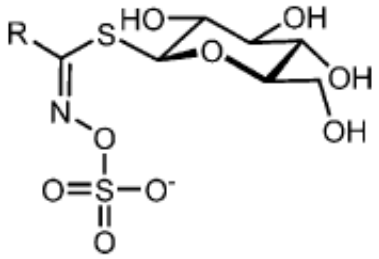
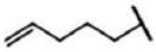
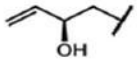
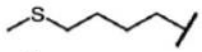
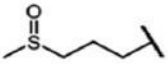
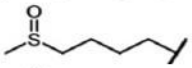
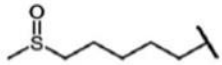
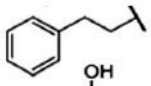
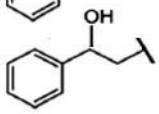
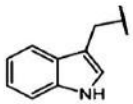
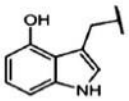
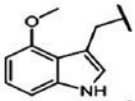
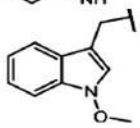


Figure 1.2.3. Basic structure of glucosinolate, from Hanschen et al. (2014).

There are already more than 200 known GLS, which are classified into aliphatic, aromatic and indolic GLS according to the structure of their side chain resulting from amino acid precursor (Hanschen et al., 2014). The modifications of the R group, including hydroxylation, methylation, desaturation, glycosylation, acylation and sulphur substitution, are the basis for further subdivision (Clarke, 2010; Hanschen et al., 2014). All GLS that were identified in broccoli cv. Marathon in at least one of the studies by Hansen et al. (1995), Rosa and Rodrigues (2001), Vallejo et al. (2003) and Aires et al. (2011) are listed in Table 1.2.3.1. However, only glucoraphanin (GRA), glucobrassicin and neoglucobrassicin were mostly predominant, while other GLS were found in contents lower than 1 μmol g^{-1} dry matter.

Table 1.2.3.1. Glucosinolates found in broccoli, adapted from Hanschen et al. (2014).

Trivial name	Chemical name	Structure of R side chain
Aliphatic glucosinolates		
Alkenyl		
Gluconapin	But-3-enyl	
Glucobrassicanappin	Pent-4-enyl	
Hydroxyalkenyl		
Progoitrin	2*R* -2-Hydroxybut-3-enyl	
Sulfur containing		
Glucoerucin	4-Methylthiobutyl	
Glucoiberin	3-Methylsulfinylpropyl	
Glucoraphanin	4-Methylsulfinylbutyl	
Glycoalyssin	5-Methylsulfinylpentyl	
Aromatic glucosinolates		
Gluconasturtiin	2-Phenylethyl	
Glucobarbarin	2-Hydroxy-2-phenylethyl	
Indolic glucosinolates		
Glucobrassicin	Indol-3-ylmethyl	
4-Hydroxyglucobrassicin	4-Hydroxyindol-3-ylmethyl	
4-Methoxyglucobrassicin	4-Methoxyinol-3-ylmethyl	
Neoglucobrassicin	1-Methoxyindol-3-ylmethyl	

Biosynthesis of GLS includes three stages: side-chain elongation of precursor amino acids, formation of the core structure of GLS and finally secondary modifications of the side chain group. GLS can be synthesised from eight amino acids but only methionine, tryptophan and phenylalanine give rise to the major aliphatic, indolic and aromatic GLS, respectively, in *Arabidopsis thaliana*. Precursors of some aliphatic and aromatic GLS are subjected to side chain elongation, while others directly proceed to another step of the GLS biosynthesis, which begins with the oxidation to aldoximes. The oxidation is catalysed by cytochrome P450 monooxygenases that are specific to the side chain and belongs to the CYP79 family. Then another oxidation is conducted by cytochromes P450 of the CYP83 family, which converts aldoximes to *aci*-nitro compounds or nitrile oxides. These compounds react with thiols to produce *S*-alkyl-thiohydroximates, which are cleaved by a C-S lyase into ammonia, pyruvate and thiohydroximic acid. The latter is *S*-glucosylated by *S*-glucosyltranferase to form desulfo-GLS, which finally are sulphated to GLS by sulfotransferases (Grubb and Abel, 2006; Halkier and Gershenzon, 2006). The final step of GLS synthesis includes oxidations, eliminations, alkylations and esterifications of the side chain (Grubb and Abel, 2006). The GLS are present in all plant's organs, however with different profiles and concentration levels, which also depend on plant developmental stage (Hanschen et al., 2014). In broccoli GLS are synthesized mainly in seeds, leaves and flower buds, with the highest total GLS level found in seeds followed by flower buds and leaves (Sotelo et al., 2014). The synthesis of GLS is therefore influenced by genotype and developmental factors but also by environmental and agronomic conditions (Hanschen et al., 2014; Sotelo et al., 2014). It has been found that nitrogen and sulphur supply, water availability, temperature and radiation during plant growth affect GLS accumulation (Hanschen et al., 2014). In addition, wounding and methyl jasmonate seems to induce some indolic GLS synthesis by upregulating specific CYP79 genes (Halkier and Gershenzon, 2006). Also, pathogen and herbivore attack through salicylates and jasmonates as

response signalling molecules, respectively, have been found to change GLS profile (Kliebenstein et al., 2005; Halkier and Gershenzon, 2006). Another factor that is suggested to trigger GLS synthesis through jasmonates and salicylates is UV-B irradiation (Hanschen et al., 2014).

In plant cells, GLS are stored in vacuoles and are separated from myrosinase, a β-thioglucosidase present in myrosin cells. Upon cell rupture GLS are mixed with myrosinase, which catalyses GLS hydrolysis to form several end-products including the most common isothiocyanates, thiocyanates, nitriles, epithionitriles and oxazolidines (Bones and Rossiter, 2006; Grubb and Abel, 2006). The final composition of GLS hydrolysis products depends on various chemical conditions such as pH and the presence of metal ions and proteins (Bones and Rossiter, 2006). For instance, epithiospecifier protein (ESP) promotes the formation of nitriles and epithionitriles from alkyl and alkenyl GLS, respectively, while its absence leads to spontaneous formation of isothiocyanates from any GLS (Kliebenstein et al., 2005; Hanschen et al., 2014). The biological functions of GLS are determined mostly by action of their breakdown products. It has long been suggested that their major role is involvement in plant defense by acting as toxic or deterrent agents against herbivores and pathogens. However, the same compounds have been found to attract some adapted insects, which indicates their impact on plant-insect interactions (Kliebenstein et al., 2005; Halkier and Gershenzon, 2006). The GLS breakdown products, isothiocyanates in particular, have been found to promote human health. Among isothiocyanates the most extensively studied was sulforaphane, a hydrolysis product of GRA, one of the major GLS in broccoli. Many studies have shown its anticarcinogenic properties such as the ability to block tumour initiation and promotion, and to suppress progression, angiogenesis and metastasis (Juge et al., 2007; Traka and Mithen, 2009; Dinkova-Kostova and Kostov, 2012).

1.3. Visible light and UV-B irradiation and their effects on plants

Both visible light and UV-B radiation are parts of sunlight, an electromagnetic radiation provided by the Sun. Visible light has wavelengths in the range of 400 – 700 nm, while UV-B is in the range of 280 – 315 nm. Sunlight is filtered by atmosphere and consequently most of the UV-B radiation is absorbed by stratospheric ozone layer. However, the amount of UV-B radiation at the Earth's surface varies and depends mostly on the latitude, season and time of the day, thus the factors that affect solar angle (Jenkins, 2009). Sunlight provides energy for plants and controls many growth and developmental processes including seed germination, photomorphogenesis, shade avoidance, phototropism, chloroplast movement, circadian rhythms and induction of flowering. Plants, which are able to sense light quantity, quality, direction and duration, developed four different photoreceptors namely cryptochromes, phototropins, phytochromes and UVR8 photoreceptor. The first two absorb blue and UV-A wavelengths, phytochromes are specific for far-red and red light whereas UVR8 perceives UV-B radiation (Jiao et al., 2007; Jenkins, 2014).

Despite being only a small part of sunlight, UV-B radiation alone has been found to influence plant growth and development and to modify metabolism by inducing flavonoid synthesis. The induction of flavonoid biosynthesis begins with monomerization of UVR8 and its interaction with the multifunctional E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1), which in turn activates two transcription factors ELONGATED HYPOCOTYL 5 (HY5) and MYB12. Finally, these transcription factors upregulate expression of genes encoding enzymes involved in flavonoid synthesis including CHS, CHI, F3'H, F3H, FLS and dihydroflavonol 4-reductase (DFR) (Schreiner et al., 2012; Jenkins, 2014; Gaudinier et al., 2015). Moreover, it has been shown that UV-B radiation through UVR8 influences many other genes in *Arabidopsis thaliana* than those involved in flavonoid biosynthesis. Among them were genes responsible for DNA repair and reduction of

oxidative damage, which suggests the role of UVR8 in acclimating plants to increasing UV-B radiation (Jenkins, 2014). High levels of UV-B radiation and/or with shorter wavelengths have negative effects on plants since they may lead to damage of DNA, proteins and membrane lipids, as well as to inhibition of photosynthesis and protein synthesis and finally to necrosis (Jenkins, 2009). Nevertheless, UV-B irradiation has been found to induce accumulation of not only flavonoids but also carotenoids and GLS. Therefore it is considered as a tool to enhance the contents of health-related compounds in plants both pre- and postharvest (Cisneros-Zevallos, 2003; Schreiner et al., 2012).

Both flavonoids and AA are involved in plant's photoprotection since they scavenge ROS produced after exposure to excess light. In addition flavonoids localized in epidermal tissues absorb damaging radiation (Takahashi and Badger, 2011; Agati et al., 2013). Therefore the level of solar radiation influences the content of these compounds in plants, that has been confirmed by several studies (Jaakola et al., 2004; Gliszczyńska-Świgło et al., 2007; Agati et al., 2009; Jin et al., 2009; Massot et al., 2012; Majer et al., 2014). Moreover, visible light during growth, as compared to growth in the dark, increased not only phenolic compounds and vitamin C but also GLS contents in broccoli sprouts (Pérez-Balibrea et al., 2008). The synthesis of GLS has been shown to be regulated by light in *Arabidopsis* (Huseby et al., 2013).

1.4. The effect of postharvest factors on health-related compounds

Harvested fruits and vegetables are still living plant portions, which continue to perform almost all metabolic processes including respiration and transpiration. To slow down these two processes is crucial for maintaining external quality of *Brassica* crops, which are expected to be at turgid state when marketed. For this purpose the application of low temperature, high relative humidity and packaging during storage is of great importance especially for broccoli, which is characterized as a produce with high respiration rate (Dixon, 2007; Wills, 2007).

Therefore it is recommended that broccoli, which is not sensitive to cooling, should be stored at 0 °C with relative humidity more than 95% (Chen et al., 2008). However, due to high costs of maintaining such a low temperature, the application of 4 °C during transport and wholesale of broccoli is still a common procedure. Moreover, the temperature during retail display of fruits and vegetables has been observed to vary significantly in both refrigerated and non-refrigerated displays affecting both produce quality and rate of its waste (Nunes et al., 2009).

Postharvest factors such as handling during harvest, conditions during storage and transport, the type of packaging, the way of industrial or consumer processing and finally the use of postharvest treatments including UV irradiation, influence not only visual quality of *Brassica* vegetables but also the contents of health-related compounds (Lee and Kader, 2000; Tomás-Barberán and Espín, 2001; Verkerk et al., 2009).

1.4.1. Storage period and temperature

In general vitamin C in fruits and vegetables degrades gradually during storage with increasing storage period or temperature (Lee and Kader, 2000). That is true also for *Brassica* vegetables, of which vitamin C content is usually not changed during short term storage at temperatures below 8 °C but degrades after storage for a couple of weeks at 1 – 2 °C (Domínguez-Perles et al., 2014). In packed broccoli heads the AA content was stable during storage for 6 days at 4 °C but decreased by 29% after storage at 15 °C for the same period (Nath et al., 2011). However, another study reported that 6 days of storage at 1 - 2 °C led to 32% loss of AA while 27 days to 71% loss, but in broccoli heads stored unpacked (Fernández-León et al., 2013b). In contrast, only 13% decrease of vitamin C was reported in packed broccoli inflorescences after storage for 7 days at 1 °C followed by 3 days at 15 °C (Vallejo et al., 2003). However, in that study the AA level decreased by 38% and DHA increased three times during storage. The DHA was also observed to increase after 2 days of storage at 20 °C

in broccoli florets and then decreased reaching the beginning level after 7 days of storage, while AA gradually decreased during storage resulting in 50% loss (Lemoine et al., 2010). Moreover, AA level in broccoli florets was stable only for 12 h of storage at 20 °C and then rapid decline was observed with 83% loss after 3 days. This decline was accompanied by reduced expression of chloroplastic genes encoding enzymes involved in AA synthesis, oxidation and regeneration (Nishikawa et al., 2003).

Flavonol contents were found to be stable in curly kale during storage at 1 °C for 6 weeks (Hagen et al., 2009) and in broccoli heads stored at 1 °C or 4 °C for up to 28 days (Winkler et al., 2007). However, other authors reported that storage for 6 – 7 days at low temperatures in the range of 1 – 4 °C significantly influenced flavonoid contents in *Brassica* vegetables and resulted in their increase (Starzyńska et al., 2003; Harbaum et al., 2008), decrease (Vallejo et al., 2003) or both (Kałużewicz et al., 2012) depending on the season. Moreover, storage for 3 days at higher temperature, 15 °C or 8 - 20 °C with cold pre-storage led to 59% decrease (Vallejo et al., 2003) or no change (Winkler et al., 2007), respectively, in flavonol levels in broccoli. Storage at 20 °C for 3 or 6 days, but without pre-storage, was observed to increase flavonoid contents in *Brassica* vegetables (Starzyńska et al., 2003; Harbaum et al., 2008). The increase of flavonoids in broccoli during storage was suggested to be a response to enhanced ROS production by stress conditions (Starzyńska et al., 2003).

Total GLS or GRA levels in broccoli were shown to be influenced by storage temperature since they were stable for 5 - 7 days at 4 °C but reduced after the same period at 20 °C (Rodrigues and Rosa, 1999; Rangkadilok et al., 2002). However, the opposite was found by other authors, who reported that total and individual GLS levels were not changed in broccoli during storage for 7 days at 12 – 22 °C but decreased during storage for 7 days at 4 – 8 °C (Song and Thornalley, 2007). Moreover, storage for 3 days at temperatures 8 - 20 °C with cold pre-storage for 2 - 28 days at 1 °C or 4 °C resulted in stable GRA levels (Winkler et al.,

2007) or reduced by 80% total GLS levels in broccoli (Vallejo et al., 2003). In addition, a 42% increase in total GLS was found for broccoli after storage at 10 °C for 7 days (Hansen et al., 1995).

1.4.2. Radiation treatments

Fluorescent light (21.8 or 24 μmol m^{-2} s^{-1}) during storage for 7 – 10 days at low temperature (1 or 7 °C) has previously been shown to protect against AA degradation and to increase total phenols in *Brassica* vegetables (Noichinda et al., 2007; Zhan et al., 2012; Zhan et al., 2014). Exposure to fluorescent light of 12 – 13 μmol m^{-2} s^{-1} for 2 days at 25 °C resulted in increased total phenol content but had no effect on total GLS level in broccoli florets (Jin et al., 2015).

A combined treatment of UV-B irradiation (0.20 W m^{-2}) and visible light (25 – 30 μmol m^{-2} s^{-1}) applied 12 h per day during storage for 10 days at 10 °C has previously been found to enhance phenolic compound and AA contents in apple peel (Hagen et al., 2007). Both total phenols and AA content in broccoli florets were also increased after exposure to UV-C irradiation (8 kJ m^{-2}) applied before storage for 21 days at 4 °C (Lemoine et al., 2007). However, a 5 min of UV-B irradiation (98 μmol m^{-2} s^{-1}, 21 W m^{-2}) for 3 days at 10 °C resulted in increased levels of flavonoids in spinach, radish sprouts and parsley determined after 6 days of storage (Kanazawa et al., 2012). In that study a single dose of this irradiation was also shown to enhance expression of 5 genes involved in flavonoid biosynthesis in parsley, including PAL, CHS and FNS. In addition, total phenols and total flavonoids accumulated more in tomato during storage for 37 days at 14 °C with previous exposure to UV-B irradiation of 20 or 40 kJ m^{-2} at a fluency rate of 6 W m^{-2} (Liu et al., 2011). Moreover, low dosage of postharvest UV-B irradiation (0.075 W h m^{-2}, 8.2 W m^{-2}) was found to enhance total phenols and glucotropaeolin content in nasturtium inflorescences after 2 h of adaptation at 20 °C (Schreiner et al., 2009).

1.5. Background

The quality of *Brassica* crops is not only determined by their visual quality and flavour, but also by their nutritional value (Dixon, 2007). The latter is related to the presence of potentially beneficial compounds for human, including minerals, vitamins, dietary fibre and plant secondary metabolites. Broccoli is a good source of all these compounds and the activity of some of them contribute to recognized health promoting properties of this vegetable. It is however becoming clearer, that the effect of broccoli consumption on cancer prevention and progression, is not related to a single compound, but to a variety of bioactive compounds acting through different pathways (Ferguson and Schlothauer, 2012). Therefore, in order to make a health claim for broccoli that is marketed, care should be taken to preserve all bioactive compounds.

The content of health-related compounds in freshly harvested broccoli is influenced by various postharvest factors. The effect of storage conditions on especially flavonols and GLS in broccoli is still not clear due to contradictory results of previous studies on this matter. Moreover, to our knowledge there are only two studies that analysed the contents of health-related compounds in broccoli applying an experimental approach that simulated real storage conditions from producer to consumer. The first study (Winkler et al., 2007) examined only GRA and flavonol levels, while the second (Vallejo et al., 2003) included all GLS, flavonols, hydroxycinnamoyl derivatives and vitamin C but applied only single levels of pre-storage and storage periods and temperatures. In both studies broccoli heads were stored in the dark, which is not typical for display of this vegetable. Therefore, to fully understand the effect of conditions during transport, wholesale distribution and retail on health-related compounds in broccoli, more research is needed.

Visible light and UV-B irradiation have been found to influence vitamin C, flavonol and GLS contents in *Brassica* plants. However, the evidence on the effect of postharvest light

and UV-B irradiation on these compounds in broccoli, in particular at temperature common during marketing, is scarce or even does not exist at all. Moreover, the knowledge on how temperature influences the outcome of these radiation treatments is limited.

2. Aim of the study

The main purpose of this thesis work was to evaluate the effect of storage time, temperature and radiation treatments on health-related compounds in broccoli flower buds using a multi-level experimental approach simulating mapped commercial storage conditions from harvest until consumer purchase. In the experiment two lengths of pre-storage period (PP) at two temperatures (PT) were applied to simulate transport with wholesale distribution, followed by storage at two temperatures (ST) and with four different radiation treatments (R), including three levels of visible light and darkness as a control, to simulate retail. A combination of visible light with UV-B irradiation was applied, as a fifth radiation treatment (R) during storage, to test whether it has a potential to alter the contents of health-related compounds in broccoli flower buds.

The specific objectives of this thesis work were:

- To examine how the levels of vitamin C, AA and DHA, and the DHA/AA ratio in broccoli flower buds are affected by postharvest temperature and radiation treatments (Paper I).
- To study the combined and separate effects of storage time, temperature and radiation treatments on flavonol levels in broccoli flower buds (Paper II).
- To investigate the kinetics of epidermal flavonol accumulation during postharvest treatments using non-destructive method based on chlorophyll fluorescence measurements (Paper II).
- To evaluate how total and individual GLS levels in broccoli flower buds, and in floret stalks of selected samples, are affected by postharvest temperature and radiation treatments (Paper III).
- To suggest appropriate conditions during transport, wholesale distribution and retail to preserve health-related compounds in broccoli (Paper I-III).

3. Materials and methods

3.1. Plant material and storage experiment

Broccoli (*Brassica oleracea* L. var. *italica*, cv. Marathon) was grown in the experimental field of the Norwegian University of Life Sciences (59° 39′ N, 10° 45′ E) from July till September 2009 as described in detail in Paper I. Broccoli heads were hand-harvested at optimum growth stage according to commercial standards. Similar in size 360 broccoli heads with no sign of damage were selected and pre-cooled in a storage room at 0 °C immediately after harvest. Apart from eight broccoli heads comprising control at harvest, all inflorescences were packaged with polyvinylchloride food film and placed in plug trays inside reusable plastic containers (Figure 3.1.1.A). There were eight broccoli heads in each container that were biological replicates for each postharvest treatment. A 10 cm diameter area on top of every broccoli head was marked non-destructively with pins and all heads were individually numbered (Figure 3.1.1.B).

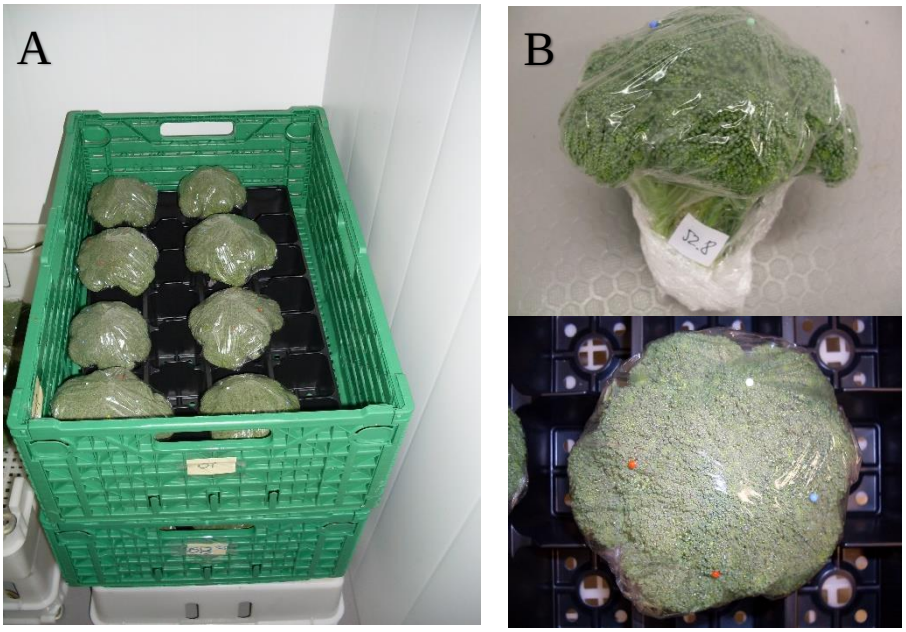


Figure 3.1.1. Pre-storage of broccoli (A) and examples of broccoli heads prepared for the experiment (B).

All details regarding experiment conducted in the study are included in Paper I. Briefly, film-wrapped broccoli heads were pre-stored at 0 °C or 4 °C for four or seven days in darkness (Figure 3.1.1.A) and they were stored for another three days at 10 °C or 18 °C. During storage different radiation treatments were applied 12h per day: visible light of 0, 13, 19 and 25 µmol m^{-2} s^{-1} (Figure 3.1.2.A) and a combination of 19 µmol m^{-2} s^{-1} visible light and UV-B irradiation in the range from 19 to 22 kJ m^{-2} d^{-1} (0.22-0.25 W m^{-2}) (Figure 3.1.2.B).

Figure 3.1.2. Visible light (A) and a combination of visible light with UV-B irradiation (B) treatments of broccoli during storage.

The temperature and visible light levels during storage were chosen according to the results of a survey conducted in seven Norwegian retail stores. Those results showed that minimal and maximal temperature at the place of broccoli display was 10 °C and 18 °C, respectively. Visible light level varied between 1000 and 2000 lux.

3.2. Chemical analyses

3.2.1. Sample preparation

Florets from a 10 cm diameter top area of individual broccoli heads were cut off from the main stem and immediately frozen in liquid nitrogen. Flower buds were scraped off the stalks and separated from the small branches using a sieve with 4 mm diameter holes under freezing conditions. The buds and stalks were stored at -80 °C in separate 50 mL polypropylene tubes until analyses. Five out of eight replicates (n=5) for each treatment were randomly selected for vitamin C (Paper I), flavonol (Paper II) and glucosinolate (Paper III) analysis in flower buds. In addition, glucosinolate analysis was conducted in floret stalks for selected treatments (Paper III).

3.2.2. Vitamin C

The contents of L-ascorbic acid (AA), L-dehydroascorbic acid (DHA) and vitamin C, as a sum of both acids, were determined according to Davey et al. (2003) and Karlsen et al. (2005) with some modifications described in Paper I. Briefly, the AA and DHA were extracted from frozen broccoli flower buds, then DHA was reduced to AA in order to obtain total AA (vitamin C) content, or AA alone was analysed in extracts. Separation, detection and quantification of AA and total AA were performed using HPLC with external standard. DHA content was determined indirectly by subtracting AA content from total AA content.

3.2.3. Flavonols

Flavonols were extracted from freeze-dried broccoli flower buds as described by Steindal et al. (2013) with some modifications described in Paper II. Then acid hydrolysis of flavonol glycosides and HPLC analysis of flavonol aglycons were performed according to Hagen et al. (2009) with some modifications described in Paper II. Identification and

quantification of flavonol aglycons in extracts of broccoli flower buds were based on external standards.

3.2.4. Glucosinolates

Glucosinolates (GLS) were extracted from freeze-dried broccoli flower buds or floret stalks as described by Steindal et al. (2013) with some modifications indicated in Paper III. The same extracts of broccoli flower buds were used for flavonol and GLS analysis. After extraction, desulfation procedure was conducted according to International Standard Method: ISO 9167-1:1992(E) (ISO, 1992) with some modifications described in Paper III. The desulfated GLS in extracts of broccoli flower buds were identified using LC-MS and quantification of GLS in broccoli flower buds or floret stalks was conducted by HPLC analysis as described in Paper III. Quantification of desulfated GLS was based on relative response factors towards internal standard.

3.3. Non-destructive chlorophyll fluorescence measurements

Chlorophyll fluorescence (ChlF) measurements were conducted using a hand-held multi-parametric optical sensor Multiplex® 3 on the marked area on top of the broccoli heads as described in Paper II. The ChlF was measured after harvest for all broccoli heads and three times during storage for all biological replicates (n=8) for every treatment. An equation given by Hagen et al. (2006) and presented in Paper II was used to calculate relative UV-A epidermal absorbance (A_{UV}). In order to illustrate the changes in A_{UV} during storage, calculated A_{UV} data were normalized as described in Paper II.

3.4. Statistical analysis

The design of the experiment in the study was a four factorial fixed effect model. Factors were as follows: pre-storage period (two levels), pre-storage temperature (two levels),

storage temperature (two levels), and light treatment (five levels). In addition, a fifth factor, storage period with three levels, was introduced in Paper II for A_{UV} data. General linear model (GLM) and Tukey's multiple comparisons test with significance level $\alpha = 0.05$ were applied to study the effects of factors, significance of interactions between factors and differences between levels of each factor (Papers I-III). The differences between treatments were tested using one way analysis of variance (ANOVA) with Tukey's multiple comparisons test (Papers I-III). Nonlinear regression was applied to study the relationship between A_{UV} data and flavonol data obtained using HPLC (Paper II). Principal component analysis (PCA) was conducted to get an overview of variations between the samples and the variables responsible for this variation.

4. Main results and discussion

4.1. Storage period and temperature

The present study has demonstrated that the contents of vitamin C, AA, DHA (Paper I), flavonols (Paper II) and GLS (Paper III) in broccoli flower buds were not significantly changed during pre-storage at both temperatures 0 °C or 4 °C for both 4 and 7 d. Even the 20% loss of vitamin C observed after 7 d of pre-storage at both temperatures and a 2-fold increase in DHA level after pre-storage for 4 d at 0 °C were not significant (Paper I). These results are in accordance with stable AA levels reported for packed broccoli heads during storage for 6 – 7 d at 4 °C or 1 °C (Vallejo et al., 2003; Nath et al., 2011) and with general finding that vitamin C is maintained in *Brassica* vegetables during short term storage at temperatures below 8 °C (Domínguez-Perles et al., 2014). Moreover, the levels of total and individual flavonols or GLS have previously been observed to be stable in *Brassica* vegetables during short or long term storage (2 d – 6 weeks) at low temperatures in the range of 1 – 4 °C (Rodrigues and Rosa, 1999; Winkler et al., 2007; Hagen et al., 2009; Fernández-León et al., 2013a).

Our study demonstrates that storage at higher temperature (18 °C) resulted in reduced vitamin C and AA contents by 40 – 50 % in broccoli flower buds, whereas lower temperature (10 °C) led to higher retention of these compounds during storage (Paper I). This is in accordance with the general finding that vitamin C in fruits and vegetables degrades during storage with increasing temperature (Lee and Kader, 2000). Moreover, similar AA losses were reported for broccoli after storage at 15 °C or 20 °C with (Fernández-León et al., 2013b) or without (Lemoine et al., 2010; Nath et al., 2011) cold pre-storage. In the present study total and individual GLS were found to be stable in broccoli flower buds during storage at both temperatures 10 °C or 18 °C (Paper III). No losses of these compounds were observed and no significant changes for most treatments used in the experiment. GRA levels were found to be

stable in broccoli during storage for three days at 8 °C, 15 °C or 20 °C with pre-storage at 1 °C or 4 °C (Winkler et al., 2007). However, in the present study two groups of broccoli heads had significantly increased contents of GIB, GRA and total aliphatic GLS after storage than at harvest (Paper III). The groups comprised broccoli pre-stored for seven days at 0 °C and stored for three days at 10 °C under visible light of 13 μmol $m^{-2}s^{-1}$ and 25 μmol $m^{-2}s^{-1}$. These broccoli heads were probably still vegetative since postharvest increase in GRA level has previously been recognized as a sign of vegetative stage in broccoli (Schouten et al., 2009). Similarly to GLS, flavonol aglycons were not significantly changed during storage at both temperatures tested (10 °C or 18 °C) for almost all broccoli groups in the present study (Paper II). This was in accordance with stable flavonol levels found by Winkler et al. (2007) in broccoli during storage at both low and high temperatures with cold pre-storage. In our study both flavonol aglycons estimated by HPLC and epidermal flavonols measured non-destructively were not affected by ST. However, the latter were significantly influenced by SP with significant increases observed after 3 d of storage (Paper II). Other authors have also reported increased flavonoid contents and increased superoxide dismutase activity, an enzyme playing a key role in plant response to stress, in flower buds of broccoli during storage at 5 °C or 20 °C (Starzyńska et al., 2003). The accumulation of flavonoids was associated with the capacity of the bud tissue to scavenge free radicals. This was probably true also for the accumulation of epidermal flavonols observed in the present study. In addition ST (10 °C or 18 °C) significantly affected the levels of vitamin C, AA, DHA, DHA/AA ratio (Paper I), total and individual GLS, except for 4-Me-GB (Paper III) in broccoli flower buds. Vitamin C, AA, DHA (Paper I), total and individual GLS, except for 4-OH-GB (Paper III), were higher in flower buds of broccoli after storage at 10 °C than at 18 °C. In contrast DHA/AA ratio (Paper I) and 4-OH-GB contents (Paper III) were significantly increased after storage at 18 °C compared to 10 °C. The DHA/AA ratio has previously been observed to increase in spinach leaves during storage at 2 °C or 10

°C (Hodges and Forney, 2003; Bergquist et al., 2006). The increase was more rapid in cultivar characterized with higher rate of senescence (Hodges and Forney, 2003) and was higher at 10 °C than at 2 °C (Bergquist et al., 2006). Moreover, increasing DHA/AA ratio was recognized as a result of reduced AA synthesis or regeneration or both (Bergquist et al., 2006). Therefore broccoli heads stored at 18 °C in the present study had probably higher senescence rate and reduced capacity to cope with oxidative stress than those stored at 10 °C. In addition, the levels of indolic GLS were observed to increase in chopped broccoli during storage for 48 h at room temperature (Verkerk et al., 2001). The authors suggested that this increase was stress-related and that mechanical wounding induced indolic GLS synthesis the same way as damage by pests. The increased levels of 4-OH-GB found in broccoli flower buds during storage at 18 °C in the present study were probably a response to stress caused by high ST.

The interaction PP × ST was found significant for vitamin C and DHA and to a minor extend for AA, indicating that the effect of ST on these compounds in broccoli flower buds was influenced by PP (Paper I). Thus, the difference in vitamin C, AA and DHA contents in broccoli flower buds between heads stored at 10 °C and at 18 °C depended on PP and was higher for broccoli pre-stored for 4 d than for those pre-stored for 7 d. Moreover, significant PT × ST interaction observed for total aliphatic and individual aliphatic GLS suggested that the effect of ST on these compounds in broccoli flower buds was influenced by PT (Paper III). For broccoli pre-stored at 4 °C the effect of ST was negligible but significant for those pre-stored at 0 °C with higher levels of aliphatic GLS found after storage at 10 °C than at 18 °C.

The multi-level experimental approach applied in the study gave the possibility to evaluate the effect of PP and PT on health-related compounds in broccoli flower buds during storage. These two factors, PP and PT significantly affected the contents of vitamin C, DHA and the DHA/AA ratio in broccoli flower buds during storage (Paper I). Higher levels of these compounds and the ratio after storage were found in broccoli pre-stored for the shortest period

(4 d) or at the lowest temperature (0 °C). In addition, AA level was influenced to a minor extend by PT in broccoli flower buds during storage with higher contents observed for broccoli pre-stored at 0 °C. These findings extend already known statement that vitamin C gradually degrades during storage with increasing storage period and temperature (Lee and Kader, 2000), indicating the importance of pre-storage conditions for preservation of this compound during storage. In the present study the DHA levels and DHA/AA ratio were stable in broccoli pre-stored for 7 d during storage but increased in those pre-stored for 4 d (Paper I). Moreover, PP × PT interaction was significant for both DHA and the ratio, which indicates that they were affected by PT during storage only in broccoli pre-stored for 4 d. These results suggest a shift in ascorbate metabolism in broccoli flower buds that have appeared during pre-storage and was maintained during storage. The broccoli heads pre-stored for the shortest period (4 d) probably had higher rate of AA oxidation, AA synthesis or both. Similarly, it has previously been reported that the expression of chloroplastic genes involved in AA synthesis, oxidation and regeneration changed in broccoli florets during storage at 20 °C (Nishikawa et al., 2003). In that study, the expressions of genes encoding AA peroxidase, MDHA reductase and DHA reductase were increasing for up to 6 h during storage followed by sharp declines, while the expression of *L*-galactono-1,4-lactone dehydrogenase was gradually decreasing. In the present study the contents of epidermal flavonols (Paper II), total and almost all individual GLS, except for 4-Me-GBS (Paper III), were significantly influenced by PP in broccoli flower buds during storage. Higher levels of these compounds after storage were found in broccoli pre-stored for 7 d than in those pre-stored for 4 d. Therefore the increase observed for epidermal flavonols during storage began already during pre-storage, which is probably true also for GLS. Moreover, the PP × PT interaction was found significant for flavonol aglycons (Paper II) indicating that the PP effect on these compounds during storage was influenced by PT. The flower buds of broccoli pre-stored for 7 d at 0 °C contained higher levels of flavonols after

storage than those pre-stored for the same PP but at 4 °C. In contrast, for shorter PP (4 d) higher temperature (4 °C) was found favourable. This is in accordance with the previous observation by Starzyńska et al. (2003) that accumulation of flavonoids in broccoli flower buds during storage appeared earlier at higher temperature. In addition, plants with higher ability to maintain antioxidants were associated with higher resistance towards postharvest oxidative stress (Toivonen, 2004). Therefore we could assume that flower buds of broccoli pre-stored for 7 d at 0 °C are more resistant to oxidative stress during storage than the ones pre-stored at 4 °C for the same period.

4.2. Radiation treatments

The present study have shown that radiation treatments (R) including light and UV-B irradiation significantly affected quercetin and epidermal flavonols in broccoli flower buds during storage (Paper II). The highest contents of these compounds were found in flower buds of broccoli exposed to the combination of visible light (19 μmol m^{-2} s^{-1}) and UV-B radiation during storage. However, in general this postharvest treatment resulted in contents of flavonols and epidermal flavonols that were not significantly different from the ones after storage in the dark or under visible light of the highest level (25 μmol m^{-2} s^{-1}). Similarly, storage in the dark for 4 d at 20 °C has previously been shown to increase flavonoid levels in broccoli florets more than storage at the same temperature and for the same time but under UV-C exposure (Costa et al., 2006). In addition, visible light has been reported to increase total phenols or both total phenols and PAL activity in broccoli and cauliflower during storage at 7 °C (Zhan et al., 2012; Zhan et al., 2014). Nevertheless, in the present study the combination of visible light and UV-B radiation during storage increased all flavonol levels in one group of broccoli heads as compared with the contents after pre-storage (Paper II). The group comprised broccoli heads pre-stored for 4 d at 4 °C and stored at 10 °C. In addition, for the same broccoli heads the

highest increase in epidermal flavonols was observed after storage. Moreover, the combination of visible light and UV-B radiation increased epidermal flavonols significantly more than other radiation treatments under most conditions in the present study. These findings confirm the potential of postharvest UV-B irradiation to increase flavonoid contents in broccoli and are in agreement with previous studies showing the same for other vegetables including spinach, radish sprouts, parsley, tomato and white asparagus (Liu et al., 2011; Eichholz et al., 2012; Kanazawa et al., 2012). However, in the present study the significant increases of flavonol levels appeared when treatment with a combination of visible light and UV-B radiation was applied at a certain temperature (10 °C) and after pre-storage for 4 d at 4 °C. Moreover, several multiple factor interactions that included R were found significant for quercetin (PT × ST × R, PP × PT × R, and PP × PT × ST × R) and for epidermal flavonols (PP × R, PT × R, ST × R, PP × PT × R, PT × ST × R, PP × PT × ST × R) in broccoli flower buds after storage (Paper II). This indicates that the effect of R was influenced by the temperature during that treatment (ST) and also by PT, PP and by the combinations of these factors. In addition, the accumulation of epidermal flavonols in flower buds of broccoli exposed to UV-B irradiation during storage began already during the first day at 18 °C, while at 10 °C was observed later, which could be explained by increased rate of metabolism or increased stress level at higher temperature (Paper II). Similarly, the UV-C pre-treatment has been found to influence total phenol contents in broccoli florets during storage at 5 °C or 10 °C for 19 d in the dark in a temperature dependant manner (Martínez-Hernández et al., 2011). In that study, the large increase in total phenols was observed earlier at 10 °C than at 5 °C.

However, in the present study no effect of postharvest R was found for vitamin C, AA, DHA, DHA/AA ratio (Paper I), total and individual GLS (Paper III) levels in broccoli flower buds.

4.3. Principal component analysis and overall discussion

The PCA of all data revealed that the first two PCs explained 90% of total variation (Figure 4.3.1). PC 1 explained 77% of the total variation and appeared to be closely related to vitamin C and AA content. PC 2 explained 13% of the total variation and was related mainly to total GLS and total indolic GLS (Figure 4.3.1.A). Total aliphatic GLS, GRA, GBS and NGBS contributed to explained variation by PC 2 only to a minor extend, while other variables including 4-Me-GBS, 4-OH-GBS, GIB, all flavonols and DHA/AA ratio were not related to any of the two PCs. The score plot demonstrates distribution of samples along PC 1 and PC 2 and revealed that broccoli samples in the present study grouped due to PC 1 (Figure 4.3.1.B). The broccoli samples at harvest and after pre-storage appeared at the outmost right positions in the plot followed by samples stored at 10 °C, which still were characterized with positive scores and high levels of vitamin C and AA. The opposite was found for broccoli samples after storage at 18 °C, majority of which appeared on the left side of the plot and were described by negative scores and low levels of vitamin C and AA.

The PCA results confirm that vitamin C and AA were compounds that varied the most, while flavonols and GLS were in general stable during pre-storage and storage in broccoli flower buds. The ST was found to influence vitamin C and AA contents to the greatest extend. Moreover, since vitamin C and AA were the most labile compounds during storage in broccoli, they can be treated as indicators of postharvest deterioration or proper handling and processing procedures, which has been suggested by others (Davey et al., 2000; Domínguez-Perles et al., 2014). The AA is a primary antioxidant in plant cells that reacts both enzymatically and non-enzymatically with ROS (Davey et al., 2000). It can reduce many substrates due to mild electronegative properties and is involved in regeneration of other antioxidants including α-tocopherol (Davey et al., 2000) and probably also flavonols in some reactions (Agati et al., 2013). The AA plays a crucial role in plant response to postharvest oxidative stress (Toivonen,

2004) and is probably more extensively used than flavonols when the stress occurs, since the antioxidant activity of the latter compounds is mainly based on their direct reaction with ROS (Quideau et al., 2011). That is probably why AA was observed to degrade while flavonols remained stable during storage in broccoli in the present study.

Despite the general stability of flavonols and GLS during pre-storage and storage in flower buds of broccoli in the present study, at some conditions higher contents of these compounds were found than at others. For instance lowering ST from 18 °C to 10 °C was favourable for almost all GLS as well as the combination of lower PT and ST (0 °C and 10 °C) for total and individual aliphatic GLS. Moreover, it was found that prolonged pre-storage (7 d) when performed at lower temperature (0 °C) led to increased flavonol contents in broccoli flower buds after storage. In addition, changing PT from 4 °C to 0 °C or PP from 7 d to 4 d, or both was observed to increase vitamin C content in broccoli flower buds after storage. Therefore, in order to preserve high contents of health-related compounds in broccoli postharvest not only conditions during storage but also during pre-storage should be taken into consideration.

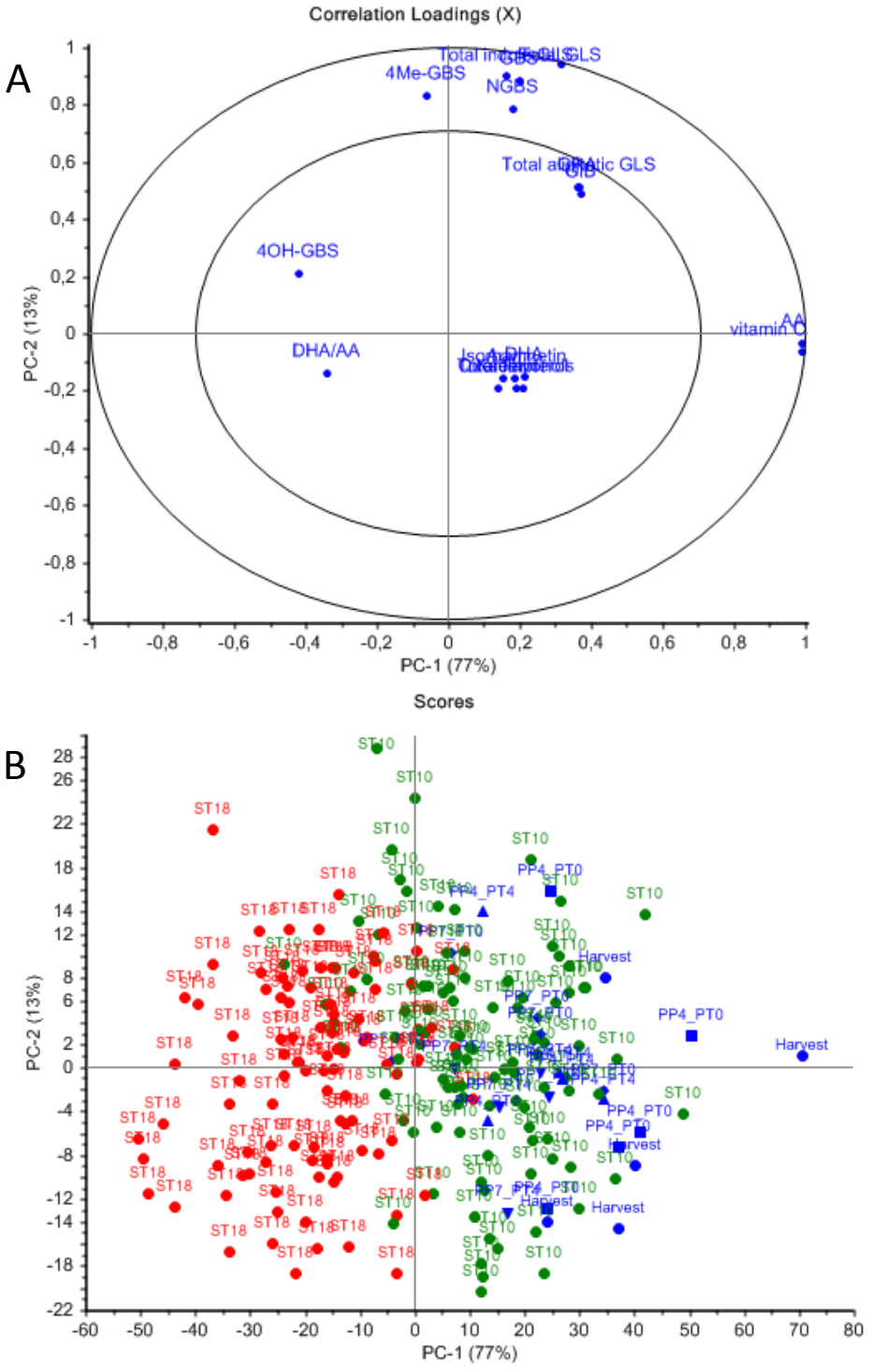


Figure 4.3.1. PCA correlation loadings (A) and score plot (B) for health-related compounds in broccoli flower buds at harvest (blue dots), after pre-storage (blue squares, triangles and diamonds) and after storage at 10 °C (green dots) or 18 °C (red dots).

5. Main conclusions and future prospective

The present study has shown that flavonol and GLS contents in broccoli flower buds were in general stable during pre-storage at low temperatures (0 °C and 4 °C) and during storage at higher temperatures (10 °C and 18 °C), while vitamin C levels remained unchanged during pre-storage but degraded during storage with increasing PP, PT and ST. However, PP and both PP and PT were found to influence almost all GLS and flavonol contents during storage, respectively. Moreover, the effect of ST on aliphatic GLS levels in broccoli flower buds was influenced by PT. Therefore, pre-storage treatment should be taken into consideration both in postharvest research and industry.

Among all the compounds analysed in the present study vitamin C and AA were the most labile in broccoli flower buds and were influenced mainly by ST. Therefore, to preserve high contents of vitamin C in broccoli the most important is to keep the temperature during retail as low as possible. However, shortening the wholesale period (to 4 d or less) and keeping the temperature close to 0 °C (instead of common 4 °C) during wholesale should also be considered. This would also preserve high contents of flavonols and GLS in broccoli since lowering of PT from 4 °C to 0 °C during wholesale longer than 4 d was favourable for flavonols and the combination of pre-storage at 0 °C and storage at 10 °C for aliphatic GLS.

In addition, the present study has demonstrated the potential of UV-B irradiation combined with visible light to increase flavonol contents in broccoli flower buds during storage. However, the outcome of this treatment was influenced by ST, PT and PP. Moreover, the non-destructive ChlF method revealed that UV-B irradiation applied at higher temperature (18 °C) induced the accumulation of epidermal flavonols in broccoli flower buds earlier, already during the first day of storage. These findings could provide a basis for future studies, which focus on developing postharvest treatments including UV-B irradiation to increase

flavonol contents in plant-derived food products. It would also be interesting to find out which flavonol glycosides in broccoli are the most UV-B responding. Moreover, results from the previous studies indicate that the combination of visible light and UV-B irradiation can influence also vitamin C and GLS contents in broccoli. However, future research is needed to find out the most effective radiation treatment by testing different light or UV-B intensities at different temperatures.

The results of the present study will hopefully stimulate industry to improve postharvest handling procedures in order to preserve health-related compounds in broccoli. Moreover, since AA content appears to be important for storability of broccoli, it would be interesting to test whether high contents of AA after pre-storage extend shelf life of broccoli. Future research could also focus on elucidating the mechanism (the changes in expression of certain genes and/or in enzyme activities) based on which conditions during pre-storage affect ascorbate, flavonol and GLS metabolism during storage.

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Vitamin C in broccoli (*Brassica oleracea* L. var. *italica*) flower buds as affected by postharvest light, UV-B irradiation and temperature

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ABSTRACT

In this study, the changes in vitamin C, L-ascorbic acid (AA) and L-dehydroascorbic acid (DHA) levels in broccoli flower buds were examined during pre-storage and storage periods, simulating refrigerated transport with wholesale distribution and retail, respectively. Broccoli heads were pre-stored for 4 or 7 days at 0 °C or 4 °C in the dark and then stored for 3 days at 10 °C or 18 °C. During storage the broccoli heads were exposed for 12 h per day to three different levels of visible light (13, 19 or 25 μmol m^{-2} s^{-1}) or a combination of visible light (19 μmol m^{-2} s^{-1}) and UV-B irradiation (20 kJ m^{-2} d^{-1}), or they were stored in the dark. The vitamin C content in broccoli flower buds during storage was significantly affected by pre-storage period and temperature. Higher vitamin C levels in flower buds after storage were observed for broccoli heads pre-stored for 4 days or at 0 °C as compared to those pre-stored for 7 days or at 4 °C. Storage temperature also affected vitamin C in broccoli flower buds, with higher levels observed for broccoli stored at 10 °C than at 18 °C. Hence, vitamin C in broccoli flower buds was demonstrated to decrease together with increasing pre-storage period, pre-storage temperature and storage temperature. AA in broccoli flower buds was influenced mainly by storage temperature and to a minor extent by pre-storage temperature. The DHA level and DHA/AA ratio were stable in flower buds of broccoli pre-stored for 7 days, whereas increasing tendencies for both DHA level and ratio were observed after pre-storage for 4 days. These results indicate a shift in the ascorbate metabolism in broccoli flower buds during storage at low temperatures, with its higher rate observed for broccoli pre-stored for shorter time. There were no effects of the light and UV-B irradiation treatments on vitamin C, AA and DHA levels in broccoli flower buds.

1. Introduction

Broccoli (*Brassica oleracea* L. var. *italica*) is a cruciferous vegetable with increasing popularity, the world production of which has doubled the last two decades (FAOSTAT, 2013). Broccoli heads are rich in minerals and vitamins together with fiber and health related secondary metabolites such as flavonols, carotenoids and glucosinolates (Moreno et al., 2006; Podsedek, 2007; Jeffery and Araya, 2009; Bjorkman et al., 2011). Consumption of cruciferous vegetables such as broccoli has been shown to reduce the risk of several cancers and cardiovascular disease mortality in epidemiological studies (Zhang et al., 2011; Bosetti et al., 2012; Liu et al., 2013; Liu and Lv, 2013). However, broccoli is a highly perishable commodity with refrigerated storage time of maximum 2 to 4 weeks (Nunes, 2008) due to rapid senescence, with degradation of chlorophyll within sepals as a visual sign, and loss of membrane lipids and proteins (Page et al., 2001).

Vitamin C, comprising L-ascorbic acid (AA) and its oxidized form L-dehydroascorbic acid (DHA), is the major antioxidant in *Brassica* vegetables (Davey et al., 2000; Podsedek, 2007). AA is structurally aldono-1,4-lactone of hexonic acid, which can easily oxidize in solutions to monodehydroascorbate. *In vivo* this oxidation product can be reduced back to AA enzymatically or can spontaneously disproportionate to AA and DHA (Davey et al., 2000). As a result, DHA, which may easily be converted to AA in the human body, comprise about 10% of the vitamin C amount in many horticultural crops (Lee and Kader, 2000). AA is characterized by three main biological activities in humans: as an enzyme cofactor, a free-radical scavenger and as an electron donor or acceptor (Davey et al., 2000). Moreover, it is well known that AA enhances the bioavailability of iron and calcium in the gastrointestinal tract (Patil et al., 2009). In plants, AA is essential in photosynthesis, being a cofactor of a

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key enzyme involved in H_2O_2 detoxification and acting as an electron acceptor in the photosynthetic electron transport chain. AA is also involved in plant growth, in defense against atmospherically induced oxidative stress and in wound healing in plants (Davey et al., 2000).

The bioactive compounds in fruit and vegetables are influenced by both pre- and postharvest factors on the way from producer to consumer. Of the pre-harvest factors, cultivar selection appears to be the most important for the vitamin C level (Lee and Kader, 2000). After harvest, vitamin C contents in fruit and vegetables have been observed to decrease gradually with increasing storage temperature or duration (Lee and Kader, 2000). For broccoli, a small decrease in vitamin C content, but with a relatively large decrease in AA and a threefold increase in DHA content, was observed during storage at both lower and higher temperature (Vallejo et al., 2003a,b). However, to our knowledge there are only three studies that have analyzed the changes in both AA and DHA levels in broccoli during storage. In two studies, broccoli florets or broccoli heads were stored at 20 °C for 7 (Lemoine et al., 2010) or 3 (Nishikawa et al., 2003) days. In the third study, conducted by Vallejo et al. (2003a), broccoli heads were pre-stored at 1 °C for 7 days and then stored for 3 more days at 15 °C, applying only single levels of pre-storage and storage periods and temperatures.

Evidence for an incident light effect during postharvest storage on vitamin C content in *Brassica* vegetables is scarce. Two studies showed that light during cold storage could protect against vitamin C loss in broccoli (Zhan et al., 2012) and in Chinese kale (Noichinda et al., 2007). To our knowledge, the effect of postharvest light exposure on vitamin C contents in broccoli at higher temperature, which is common during retail display, has not yet been studied.

High levels of UV irradiation, an abiotic environmental stress factor, lead to increased concentrations of antioxidants in crop plants and is considered as a tool for enhancing contents of bioactive compounds in fresh fruit and vegetables after harvest (Cisneros-Zevallos, 2003; Schreiner and Huyskens-Keil, 2006). UV exposure alters expression of genes involved in antioxidant biosynthesis, enzyme activity and causes oxidative stress in crop plants (Wang and Frei, 2011). The research on the effect of pre- and postharvest UV irradiation has focused mainly on phenolics, carotenoids and glucosinolates in fruit and vegetables (Schreiner et al., 2012), while results regarding vitamin C are scarce: UV-B and UV-C treatments were found to enhance ascorbic acid contents in apple peel (Hagen et al., 2007) and in broccoli (Lemoine et al., 2007), respectively.

The purpose of the present study was to examine how vitamin C, AA and DHA levels in broccoli flower buds are affected by postharvest light, UV-B irradiation and temperature in a multi-level design simulating mapped commercial storage conditions from harvest until consumer purchase. Two lengths of pre-storage period at two temperatures were applied to simulate transport with wholesale distribution, followed by storage at two temperatures and with four different light treatments, including three levels of visible light and darkness as a control, to simulate retail. A combination of visible light with UV-B irradiation was applied, as a fifth treatment during storage, to test whether it has a potential to increase the vitamin C content in broccoli flower buds or prevent loss.

2. Materials and methods

2.1. Chemicals

L-Ascorbic acid, L-dehydroascorbic acid, Trizma® Base (tris-(hydroxymethyl)aminomethane), TCEP (tris[2-carboxyethyl]-phosphine hydrochloride), dodecyltrimethyl ammonium chloride, *ortho*-phosphoric acid and *meta*-phosphoric acid of analytical grade were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Disodium-dihydrogen-EDTA (Titriplex® III), sodium dihydrogen phosphate monohydrate of analytical grade and acetonitrile of HPLC grade were from Merck KGAa (Darmstadt, Germany). Milli-Q quality water (Millipore, Bedford, MA, USA) was used for chemical analysis.

2.2. Plant material and storage experiment

Seeds of broccoli (*B. oleracea* L. var. *italica*, cv. Marathon) were sown in polypropylene plug trays with 77 cells filled with commercial soil comprising 86% of peat, 10% of sand and 4% of granulated clay. Four week old seedlings were transplanted into the experimental field of the Norwegian University of Life Sciences (59°39′N, 10°45′E). The soil in the field was silty loam. Before transplanting the field was fertilized with 21 g N, 8 g P and 28 g K per square meter. The experimental field was also treated with the herbicide Lentagran® (Belchim Crop Protection NV/SA, Londerzeel, Belgium; 80 g dissolved in 20 L of water) before transplanting. The plants were spaced with 40 cm between the plants and 60 cm between the rows and they were covered with light and water permeable material (Wondermesh® insect net, Wondermesh Ltd., Garvock Laurencekirk, Scotland) until the last week before harvest. After seven weeks, broccoli heads were hand-harvested at commercial maturity. Inflorescences were sorted and selected for uniform size and lack of defects.

A total of 360 broccoli heads, with a head weight of 236 ± 58 g (mean ± SD), were pre-cooled in a storage room at 0 °C immediately after harvest. Eight broccoli heads comprised the control sample at harvest and they were prepared for chemical analysis. The remaining broccoli heads were wrapped with 0.01 mm polyvinylchloride food film with about 90% transparency at 290–800 nm (Toro Clingfilm, Rieber & Søn, Bergen, Norway) and put into polypropylene plug trays, which were placed in standard reusable plastic containers with the dimensions 60 × 40 cm (Green Plus, IFCO SYSTEMS GmbH, Pullach, Germany). There were eight broccoli heads in each container that were biological replicates for each postharvest treatment. The broccoli heads were numbered and 10 cm diameter areas of the top of the heads were marked non-destructively with pins to indicate which florets to be analyzed. Half the number of such prepared broccoli heads was pre-stored in the dark at 0 °C and the other half at 4 °C for the same periods of time. After 4 or 7 days of pre-storage, samples of eight broccoli heads were collected and prepared for chemical analysis, while the remaining broccoli heads were stored for three more days. Different temperature and light conditions during the 3 day storage period were applied; two different temperatures, 10 °C or 18 °C, and five different light treatments.

The light treatments were applied 12 h per day as follows: visible light of 0, 13, 19 and 25 $\mu mol\ m^{-2}\ s^{-1}$ and a combination of 19 $\mu mol\ m^{-2}\ s^{-1}$ visible light and UV-B irradiation in the range from 19 to 22 $kJ\ m^{-2}\ d^{-1}$ (0.22–0.25 $W\ m^{-2}$). The level of UV-B irradiation chosen in the study was according to Hagen et al. (2007), who found the treatment effective in increasing the content of ascorbic acid in apple peel. Visible light was provided by cool white light L 58W/840 fluorescent tubes (Osram GmbH, Munich, Germany), and UV-B irradiation by QUV UV-B 313 nm fluorescent tubes (Q-Lab Corp.,Westlake, OH, USA). The irradiation levels were obtained by regulating the distance between the lamps and broccoli heads. The level of visible light was measured using LI-250 quantum photometer (LI-COR Corp., Lincoln, NE, USA) and Lu-Ex 02 digital luxmeter (Ecom Rolf Nied GmbH, Assamstadt, Germany). UV-B irradiance was determined using Skye RS232 meter equipped with SKU430 sensor (Skye instruments Ltd., Powys, UK) and it was in the range from 0.22 to 0.25 $W\ m^{-2}$. Additionally, 0.18 mm cellulose diacetate foil (Rachow, Hamburg, Germany) was placed

above broccoli heads treated with both visible light and UV-B radiation to exclude possible UV-C stray light. The surface temperature of broccoli heads during irradiation treatments was measured using Raynger® MX4™ noncontact infrared thermometer (Raytek® GmbH, Berlin, Germany). Room temperatures were logged every hour with average measured values of 10.4 ± 0.4 or 18.3 ± 0.3 °C. Broccoli heads were weighed before and after the experiment.

All temperature and visible light levels used in the storage part of the experiment were chosen according to the results of a survey conducted in seven Norwegian retail stores. The surface temperature of broccoli heads during display, measured with the Raynger® MX4™ noncontact infrared thermometer, was found to be in the range from 10 °C to 18 °C. The visible light level varied between 1000 lx and 2000 lx in the different stores, as determined just above the area of broccoli display using the Lu-Ex 02 digital luxmeter.

2.3. Sample preparation

Florets from the previously marked top area of individual broccoli heads were cut off from the main stem and immediately frozen in liquid nitrogen. Flower buds were scraped off the stalks and separated from the small branches using a sieve with 4 mm diameter holes under freezing conditions. The weight of flower buds per floret was 24.8 ± 3.7 g (mean ± SD, $n = 214$). The mean bud mass fraction of the florets was 38.7% (range 25.9–44.2%). The buds were placed in airtight 50 mL polypropylene tubes (VWR International, Arlington Heights, IL, US) and stored at −80 °C until analyses. Five out of eight replicates ($n = 5$) for each treatment were randomly chosen for vitamin C analysis.

2.4. Vitamin C determination

L-Ascorbic acid (AA) and L-dehydroascorbic acid (DHA) contents in broccoli flower buds were determined with some modifications according to the methods described by Davey et al. (2003) and Karlsen et al. (2005). Approximately 2 g of frozen material was placed into a polypropylene tube containing 5 mL ice-cold extraction solution with 6% *meta*-phosphoric acid and 2 mM disodium-dihydrogen-EDTA. Then additional 5 mL of the solution was added immediately and the whole mixture was homogenized using a Polytron PT 3000 homogenizer (Kinematica AG, Luzern, Switzerland) for 30 s at 24.000 rpm. The homogenate was filtered through a folded filter paper (Grade 595 1/2, Schleicher & Schüll GmbH, Dassel, Germany) and the solid residue was rinsed with 5 mL of the cold extraction solution. The filtrate was additionally clarified using 0.45 μm Millex-HV filters (Merck Millipore Ltd., Cork, Ireland). In order to perform DHA reduction, 100 μL of the clear filtrate was mixed with 50 μL reducing solution containing 800 mM Trizma Base and 5 mM TCEP (pH 9.0). After 20 min incubation time at room temperature in the dark, the sample volume was made up to 500 μL with 1% *ortho*-phosphoric acid solution containing 1 mM disodium-dihydrogen-EDTA. Samples for AA determination were prepared by diluting 100 μL of the clear filtrate with 400 μL 0.87% *ortho*-phosphoric acid solution containing 100 mM Trizma Base and 1 mM disodium-dihydrogen-EDTA. HPLC analysis was performed on an Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany), equipped with a quaternary pump, an in-line degasser, a thermo-stated autosampler (4 °C), a column heater and a photodiode array detector. Chromatographic separation was performed on a monolithic HPLC column; Chromolith® Performance RP-18e (100 mm × 4.6 mm i.d., Merck KGaA, Darmstadt, Germany) fitted with a Chromolith® RP-18e guard cartridge (5 mm × 4.6 mm i.d., Merck KGaA) and operated at 25 °C. The mobile phase consisted of 50 mM NaH_2PO_4, 2.5 mM dodecyltrimethyl ammonium chloride and 1.25 mM disodium-dihydrogen-EDTA in water with 2% (v/v) addition of acetonitrile. The pH of the mobile phase was adjusted to 4.7 using 0.1 M sodium hydroxide solution. The run time was 10 min, injection volume 10 μL and flow rate was set to 1.0 mL min^{-1}. Detection of AA was carried out at 264 nm and quantification was performed using external standard calibration curves in the concentration range 30–100 μg mL^{-1}. DHA content was determined indirectly by subtracting measured AA content from total AA (vitamin C) content obtained after DHA reduction. The results were expressed as mg ascorbic acid, dehydroascorbic acid or vitamin C per 100 g of fresh matter (FM) of broccoli flower buds.

2.5. Statistical analyses

The experimental design chosen for the study was a four factorial fixed effect model, where factors used were as follows: pre-storage period (two levels), pre-storage temperature (two levels), storage temperature (two levels) and light treatment (five levels). One way analysis of variance (ANOVA), general linear model (GLM) and Tukey's multiple comparison test with significance level $\alpha = 0.05$ were performed using Minitab® 16 Statistical Software (Minitab Inc., State College, PA, USA). Standard error of the mean (SEM) was calculated for each mean value by dividing obtained standard deviation by square root of the number of biological replicates analyzed ($n = 5$).

3. Results

The vitamin C content in flower buds of freshly harvested broccoli was 96.52 mg 100 g^{-1} FM on average, 95% and 5% of which constituted AA (91.43 mg 100 g^{-1} FM) and DHA (5.09 mg 100 g^{-1} FM), respectively (Table 1, Fig. 1). After 4 days of pre-storage at 0 °C the vitamin C content remained stable, whereas a small decrease was observed at 4 °C resulting in an average vitamin C content of 83.7 mg 100 g^{-1} FM (Fig. 1A). Seven days of pre-storage at both temperatures, 0 and 4 °C, led to about 20% loss of vitamin C content, which was still not statistically significant (Fig. 1B). However, pre-storage period (PP) and pre-storage temperature (PT) were found to be highly significant factors influencing the final vitamin C content (Table 2). This means that changing pre-storage temperature from 0 to 4 °C or pre-storage period from 4 to 7 days, or both, resulted in decreased vitamin C content in broccoli flower buds after storage.

The vitamin C content was even more reduced after 3 days storage at 10 °C or 18 °C. Broccoli heads stored at 10 °C had significant vitamin C content reductions, compared with the samples collected at harvest, for 9 and 3 out of 10 differently irradiated groups after earlier pre-storage at 4 °C and 0 °C, respectively (Fig. 1A and B). On the other hand, all groups of broccoli heads stored at 18 °C had significantly lower content of vitamin C in flower buds than samples collected at harvest; the decrease was about 40% (Fig. 1A and B). Thus, storage temperature (ST) did have an influence on the vitamin C content (Table 2).

An interaction between pre-storage period and storage temperature (PP × ST) was found significant for vitamin C, as indicated in Table 2. This means that the difference in vitamin C content in flower buds between broccoli heads stored at 10 °C and 18 °C depended on the pre-storage period. The difference was higher for broccoli heads pre-stored for the shorter period (4 days). None of the light treatments applied affected the levels of vitamin C, AA or DHA significantly (Table 2). However, some multiple-factor interactions that included light treatment were found significant (Table 2).

For broccoli heads pre-stored for 7 days, the changes in vitamin C content in flower buds mirror changes in AA, due to a stable and low level of DHA around 5 mg 100 g^{-1} fresh matter (Table 1). However, when pre-stored for 4 days at 0 °C, the DHA level increased 2-fold. Although this difference was not significant, further storage at 10 °C led to a further increase, resulting in a more than

Table 1
L-Ascorbic and L-dehydroascorbic acid contents (mg 100 g^{-1} FM) in flower buds of broccoli pre-stored for 4 days or 7 days at 0 °C or 4 °C and differently light and UV-B treated during 3 days of storage at 10 °C or 18 °C[a].

Pre-storage period	Pre-storage temperature (°C)	Storage temperature (°C)	Harvest	Pre-storage	Light and UV-B treatments				
					13 μmol m^{-2} s^{-1}	19 μmol m^{-2} s^{-1}	25 μmol m^{-2} s^{-1}	UV-B + 19 μmol m^{-2} s^{-1}	Dark
L-Ascorbic acid									
4 days	0	10	91.43 a	83.53 ab	77.57 a–c[b]	77.02 a–c	74.85 a–c	70.00 b–e	64.64 b–g
		18			43.03 ij	45.59 g–j	41.21 j	50.09 e–j	43.94 h–j
	4	10		76.44 a–c	61.37 c–i	65.67 b–f	69.86 b–e	70.61 b–d	63.77 b–h
		18			53.53 d–j	47.99 f–j	50.76 d–j	39.14 j	44.24 h–j
7 days	0	10	91.43 a	72.49 ab	68.37 b–e	64.27 b–f	70.98 ab	70.28 bc	68.98 b–d
		18			40.91 h	47.80 e–h	49.69 c–h	57.01 b–h	57.17 b–h
	4	10		74.14 ab	60.78 b–h	61.83 b–g	66.30 b–f	64.58 b–f	65.83 b–f
		18			46.01 f–h	49.49 d–h	48.63 d–h	48.31 d–h	42.57 gh
L–Dehydroascorbic acid									
4 days	0	10	5.09 e	10.86 a–e	14.13 ab	12.78 a–d	13.24 a–c	15.15 a	13.03 a–d
		18			11.40 a–e	10.64 a–e	9.32 a–e	10.49 a–e	8.52 a–e
	4	10		7.30 b–e	8.31 a–e	10.89 a–e	7.46 b–e	8.30 a–e	9.39 a–e
		18			5.88 de	7.58 b–e	6.59 c–e	7.10 b–e	6.23 c–e
7 days	0	10	5.09 a	5.54 a	5.03 a	4.85 a	5.11 a	5.35 a	6.10 a
		18			5.41 a	4.65 a	6.27 a	6.32 a	5.86 a
	4	10		4.83 a	5.84 a	6.58 a	7.21 a	6.46 a	5.80 a
		18			5.53 a	4.72 a	4.73 a	5.99 a	5.61 a

[a] Data presented as mean values ($n = 5$).
[b] Values for each compound within four adjacent rows (obtained for broccoli with the same pre-storage period) followed by different letter are significantly different at $P \leq 0.05$.

2.5-fold higher level than at harvest. The DHA content in flower buds of broccoli heads pre-stored at 4 °C was not significantly different from the contents at harvest and after pre-storage. Thus the interaction between pre-storage period and pre-storage temperature (PP × PT) was highly significant for DHA content. In addition, the DHA content in broccoli flower buds after storage was affected by pre-storage period, pre-storage temperature and storage temperature (Table 2). Of all the factors investigated, the AA content was mainly affected by storage temperature, and only to a minor extent by pre-storage temperature.

The DHA/AA ratio was stable with a value around 0.1 in flower buds of broccoli pre-stored for 7 days (Fig. 2B), whereas there was

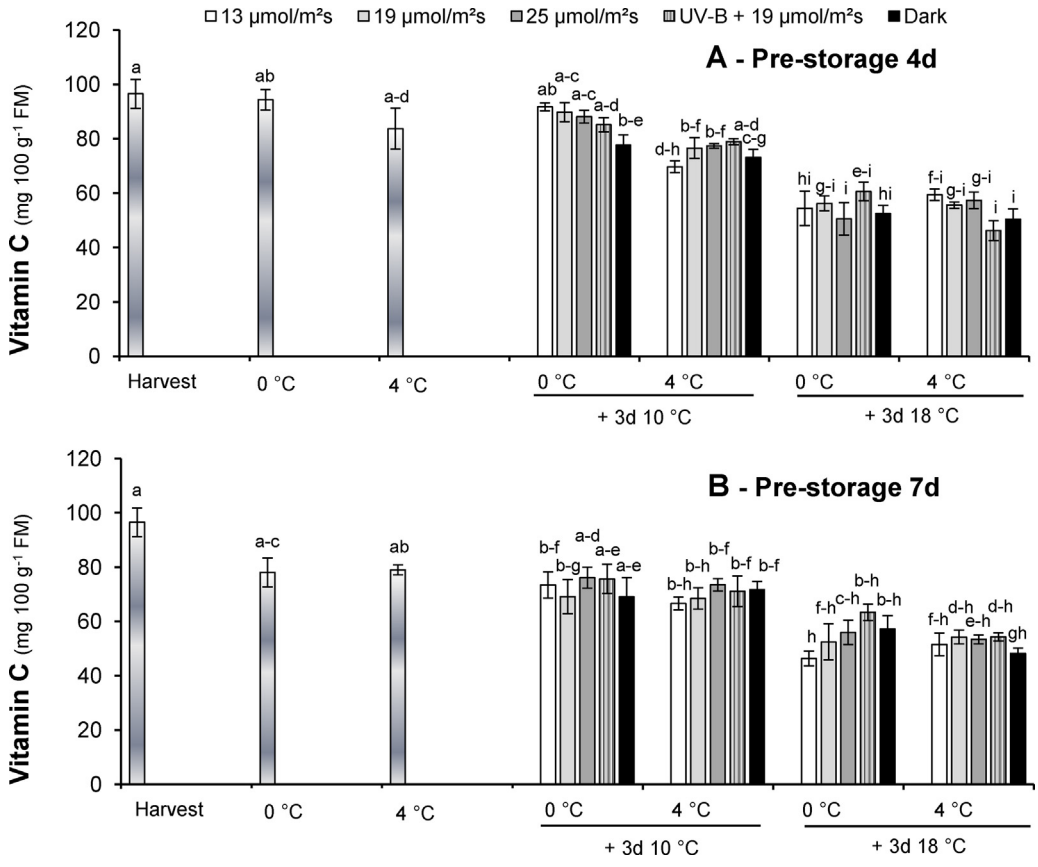

Fig. 1. Vitamin C content (mg 100 g^{-1} FM) in flower buds of broccoli pre-stored for 4 days (A) or 7 days (B) at 0 °C or 4 °C and differently light and UV-B treated during 3 days of storage at 10 °C or 18 °C. Bars represent mean values ± SEM ($n = 5$). Bars marked with different letters in each graph indicate significantly different values at $P \leq 0.05$.

Table 2
Significance of pre-storage period[a], pre-storage temperature[b], storage temperature[c], light and UV-B treatments[d] and all interactions for vitamin C, L-ascorbic acid (AA), L-dehydroascorbic acid (DHA) and the ratio DHA/AA in broccoli flower buds determined by general linear model analysis.

Source of variation	*F* values			
	Vitamin C	AA	DHA	DHA/AA
Pre-storage period (PP)	13.35 ***	0.04 NS	116.90 ***	79.41 ***
Pre-storage temperature (PT)	17.06 ***	6.59 *	23.79 ***	11.68 ***
Storage temperature (ST)	337.09 ***	287.79 ***	17.53 ***	11.58 ***
Light and UV-B treatments (LT)	0.99 NS	0.85 NS	0.36 NS	0.23 NS
PP × PT	1.02 NS	0.69 NS	33.54 ***	23.41 ***
PP × ST	13.00 ***	6.40 *	11.18 ***	0.06 NS
PP × LT	2.72 *	2.07 NS	0.86 NS	0.4 NS
PT × ST	4.33 *	4.37 *	0.00 NS	4.41 *
PT × LT	0.85 NS	0.72 NS	0.69 NS	1.06 NS
ST × LT	0.28 NS	0.35 NS	0.15 NS	0.57 NS
PP × PT × ST	4.83 *	2.55 NS	3.51 NS	0.45 NS
PP × PT × LT	1.15 NS	0.88 NS	0.70 NS	0.5 NS
PP × ST × LT	0.70 NS	0.76 NS	0.25 NS	0.56 NS
PT × ST × LT	3.85 **	4.29 **	0.31 NS	1.65 NS
PP × PT × ST × LT	0.61 NS	0.73 NS	0.65 NS	0.97 NS

NS, *, **, *** Non-significant or significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.
[a] Two levels: 4 and 7 days.
[b] Two levels: 0 °C and 4 °C.
[c] Two levels: 10 °C and 18 °C.
[d] Five levels: 13 μmol m^{-2} s^{-1}, 19 μmol m^{-2} s^{-1}, 25 μmol m^{-2} s^{-1}, UV-B + 19 μmol m^{-2} s^{-1} and the dark.

a tendency of increasing ratio after 4 days of pre-storage (Fig. 2A). GLM analysis showed that pre-storage period and temperature affected the DHA/AA ratio in broccoli flower buds significantly (Table 2). After storage, broccoli buds pre-stored at 0 °C showed a higher DHA/AA ratio than broccoli buds pre-stored at 4 °C, but only for plants with the shorter (4 days) pre-storage period (Fig. 2). This is in accordance with the high statistical significance for the interaction between pre-storage period and temperature for the DHA/AA ratio (Table 2). An increase of the storage temperature from 10 °C to 18 °C gave a significant increase of the DHA/AA ratio by 19% on average. Although no effect of light treatments on the DHA/AA ratio was found in general (Table 2), three groups of

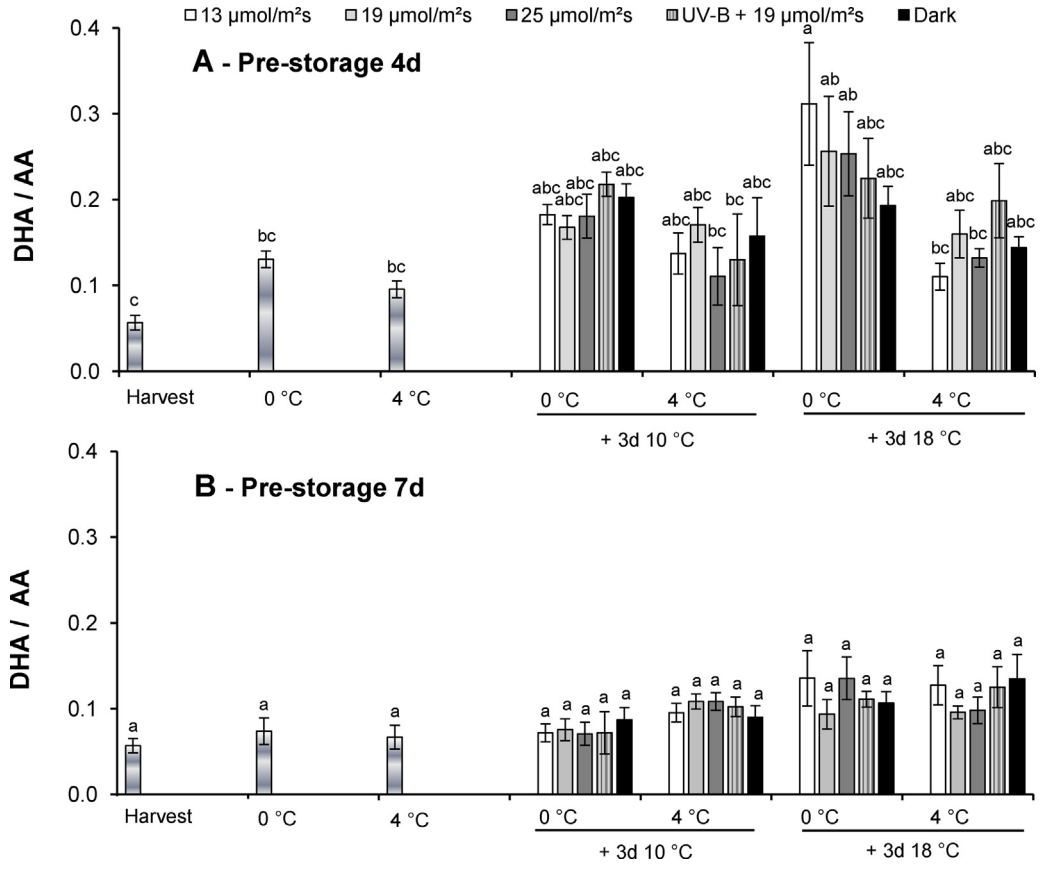

Fig. 2. Ratio of L-dehydroascorbic acid and L-ascorbic acid contents (DHA/AA) in flower buds of broccoli pre-stored for 4 days (A) or 7 days (B) at 0 °C or 4 °C and differently light and UV-B treated during 3 days of storage at 10 °C or 18 °C. Bars represent mean values ± SEM ($n = 5$). Bars marked with different letters in each graph indicate significantly different values at $P \leq 0.05$.

broccoli heads that were exposed to visible light during storage at 18 °C after pre-storage at 0 °C had a significantly higher ratio than freshly harvested broccoli (Fig. 2A).

4. Discussion

Changes in vitamin C, AA and DHA levels in broccoli flower buds were examined during two different pre-storage periods at two different temperatures, as well as during storage at two different temperatures and different light treatments. To our knowledge, this is the first time such an experimental approach has been applied to study the effects of commercially relevant storage conditions on vitamin C and its components in broccoli. This experimental design gave the possibility to evaluate not only the effect of storage temperature on vitamin C, AA and DHA levels in broccoli flower buds, but also the influence of pre-storage period and pre-storage temperature on these compounds during storage. Another novelty of this study was to examine the effect of postharvest light and UV-B irradiation at temperatures commonly applied during retail display. In addition, analysis of not only vitamin C, but also AA and its oxidized form DHA, together with data for the DHA/AA ratio, has increased the knowledge of ascorbate metabolism in broccoli flower buds during pre-storage and storage periods. In a previous study, flower buds have been shown to have a higher content of vitamin C than the floret stalks of the broccoli cultivar 'Lord' (unpublished results). This uneven distribution of vitamin C between flower buds and floret stalks together with the observed large variation in the mass fraction of flower buds in the florets, were the reasons why flower buds were chosen as the plant material in the present study.

The content of vitamin C in broccoli flower buds at harvest was 23% higher than the level determined by Vallejo et al. (2002) in florets of the same cultivar of broccoli. This difference was expected since vitamin C, as previously mentioned, was found to be unevenly distributed between flower buds and stalks with higher content in flower buds. Both vitamin C and AA levels in broccoli are influenced by cultivar and growing conditions (Vallejo et al., 2003a,b). Therefore it is not surprising that other authors have found both higher (Nath et al., 2011) and lower (Lemoine et al., 2010; Fernandez-Leon et al., 2013) AA levels in florets and heads of different broccoli cultivars at harvest. DHA levels in 'Marathon' broccoli in two earlier studies (Vallejo et al., 2003a,b) were reported to be 2.7 or 3.1–4.8 folds higher than those found in the present study at harvest, probably due to different growing conditions and/or plant material used for analysis. A much higher DHA/AA ratio than a normal, basic level of about 0.1 could be due to various stress before, during or after harvest.

In the present study, vitamin C and AA contents of broccoli flower buds were relatively stable during pre-storage at 0 °C or 4 °C for 4 or 7 days. Similarly, other studies with broccoli heads in packaging with high gas permeability reported either lack of change in AA content after storage at 4 °C for 6 days (Nath et al., 2011) or a 2.4% decrease after storage at 1 °C for 7 days (Vallejo et al., 2003a,b). In the latter study, there was no change in vitamin C content during the storage period, whereas the DHA content almost tripled. In contrast, Fernandez-Leon et al. (2013) observed 34% loss of AA content in unpackaged broccoli heads stored for 2 days at 1–2 °C, but the DHA content was not analyzed.

Despite the stability during cold pre-storage, flower buds of broccoli pre-stored at the lowest temperature (0 °C) or for the shortest period (4 days) had higher vitamin C levels at the end of the experiment than those pre-stored at a slightly higher temperature or for a longer period (4 °C, 7 days). This is in agreement with a general finding that vitamin C in plant products continuously degrades during storage, and that the rate of decrease is faster with increased storage temperature (Lee and Kader, 2000). Our results extend this finding and indicate that it is valid also for cold pre-storage. Therefore the rate of vitamin C decrease in broccoli flower buds during storage depends not only on storage temperature, but also on pre-storage temperature and pre-storage period. The stable levels of DHA and DHA/AA after 7 days of pre-storage compared to the elevated levels after 4 days of pre-storage suggest that the ascorbate metabolism in broccoli changed during the cold pre-storage period. Similarly, it has been shown that the expression of genes involved in ascorbate oxidation and regeneration changed in harvested broccoli florets, however during storage at 20 °C (Nishikawa et al., 2003). In our study, broccoli with shorter pre-storage period probably maintained a higher rate in ascorbate metabolism, including AA oxidation, AA synthesis or both. AA oxidation can be catalyzed by ascorbate peroxidase, with simultaneous reduction of hydrogen peroxide, or by ascorbate oxidase, which also reduces molecular oxygen to water. The activity of the second enzyme has previously been found to be positively correlated with DHA accumulation (Davey et al., 2000), and reported to increase followed by a decrease during storage in broccoli (Raseetha et al., 2013). According to this, differences in DHA content and DHA/AA ratio between broccoli florets with different pre-storage periods in our study could possibly be due to different levels of ascorbate oxidase activity.

Our study clearly demonstrates that storage temperature had a significant effect on vitamin C, the AA and DHA levels and the ratio DHA/AA in broccoli flower buds. Storage at higher temperature (18 °C) caused significant losses of vitamin C and AA, both of which were in the range 40–50% and 30–40% as compared to freshly harvested and already pre-stored plants, respectively. Storage at 18 °C also increased the DHA/AA ratio, whereas a lower storage temperature (10 °C) resulted in higher retention of AA and DHA with a lower DHA/AA ratio. These findings are in accordance with other studies, which reported AA losses in the same range as ours in broccoli after storage at higher temperature with (Fernandez-Leon et al., 2013) or without (Lemoine et al., 2010; Nath et al., 2011) pre-storage in the cold. An increase in the DHA/AA ratio during storage was also observed for spinach (Hodges and Forney, 2003; Bergquist et al., 2006;). On the other hand, Vallejo et al. (2003a) reported only 13% reduction in vitamin C content in broccoli after pre-storage at 1 °C followed by storage at 15 °C for 10 days in total. However, in that study the AA level in broccoli decreased by 38% and DHA increased three times during storage.

AA has a capacity to scavenge reactive oxygen species (ROS). When production of ROS, including superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and more toxic hydroxyl radical ($OH^{\bullet}$), exceeds the ability of plant scavenging system to repel them, oxidative stress occurs. AA can scavenge ROS directly, or indirectly as a substrate of ascorbate peroxidase, which reduces H_2O_2. The H_2O_2 reduction with simultaneous AA oxidation begins a series of enzymatic reactions in the ascorbate–glutathione cycle (Davey et al., 2000; Hodges et al., 2004; Toivonen, 2004). However, extensive ROS accumulation in broccoli florets stored at higher temperature has been shown to suppress the ascorbate–glutathione cycle resulting in ascorbate degradation (Raseetha et al., 2013). It was previously reported that this degradation was mainly caused by a decline in the ascorbate regenerating system including monodehydroascorbate reductase and dehydroascorbate reductase activity (Nishikawa et al., 2003). Most probably, in our experiment this occurred in broccoli florets during storage at 18 °C and could explain the observed ascorbate losses and the increased DHA/AA ratio levels. Moreover, since lower storage temperature (10 °C) better preserved ascorbate content in broccoli, and degradation of this compound is a recognized indicator of oxidative stress (Toivonen, 2004), we could assume that lowering storage temperature can lead to reduced level of oxidative stress in broccoli. Additionally, the lower DHA/AA ratio in broccoli stored

at 10 °C indicates a lower senescence rate (Hodges and Forney, 2003).

No effect of postharvest light treatment on vitamin C, AA and DHA contents in broccoli flower buds was observed. Similarly, Martinez-Sanchez et al. (2011) reported that exposure to visible light during storage did not change vitamin C content in Romaine lettuce. This finding was however contrary to other studies, which report the ability of visible light treatment to reduce the degradation of ascorbic acid levels in broccoli (Zhan et al., 2012) and in Chinese kale (Noichinda et al., 2007) during storage. The light treatments in those studies were applied under lower temperatures (1 °C and 7 °C) than in our experiment, and that could have influenced the light treatment effect. Moreover, this positive effect of light treatment was observed in differently prepared plant material than ours. For instance, Zhan et al. (2012) used separated florets that could show different response to visible light due to cutting stress than intact broccoli heads used in our experiment.

The levels of vitamin C, AA and DHA in broccoli flower buds were not influenced by the combination of visible light and UV-B irradiation during storage. This was in accordance with Kanazawa et al. (2012), who found no change in ascorbic acid content in parsley upon short-term irradiation with both UV-A and UV-B applied for 3 days during 6 days of refrigerated storage. Similarly, UV-C irradiation treatments performed before storage had no effect on ascorbic acid content in mandarin segments stored at 4 °C for up to 12 days (Shen et al., 2013). On the contrary, other studies have reported an increase in ascorbic acid content in apple peel after irradiation with a combination of visible light and UV-B (Hagen et al., 2007), or a delay in ascorbic acid degradation in broccoli florets stored for 21 days at 4 °C after UV-C treatment (Lemoine et al., 2007). The irradiation treatment conducted by Hagen et al. (2007) is similar to the one in the present study and applied at the same temperature (10 °C), however performed for longer time and on other plant material, which can explain the diverging results obtained. Lemoine et al. (2007) applied a considerably different irradiation procedure including UV-C irradiation, which probably caused distinct response in the broccoli florets. Significant discrepancies in the ascorbic acid content between irradiated and non-irradiated broccoli florets were shown after 21 days of storage at 4 °C, but not after 7 or 14 days (Lemoine et al., 2007).

In conclusion, the present study has demonstrated that although vitamin C in broccoli flower buds was stable during pre-storage, it degraded during storage as a function of increasing pre-storage period, pre-storage temperature and storage temperature. Addition of visible light with or without UV-B irradiation at temperatures commonly applied during retail (10 °C and 18 °C), did not change the rate of vitamin C degradation. Therefore, to preserve high contents of vitamin C in broccoli, it is recommended to shorten the wholesale period (to 4 days or less), keep the temperature close to 0 °C (instead of common 4 °C) during wholesale and, most importantly, keep the temperature during retail as low as possible between non-freezing temperature and room temperature. Interestingly, ascorbic acid content in broccoli flower buds was affected mainly by storage temperature and only to a minor extent by the investigated pre-storage temperatures. The increased DHA level and the DHA/AA ratio after storage when broccoli was pre-stored for 4 days at both 0 °C and 4 °C, compared with the stable values in broccoli pre-stored for 7 days suggest a shift in the ascorbate metabolism with a higher rate after shorter pre-storage period. Therefore, the vitamin C content of broccoli pre-stored for shorter time (4 days) will probably be more susceptible to various postharvest handling procedures than broccoli pre-stored longer (7 days) at low temperature.

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Paper II

Flavonols in broccoli (*Brassica oleracea* L. var. *italica*) flower buds as affected by postharvest temperature and radiation treatments

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Abstract

The aim of this study was to evaluate the effects of postharvest temperature, light and UV-B irradiation on the flavonol content in broccoli using a multi-level design simulating commercial storage conditions from harvest until consumer purchase. The flavonol content in broccoli flower buds was examined during pre-storage and storage of broccoli heads, representing refrigerated transport with wholesale distribution and retail, respectively. Broccoli heads were pre-stored for four or seven days at 0 or 4 °C in the dark and then stored for three days at 10 or 18 °C. During storage, the broccoli heads were exposed for 12 h per day to three different levels of visible light (13, 19 or 25 μmol m^{-2} s^{-1}) or a combination of visible light (19 μmol m^{-2} s^{-1}) and UV-B irradiation (20 kJ m^{-2} d^{-1}), or they were stored in the dark. The contents of flavonols (quercetin, kaempferol and isorhamnetin), measured by HPLC as aglycons in flower bud extracts, were not significantly influenced by neither pre-storage period, pre-storage temperature nor storage temperature. However, the interaction between pre-storage period and pre-storage temperature was found significant for flavonol contents during storage, with higher contents observed for broccoli pre-stored shorter (4 d) at 4 °C and pre-stored longer (7 d) at 0 °C. The levels of epidermal flavonols, monitored with non-destructive repeated measurements of the broccoli heads during storage, were observed to increase with prolonged pre-storage and storage periods. General linear model analysis revealed that radiation treatments significantly affected both quercetin content and epidermal flavonol levels in broccoli flower buds during storage, with the highest levels observed after a combination of visible light and UV-B irradiation treatment. However, the outcome of this postharvest treatment was influenced by a combination of factors including pre-storage period, pre-storage temperature and storage temperature. For one group of broccoli heads, that were pre-stored for four days at 4 °C and exposed to visible light with UV-B irradiation during storage at 10 °C, a significant 3.5-fold increase in the level of all flavonols was observed as compared with after pre-storage. The non-

destructive measurements demonstrated that accumulation of epidermal flavonols upon UV-B exposure was initiated earlier in broccoli heads stored at higher temperature (18 °C), that is, already during the first day of storage.

Keywords: epidermal flavonols, chlorophyll fluorescence, UV-A absorbance, storage period, visible light, UV-B irradiation

1. Introduction

The flavonols comprise one of the major groups of flavonoids, a ubiquitous class of phenolic compounds. Dietary flavonoids are associated with reduced risk of chronic diseases and age-related disorders including cardiovascular diseases, neurodegeneration and carcinogenesis (Quideau et al., 2011; Bondonno et al., 2015; Ivey et al., 2015). The health-promoting effects of flavonoids are mainly based on their specific interactions with proteins such as enzymes, transcription factors and receptors, while non-specific antioxidant properties through free radical scavenging or metal chelation appear to be less important (Fraga et al., 2010; Quideau et al., 2011; Del Rio et al., 2013). In plants, flavonoids act as antioxidants, photoreceptors and UV radiation protectors (Pietta, 2000), and they are also involved in plants' general response to stress, for which flavonols appear to be of most importance (Winkel-Shirley, 2002). The flavonols are the most widespread flavonoids in plants, including quercetin, kaempferol, isorhamnetin and myricetin as the most common (Crozier et al., 2009).

The phenolic composition of fruits and vegetables is influenced by genetic and different agronomic and environmental factors and may be affected by postharvest factors including handling during harvest, transport and storage conditions (Tomás-Barberán and Espín, 2001; Cartea et al., 2011). Among postharvest factors affecting flavonol content, the influence of storage is still not clear. In *Brassica* vegetables some studies show no change in flavonol contents during cold storage (Winkler et al., 2007; Hagen et al., 2009), while others found increase (Harbaum et al., 2008), decrease (Vallejo et al., 2003), or even both depending on the

cultivation season (Kałużewicz et al., 2012). High temperature during storage seems to cause an increase in flavonoid contents in *Brassica* vegetables (Starzyńska et al., 2003; Harbaum et al., 2008; Kałużewicz et al., 2012). Evidence for an incident light effect during storage on flavonol contents in *Brassica* vegetables is scarce. Two studies show that fluorescent light during storage at 7 ºC increase total phenols in broccoli and cauliflower florets (Zhan et al., 2012; Zhan et al., 2014), whereas a third study found only a slight increase in total phenols in broccoli florets after low intensity (12 – 13 μmol m^{-2} s^{-1}) fluorescent light treatment conducted at 25 °C (Jin et al., 2015). To our knowledge, the effect of postharvest light exposure on flavonol content in broccoli, conducted at temperatures that are common during retail display, has not yet been reported.

UV-B irradiation influences plant growth, morphology and biochemical composition (Jenkins, 2014). The plants' response towards UV-B irradiation varies between acute stress and regulatory photomorphogenic responses, including the expression of genes involved in UV-protection and flavonoid accumulation (Jenkins, 2009). Postharvest UV-B irradiation has previously been found to enhance phenolic compounds in apple peel (Hagen et al., 2007), spinach, radish sprouts, parsley (Kanazawa et al., 2012), tomato (Liu et al., 2011) and asparagus (Eichholz et al., 2012). However, little is known about how temperature influences the outcome of this postharvest treatment.

Chlorophyll fluorescence (ChlF) excitation can be used as a tool for non-destructive estimation of epidermal phenolic compounds in plants (Bilger et al., 2001; Agati et al., 2011). The method is based on the optical properties of phenolic compounds, and the epidermal content of these compounds is estimated by the difference between ChlF excited by UV-A radiation and by visible light. Although the method estimates only epidermal phenolic compounds and gives no information on the individual compound levels, it allows for multiple measurements over time on one particular sample. Many studies have already validated the

application of the ChlF method for estimation of epidermal flavonoids in plants as reviewed by Julkunen-Tiitto et al. (2014). The epidermal flavonoids have been mostly estimated in leaves of different plant species (Hagen et al., 2009; Kuhlmann and Müller, 2009; Harbaum-Piayda et al., 2010; Morales et al., 2010; Agati et al., 2011; Rodriguez et al., 2014), but also in fruit skins, such as grape (Lenk et al., 2007; Cerovic et al., 2008) and apple skin (Hagen et al., 2006; Betemps et al., 2012). However, to our knowledge, ChlF for assessment of epidermal flavonoids in broccoli flower buds has so far been applied only by Bengtsson et al. (2006).

Broccoli (*Brassica oleracea* L. var. *italica*) is a cruciferous vegetable of high economic importance and the world production of this crop has doubled the last two decades (FAOSTAT, 2013). However, postharvest handling of this highly perishable vegetable is challenging due to its rapid senescence, with degradation of chlorophyll within flower bud sepals as a visual sign (Page et al., 2001). Therefore, broccoli can be stored for only two to four weeks in refrigerated conditions (Nunes, 2008). Broccoli has been recognized as one of the major food sources of flavonols in human diet (Hollman and Katan, 1999; Ross and Kasum, 2002; Manach et al., 2004). The flavonols are present in broccoli inflorescences as glycosides of kaempferol, quercetin and isorhamnetin with complex and less complex structures. Additionally, almost all native flavonols in broccoli are acylated with hydroxycinnamic acids including *p*-coumaric, caffeic, ferulic and sinapic acids (Vallejo et al., 2004).

The purpose of the present study was to examine how flavonol levels in broccoli flower buds are affected by storage time, temperature and radiation treatments in a multi-level design simulating mapped commercial storage conditions from harvest until consumer purchase. Two lengths of pre-storage period at two temperatures were applied to simulate transport with wholesale distribution, followed by storage at two temperatures and with four different light treatments, including three levels of visible light and darkness as a control, to simulate retail. A combination of visible light with UV-B irradiation was applied, as a fifth treatment during

storage, to test whether it has a potential to increase the flavonol contents in broccoli flower buds. The ChlF method was applied for repetitive and non-destructive measurements of epidermal flavonol levels in broccoli flower buds during the storage periods. Thus, the kinetics of flavonols accumulation during postharvest treatments was evaluated.

2. Materials and methods

2.1. Chemicals

Quercetin was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Kaempferol and isorhamnetin were obtained from Fluka Chemie GmbH (Buchs, Switzerland). Acetonitrile, methanol of HPLC grade, glacial acetic acid and hydrochloric acid of analytical grade were purchased from Merck KGAa (Darmstadt, Germany). Milli-Q quality water (Millipore, Bedford, MA, USA) was used for chemical analysis.

2.2. Plant material and storage experiment

Broccoli (*Brassica oleracea* L. var. *italica*, cv. Marathon) inflorescences were grown and the storage experiment was conducted as previously described by Rybarczyk-Plonska et al. (2014).

In brief, a total of 360 broccoli heads were pre-cooled in a storage room at 0 °C immediately after harvest. Eight broccoli heads comprised the control sample at harvest and were prepared for chemical analysis. The remaining broccoli heads were wrapped with 0.01 mm polyvinylchloride food film and placed in polypropylene plug trays. For each postharvest treatment there were eight broccoli heads representing biological replicates. The broccoli heads were numbered and 10 cm diameter areas of the top of the heads, which coincided with the area of ChlF measurement, were marked non-destructively with pins to indicate which florets to be analysed. Half the number of such prepared broccoli heads were pre-stored in the dark at 0 °C and the other half at 4 °C for the same periods. Samples were collected and prepared for

chemical analysis after four and seven days of pre-storage, and after storage for three more days. During the three-day storage period, two different temperatures, 10 °C or 18 °C, and five different radiation treatments were applied. Broccoli heads were weighed before and after the experiment.

The radiation treatments were applied 12 h per day as follows: visible light of 0, 13, 19 and 25 μmol m^{-2} s^{-1} and a combination of 19 μmol m^{-2} s^{-1} visible light and UV-B irradiation in the range from 19 - 22 kJ m^{-2} d^{-1} (0.22 - 0.25 W m^{-2}). The irradiation levels were obtained by regulating the distance between the lamps and broccoli heads. Additionally, 0.18 mm cellulose diacetate foil (Rachow, Hamburg, Germany) was placed above broccoli heads treated with both visible light and UV-B radiation to exclude possible UV-C stray light.

The temperature and visible light levels used during the three-day storage were chosen according to the results of a survey conducted in seven Norwegian retail stores. The surface temperature of broccoli heads during display, measured with a Raynger® MX4™ non-contact infrared thermometer, was found to be in the range from 10 °C to 18 °C. The visible light level varied between 1000 lux and 2000 lux in the different stores, as determined just above the area of broccoli display using a Lu-Ex 02 digital luxmeter. The level of UV-B irradiation chosen in the study was according to Hagen et al. (2007), who found the treatment effective in increasing the content of flavonols in the peel of shade-grown apples.

2.3. Chemical analyses

2.3.1. Sample preparation

Florets from the previously marked top area of individual broccoli heads were cut off from the main stem and immediately frozen in liquid nitrogen. Flower buds were scraped off the stalks and separated from the small branches using a sieve with 4 mm diameter holes under freezing conditions. The weight of flower buds per floret was 24.8 ± 3.7 g (mean ± SD, n=214). The mean bud mass fraction of the florets was 38.7% (range 25.9 – 44.2%). The buds were

placed in 50 mL polypropylene tubes (VWR International, Arlington Heights, IL, US) and stored at -80 °C until analyses. Five out of eight biological replicates (n=5) for each treatment were randomly chosen for flavonols analysis.

2.3.2. Flavonols extraction

The extraction procedure was according to Steindal et al. (2013) with some modifications. Approximately 10 g of frozen broccoli flower buds, was freeze dried using GAMMA 1-16 LSC laboratory freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until the constant weight of the material was obtained. Dry matter was determined after freeze drying and the material was ground to a fine powder in a mortar with a pestle. In order to perform phenolic compounds extraction, 200 mg of freeze dried flower buds was placed into a 15 mL polypropylene tube (VWR International, Arlington Heights, IL, US) and thermostated at 73 °C for 3 min. Then 3 mL of hot (73 °C) 70% aqueous methanol was added and the sample was hand mixed at 73 °C for 3 min. After extraction the samples were centrifuged at 4400 rpm for 15 min at 4 °C (Heraeus® Multifuge® 4KR, Thermo Electron Corp., Waltham, MA, US). The pellets were re-extracted under the same conditions as above and the supernatants obtained from two-step extraction procedure were combined. Such prepared extracts were stored at -80 °C until further acid hydrolysis.

2.3.3. Acid hydrolysis of flavonol glycosides

The acid hydrolysis of flavonol glycosides in the broccoli flower buds extracts was performed according to the method described by Hagen et al. (2009). Briefly, 2 mL of the methanolic extract was mixed with 2 mL of 2 M HCl in a headspace crimp top vial (Agilent Technologies, Waldbronn, Germany), flushed with nitrogen gas for 15 s before sealing the vial, and hydrolysed for 90 min at 94 °C. After cooling the samples on ice, the extracts were filtered

through 0.45 μm Millex-HA filters (Millipore, Molsheim, France), and the HPLC analysis was performed shortly after.

2.3.4. Quantitative HPLC analysis of flavonol aglycons

The HPLC analysis of flavonol aglycons in the acid hydrolysed extracts was conducted as described by Hagen et al. (2009), but using a Betasil RP-C18 column equipped with a C18 guard column both obtained from Thermo Fisher Scientific (Waltham, MA, USA), and a HPLC run time of 31 min. Eluent A consisted of 2% acetic acid in water, whereas eluent B consisted of 2% acetic acid in water:acetonitrile (50:50; v/v) solution. The gradient elution profile was as follows: 55-65% B in 15 min, 65-85% B in 6 min, 85-100% B in 1 min followed by 3 min 100% B, 100%-55% B in 1 min and finally 5 min 55% B as column equilibration step. Identification was performed by comparing retention times and spectral characteristics with those of the representative standards. Flavonol aglycons were quantified on a basis of external standard curves for quercetin, kaempferol and isorhamnetin, with UV detection at 370 nm. The results were expressed as milligram quercetin, kaempferol or isorhamnetin aglycons per 100 gram of dry matter of broccoli flower buds, and then recalculated as milligram per 100 gram of fresh matter.

2.4. Non-destructive chlorophyll fluorescence measurements

Chlorophyll fluorescence (ChlF) was measured using a hand-held multi-parametric optical sensor Multiplex® 3 (Force-A, Orsay, France). The ChlF measurements were conducted in triplicate on the marked area on top of the broccoli heads after harvest, at the beginning of storage (after 1 – 3 hours of storage), after one day of storage and after three days of storage, just before sampling. Every measuring session started with instrument temperature stabilization and a calibration procedure using black frame and blue standards, both obtained from Force-A. The calibration procedure was also performed at the end of each session. The

signals obtained for far-red ChlF excited with UV-A radiation (375 nm) and red light (630 nm), that is, FRF_{UV} and FRF_R respectively, were recorded and corrected by calibration data. As UV-A radiation, in contrary to red light, is absorbed by epidermal phenolic compounds, relative UV-A epidermal absorbance (A_{UV}) values were calculated according to the equation given by Hagen et al. (2006):

$$A_{UV} = \log \frac{FRF_R}{FRF_{UV}}$$

The calculated A_{UV} data were further normalized by dividing the value determined in every time point for each broccoli head by the corresponding value obtained at the beginning of storage. The normalized data were used to illustrate the changes in A_{UV} in broccoli flower buds during storage.

2.5. Statistical analyses

The experimental design chosen for the study was four and five factorial fixed effect model, for flavonol and A_{UV} data, respectively. Factors used were as follows: pre-storage period (two levels), pre-storage temperature (two levels), storage temperature (two levels), storage period (three levels, only for A_{UV} data) and light treatment (five levels). One way analysis of variance (ANOVA), general linear model (GLM) and Tukey's multiple comparisons test with significance level $\alpha = 0.05$ were performed using Minitab® 16 Statistical Software (Minitab Inc., State College, PA, USA). Nonlinear regression with fit curve option was conducted using SigmaPlot™ Version 10.0 Software (Systat Software Inc., San Jose, CA, USA). Standard error of the mean (SEM) was calculated for each mean value by dividing obtained standard deviation by square root of the number of biological replicates analysed.

3. Results

3.1. Effect of postharvest temperature on flavonol contents

At harvest, the total content of flavonol aglycons in broccoli flower buds was on average 5.15 ± 0.79 mg 100 g^{-1} fresh matter (n = 5) . Quercetin (1.79 ± 0.33 mg 100 g^{-1} fresh matter), kaempferol (2.96 ± 0.42 mg 100 g^{-1} fresh matter) and isorhamnetin (0.4 ± 0.06 mg 100 g^{-1} fresh matter) accounted for 35%, 57% and 8% of the total flavonol content, respectively (Table 1, Fig. 1). The contents of flavonols were not significantly influenced by temperature (0 °C or 4 °C) during pre-storage for four (Fig. 1) or seven days (Table 1). Furthermore, pre-storage period (PP) and temperature (PT) were not found to influence the flavonol contents in broccoli flower buds during further storage (Table 2). However, a significant interaction between these two factors (PP × PT) was found for all flavonols, indicating that a possible influence of pre-storage period on the flavonol contents after storage depends on the temperature during pre-storage (Table 2). GLM analysis revealed that storage temperature (ST), 10 or 18 °C, had no effect on flavonol contents in broccoli flower buds (Table 2). However, an interaction between pre-storage temperature and storage temperature (PT × ST) was found significant for isorhamnetin (Table 2).

3.2. Effect of postharvest radiation treatments on flavonol contents

Significantly higher contents of flavonols in flower buds, than at harvest or after pre-storage, were found in broccoli stored at 10 °C and exposed to visible light of 19 μmol $m^{-2}s^{-1}$ and UV-B irradiation, but only after four days of pre-storage at 4 °C (Fig. 1). The flower buds contained 4.63 ± 0.78, 6.86 ± 1.31 and 1.01 ± 0.26 mg 100 g^{-1} fresh matter of quercetin, kaempferol and isorhamnetin after treatment, approximately 3.5 times higher than after pre-storage. However, compared to broccoli heads at harvest this specific radiation treatment resulted in a 2.3- 2.6-fold increase of flavonols, which was significant for total flavonols (data not shown), quercetin and isorhamnetin, but not for kaempferol (Fig. 1). None of the other radiation treatments during storage altered the flavonol contents significantly (Fig. 1), including those after pre-storage for seven days (Table 1). According to GLM analysis of all

data, radiation treatments (R) during storage significantly affected the content of quercetin, for which the combination of visible light (19 μmol $m^{-2}s^{-1}$) and UV-B radiation produced higher contents than the lowest (13 μmol $m^{-2}s^{-1}$) visible light level (Table 2). A three-factor interaction between pre-storage temperature, storage temperature and radiation treatment (PT × ST × R) was found significant for all the three flavonols analysed, indicating that the effect of the radiation treatment depended on the temperature during both pre-storage and storage. In addition, a three-factor interaction (PP × PT × R) and a four-factor interaction (PP × PT × ST × R) were significant for quercetin content in broccoli flower buds after storage (Table 2).

3.3. Effects of postharvest treatments on relative UV-A epidermal absorbance

Relative UV-A epidermal absorbance (A_{UV}), calculated from non-destructive chlorophyll fluorescence measurements, and quercetin content in broccoli flower buds, determined by HPLC analysis, were in a curvilinear relationship with a R value of 0.8226 ($P < 0.001$) (Fig. 2). The R values obtained for the relationships between A_{UV} and kaempferol and isorhamnetin contents were 0.8039 ($P < 0.001$) and 0.7943 ($P < 0.001$), respectively (data not shown).

The GLM analysis revealed that pre-storage and storage period, but not temperature, significantly influenced A_{UV} levels in broccoli flower buds after storage (Table 3). Broccoli heads that were pre-stored for seven days had significantly higher A_{UV} levels than those pre-stored for four days. Also, a significant increase in A_{UV} level was observed during storage (Table 3). Radiation treatment was a significant factor influencing A_{UV} level in broccoli flower buds (Table 3). However, the A_{UV} in flower buds of broccoli treated with the combination of visible light and UV-B irradiation was in general at the same level as in broccoli stored in the dark or treated with the highest level of visible light (25 μmol m^{-2} s^{-1}). The two-factor interactions PP × R, PT × R and ST × R were found to be significant (Table 3). Additionally, the three-factor interactions PP × PT × R and PT × ST × R, and the four-factor interaction PP

× PT × ST × R were found to be significant. Thus, the effect of radiation treatment depends on the temperature during that treatment as well as on pre-storage period and pre-storage temperature. Although there was no individual effects of pre-storage and storage temperatures on A_{UV} in broccoli flower buds, the significant two-factor interactions PP × ST and PT × ST indicate that the effect of storage temperature on A_{UV} in broccoli flower buds depends on pre-storage period and pre-storage temperature.

At harvest, A_{UV} varied between 0.27 and 1.23 for all freshly harvested broccoli heads (data not shown). Due to this large variation in A_{UV} levels between biological replicates and to benefit from repeated measurements, data normalization against beginning of storage was applied to facilitate analysis of true changes in A_{UV} of broccoli flower buds during storage.

The combination of visible light and UV-B radiation was the treatment causing most changes in relative UV-A absorbance with significant increases (Figs. 3 and 4). Regardless of pre-storage period and temperature, this radiation treatment significantly increased A_{UV} of broccoli heads already during the first day at 18 °C, and later also at 10 °C. In addition, for broccoli pre-stored for four or seven days at 0 °C and then stored in the dark at 18 °C, the A_{UV} levels were significantly increased as compared to the other light treatments already after one day of storage (Figs. 3C, 4C).

When broccoli heads were pre-stored for four days at 4 °C and 0 °C, the combination of visible light and UV-B radiation during further storage at 10 °C increased A_{UV} by 15% and 11%, respectively (Fig. 3A, B). After pre-storage at 0 °C, three days of storage in the dark at 18 °C gave a 12% increase in A_{UV} (Fig. 3C). However, the level of this increase was not significantly different from the one observed upon treatment with visible light and UV-B radiation. For broccoli pre-stored at 4 °C, the combination of visible light and UV-B radiation increased A_{UV} by 10% after 3 d of storage at 18 °C (Fig. 3D).

For broccoli heads pre-stored for seven days at 0 °C, storage upon visible light with UV-B radiation and storage in the dark increased A_{UV} level to a similar extent, that is, approximately 13% at 10 °C (Fig. 4A) and 9-10% at 18 °C (Fig. 4C). The levels of these increases were, however, not significantly different from the one observed after visible light treatment at the highest level (25 µmol m^{-2} s^{-1}). When broccoli heads were pre-stored for seven days at 4 °C, A_{UV} levels increased significantly after exposure to the combination of visible light and UV-B radiation (Fig. 4B, D). The increases were approximately 9% at 10 °C and 8% at 18 °C.

4. Discussion

The flavonol aglycons that were identified in broccoli flower buds after acidic hydrolysis were in agreement with those reported by Vallejo et al. (2004) in broccoli inflorescences. The contents of quercetin and total flavonols in broccoli flower buds determined at harvest were in the same range as previously reported for broccoli (Aherne and O'Brien, 2002; Manach et al., 2004). In addition, quercetin and kaempferol contents determined at harvest were similar to those found in broccoli inflorescences of the same cultivar grown in the season with the lowest solar radiation (Gliszczyńska-Świgło et al., 2007). However, for the same cultivar there are reported both lower (Winkler et al., 2007) and higher (Bengtsson et al., 2006) contents of quercetin and kaempferol in freshly harvested heads and in flower buds of broccoli stored for 5 days at 2 °C, respectively. The differing results may be explained by differences in growing conditions, sampled plant part, or methodology.

The stable flavonol levels in broccoli flower buds during cold pre-storage (for 4 or 7 d at 0 °C or 4 °C) are in accordance with unchanged total flavonol and quercetin contents in kale stored for up to 6 weeks at 1 °C (Hagen et al., 2009), and for quercetin and kaempferol contents in broccoli florets stored for 2-28 days at 1 °C or 4 °C (Winkler et al., 2007). However, other authors have reported around 60% decrease of total flavonoids (Vallejo et al., 2003) and of

individual flavonols (Fernández-León et al., 2013) in broccoli inflorescences after storage for seven days at 1 °C or for 21 days at 1-2 °C, respectively. In the study conducted by Vallejo et al. (2003) the level of total flavonoids was determined in broccoli inflorescences, whereas in the present study flavonol levels were analysed in broccoli flower buds. Also, their broccoli was harvested in February in Spain and ours in September in Norway. Hence, differences in raw material and pre-storage conditions may explain the divergent results obtained. In the study by Fernandez-Leon et al. (2013), broccoli heads were stored unpackaged, which led to 41% weight loss at the end of the storage and this could also have had a negative impact on flavonol contents. Packaging not only prevents weight loss, but also results in better retention of phytochemicals in broccoli florets during storage (Nath et al., 2011).

The present study demonstrates that pre-storage treatment influences the flavonol content in broccoli during storage. The significant interaction between pre-storage period and pre-storage temperature indicates that prolonged pre-storage (7 d), when performed at lower temperature (0 °C), can contribute to increased flavonol content in broccoli flower buds after storage. With elevated pre-storage temperature (4 °C), shorter pre-storage (4 d) was found favourable. On the other hand, prolonged pre-storage of broccoli heads resulted in higher A_{UV} values after ended storage regardless of both pre-storage and storage temperature. Similarly, Starzyńska et al. (2003) found a significant increase in flavonoid content and superoxide dismutase (SOD) activity, the key enzyme in plant response to stress, in the flower buds of packaged broccoli inflorescences during storage at both 5 °C and 20 °C. The authors concluded that increased SOD activity was a response to stress during storage and that accumulation of flavonoids was related to the capacity of the bud tissue to scavenge free radicals. Plant commodities are exposed to diverse types of stresses during storage and flavonols have been reported to play an important role in plant response to stress (Dixon and Paiva, 1995; Winkel-Shirley, 2002; Pollastri and Tattini, 2011). The accumulation of flavonols in the epidermis of

broccoli flower buds found in the present study during pre-storage and storage may thus be stress-related. Moreover, plant products with higher resistance towards postharvest oxidative stress are characterized with a greater ability to maintain antioxidants in plant tissues (Toivonen, 2004). Therefore, it is reasonable to believe that flower buds of broccoli pre-stored for seven days at 0 °C are more resistant to oxidative stress during storage than the ones pre-stored at 4 °C for the same period.

That the radiation treatments during storage, according to GLM analysis, affected quercetin content in flower buds of broccoli and not the content of kaempferol and isorhamnetin is in agreement with other studies reporting ortho-dihydroxy B-ring substituted flavonoids to be the most light-responsive (Agati et al., 2011; Majer et al., 2014; Müller et al., 2015). It has also been reported that plants under stress conditions, including light excess, produce preferably ortho-dihydroxy B-ring substituted flavonoids, which are effective ROS scavengers (Agati et al., 2012; Majer et al., 2014). Among all radiation treatments applied in the present study, the combination of UV-B radiation and visible light increased quercetin and A_{UV} levels in broccoli flower buds the most. However, the levels of such treated broccoli heads were not significantly different from the levels obtained during storage in the dark or under visible light of the highest levels. It has been previously shown that the flavonoid level in broccoli increased more in the dark than with UV-C radiation treatment of 10 kJ m^{-2} d^{-1} during storage at 20 °C for 5 days (Costa et al., 2006). Additionally, visible light exposure during storage at 7 °C has been shown to increase total phenols content in broccoli (Zhan et al., 2012) or both total phenols content and PAL activity in cauliflower (Zhan et al., 2014). Despite this, the combination treatment of UV-B radiation and visible light, when performed at a certain temperature (10 °C) and after pre-storage for four days at 4 °C, was the only treatment that significantly increased the contents of all individual flavonols as compared with the contents determined after pre-storage. Under these specific conditions the highest increase in A_{UV} was

observed. Moreover, the combination of visible light and UV-B irradiation increased A_{UV} significantly more than other treatments under most conditions (at 10 °C after pre-storage for 4 days at 0 °C and at both temperatures after pre-storage for 7 days at 4 °C). These findings confirm that postharvest UV-B irradiation indeed has a potential to increase flavonols in plant food. Previously, UV-B irradiation of moderate dose (1.08 kJ m^{-2}) have been found to increase both quercetin diglucoside level and the activities of enzymes involved in polyphenolic metabolism, namely phenylalanine ammonia-lyase (PAL) and peroxidase (POD), in white asparagus (Eichholz et al., 2012). Short-term UV-B irradiation during refrigerated storage (10 °C) has also been reported to increase the flavonoid levels in spinach, radish sprouts and parsley and the expression of five genes involved in flavonoid synthesis in parsley (Kanazawa et al., 2012). However, in the present study, the increase of individual flavonol levels in flower buds of broccoli upon UV-B irradiation appeared only under certain conditions. Similarly, Martínez-Hernández et al. (2011) showed that changes in total phenols content in broccoli florets during storage at 5 °C or 10 °C for 19 days in the dark and after UV-C pre-treatment of different doses were influenced by storage temperature. The A_{UV} results showed that the accumulation of epidermal flavonols in broccoli flower buds upon UV-B exposure during storage appears earlier (already during the first day of storage) when applied at higher temperature (18 °C), which could be explained by a higher rate of metabolism or a higher level of stress at increased temperature. This is in agreement with Martínez-Hernández et al. (2011), who reported that the large increase in total phenols content in broccoli florets after UV-C pre-treatment appeared earlier during storage at 10 °C than at 5 °C.

The levels of A_{UV} measured by the ChlF method and the HPLC-determined flavonol contents were positively, though curvilinearly related. A curvilinear relationship between A_{UV} and flavonol or UV absorbance levels of phenolic extracts has been observed also for barley and grape leaves (Kolb and Pfündel, 2005), grape berry skin (Cerovic et al., 2008) and apple

peel (Hagen et al., 2006). The curvilinearity, which is a result of underestimation of A_{UV} in relation to flavonol concentration in the higher range, can be explained by technical artefacts, by the presence of UV-A transparent areas in epidermis or, especially for curves with low initial slope, by non-correlated presence of flavonols in the mesophyll tissue (Kolb and Pfündel, 2005). Similar low initial slopes of the relationships between flavonol levels and A_{UV} values observed in the present study indicate that flavonols in broccoli flower buds are localized not only in the sepal epidermis, but also in the mesophyll tissue. Similarly, Agati et al. (2009) reported the increase of ortho-dihydroxy B-ring-substituted quercetin and luteolin levels in both epidermal and mesophyll cells of *Ligustrum vulgare* leaves upon high irradiance sunlight treatment with or without UV irradiation. In addition, a certain difference between ChlF method and HPLC results in our study was expected, since the first method estimates all flavonols present only in the epidermis of mostly flower bud sepals, and the second method gives information on soluble flavonols from whole broccoli flower buds. Regarding this, the R value for the relationship (R = 0.82 for quercetin) between the methods was indeed satisfying. The application of the ChlF method in the present study allowed repeated measurements on the same broccoli heads over time and thereby obtaining information on the kinetics of flavonols accumulation during storage. However, to obtain accurate information on flavonol level in broccoli flower buds, the ChlF method should preferably be applied in the combination with wet-chemical analysis.

In conclusion, the present study has demonstrated that flavonol contents in broccoli flower buds were in general stable during both pre-storage at low temperatures (0 °C and 4 °C) and during storage at higher temperature (10 °C and 18 °C), simulating refrigerated transport with wholesale distribution and retail, respectively. Pre-storage period and temperature were found to influence the flavonol content during storage, which emphasise the importance of pre-storage treatment for both postharvest research and commercial industry. Therefore, to preserve

high contents of flavonols in broccoli, we recommend to apply a temperature of 0 °C instead of commonly 4 °C during wholesale longer than 4 days. The UV-B irradiation combined with visible light had a potential to increase flavonol content in flower buds of broccoli during storage. However, the outcome of postharvest UV-B irradiation on flavonols in broccoli was influenced by storage temperature, pre-storage temperature and pre-storage period. The ChlF method proved to be a valuable tool also in broccoli for following the kinetics in individual plants of epidermal flavonol content. The application of the ChlF method revealed that the accumulation of epidermal flavonols upon UV-B exposure was initiated earlier in broccoli stored at higher temperature (18 °C), already during the first day of storage.

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Figure captions

Fig. 1. Quercetin (A), kaempferol (B) and isorhamnetin (C) contents (mg 100 g^{-1} fresh matter) in flower buds of broccoli heads after pre-storage in the dark for four days at 0 °C or 4 °C and after different radiation treatments during three days of storage at 10 °C or 18 °C. Bars represent mean values ± SEM (n = 5). Bars marked with different letters in each graph indicate significantly different values at $P < 0.05$.

Fig. 2. Scatter plot of quercetin contents in flower buds of different broccoli heads (n = 220) determined by HPLC, vs. relative UV-A absorbance, calculated from chlorophyll fluorescence measurements and expressed in log[F(R)/F(UV-A)].

Fig. 3. Changes in relative UV-A absorbance (normalised data) in flower buds of broccoli pre-stored in the dark for four days at 0 °C (A, C) or 4 °C (B, D) during three days of storage with different radiation treatments at 10 °C (A, B) or 18 °C (C, D). All data points are mean values ± SEM (n = 8). Grey, vertical lines illustrate grouping information obtained after ANOVA with Tukey's multiple comparisons test (*, **, *** or no star mean significant at $P < 0.05$, $P < 0.01$, $P < 0.001$ or non-significant, respectively).

Fig. 4. Changes in relative UV-A absorbance (normalised data) in flower buds of broccoli pre-stored for seven days at 0 °C (A, C) or 4 °C (B, D) during three days of storage with different radiation treatments at 10 °C (A, B) or 18 °C (C, D). All data points are mean values ± SEM (n = 8). Grey, vertical lines illustrate grouping information obtained after ANOVA with Tukey's multiple comparisons test (*, **, *** or no star mean significant at $P < 0.05$, $P < 0.01$, $P < 0.001$ or non-significant, respectively).

Tables

Table 1
Flavonol contents (mg 100 g^{-1} fresh matter) in flower buds of broccoli heads pre-stored for seven days in the dark and then stored for three days with different radiation treatments at 10 °C or 18 °C [a].

Pre-storage temperature (°C)	Storage temperature (°C)	Harvest	Pre-storage	Radiation treatments with light (13-25 µmol m^{-2} s^{-1}) and UV-B (20 kJ m^{-2} d^{-1})				
				13	19	25	19 + UV-B	Dark
		Quercetin[b]						
0	10	1.79 ab	2.24 ab	2.91 ab	3.02 ab	3.93 a	3.11 ab	2.78 ab
	18			1.80 ab	1.63 ab	3.16 ab	2.44 ab	3.86 ab
4	10		2.52 ab	1.26 b	2.00 ab	2.35 ab	2.82 ab	3.16 ab
	18			2.73 ab	3.12 ab	1.71 ab	3.23 ab	2.85 ab
		Kaempferol						
0	10	2.96 a	3.89 a	5.24 a	5.34 a	6.66 a	4.78 a	4.62 a
	18			2.87 a	2.67 a	5.34 a	3.75 a	6.30 a
4	10		4.70 a	1.84 a	3.21 a	4.06 a	3.79 a	5.44 a
	18			4.15 a	4.45 a	2.57 a	4.21 a	4.27 a
		Isorhamnetin						
0	10	0.40 ab	0.51 ab	0.75 ab	0.83 ab	1.07 a	0.75 ab	0.69 ab
	18			0.45 ab	0.45 ab	0.78 ab	0.50 ab	0.92 ab
4	10		0.68 ab	0.27 b	0.47 ab	0.57 ab	0.54 ab	0.73 ab
	18			0.60 ab	0.69 ab	0.40 ab	0.62 ab	0.64 ab

[a] Data presented as mean values (n=5).
[b] Values for each compound followed by different letters are significantly different at $\alpha = 0.05$

Table 2
Influence of pre-storage period and temperature, storage temperature, and radiation treatments on flavonol contents in broccoli flower buds after pre-storage in the dark and then storage for three days with radiation treatments (GLM analysis).

Source of variation	Quercetin	Kaempferol	Isorhamnetin
	F value and significance level [a]		
Pre-storage period (PP)	0.92 NS	0.55 NS	2.99 NS
Pre-storage temperature (PT)	0.01 NS	0.50 NS	0.68 NS
Storage temperature (ST)	0.68 NS	1.74 NS	1.31 NS
Radiation treatments (R)	3.38 *	0.88 NS	1.15 NS
PP × PT[b]	5.15 *	6.56 *	9.97 **
PT × ST	3.22 NS	3.23 NS	4.59 *
PP × PT × R	2.90 *	2.14 NS	2.22 NS
PT × ST × R	3.23 *	3.52 **	2.52 *
PP × PT × ST × R	2.61 *	1.48 NS	1.78 NS
	Content (mg 100 g^{-1} fresh matter) [c]		
Pre-storage period			
4 days	2.54 a	4.04 a	0.56 a
7 days	2.68 a	4.26 a	0.63 a
Pre-storage temperature			
0 °C	2.62 a	4.26 a	0.61 a
4 °C	2.60 a	4.05 a	0.58 a
Storage temperature			
10 °C	2.67 a	4.35 a	0.62 a
18 °C	2.55 a	3.95 a	0.57 a
Radiation treatments			
13 µmol m^{-2} s^{-1}	2.21 b	3.71 a	0.51 a
19 µmol m^{-2} s^{-1}	2.44 ab	3.96 a	0.59 a
25 µmol m^{-2} s^{-1}	2.61 ab	4.16 a	0.62 a
UV-B + 19 µmol m^{-2} s^{-1}	3.03 a	4.42 a	0.63 a
Dark	2.77 ab	4.47 a	0.64 a

[a] NS, *, **, *** Non-significant or significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
[b] Only significant interactions are shown.
[c] Data presented as mean values (n = 40 for radiation treatments, n = 100 for pre-storage period, pre-storage temperature and storage temperature). Values that do not share a common letter are significantly different at $\alpha = 0.05$.

Table 3
Influence of pre-storage period and temperature, storage period and temperature, and radiation treatments on relative UV-A epidermal absorbance (A_{UV}) in broccoli flower buds after pre-storage in the dark and then storage for three days with radiation treatments (GLM analysis).

Source of variation	A_{UV}
	F values and significance level [a]
Pre-storage period (PP)	27.16 ***
Pre-storage temperature (PT)	2.66 NS
Storage period (SP)	3.30 *
Storage temperature (ST)	0.64 NS
Radiation treatments (R)	19.41 ***
PP × ST[b]	5.07 *
PP × R	5.71 ***
PT × ST	16.82 ***
PT × R	14.60 ***
ST × R	3.28 *
PP × PT × R	6.41 ***
PT × ST × R	8.50 ***
PP × PT × ST × R	9.31 ***
	A_{UV} level [c]
Pre-storage period	
4 days	0.817 b
7 days	0.878 a
Pre-storage temperature	
0 °C	0.857 a
4 °C	0.838 a
Storage temperature	
10 °C	0.843 a
18 °C	0.852 a
Storage period	
0 days	0.830 b
1 day	0.846 ab
3 days	0.867 a
Radiation treatments	
13 µmol m^{-2} s^{-1}	0.765 c
19 µmol m^{-2} s^{-1}	0.815 bc
25 µmol m^{-2} s^{-1}	0.858 ab
UV-B + 19 µmol m^{-2} s^{-1}	0.903 a
Dark	0.897 a

[a] NS, *, **, *** Non-significant or significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
[b] Only significant interactions are shown.
[c] Data presented as mean values (n = 192 for Light and UV-B treatments, n = 320 for Storage period and n = 480 for Pre-storage period, Pre-storage temperature and Storage temperature). Values that do not share a common letter are significantly different at $\alpha = 0.05$.

Figure 1

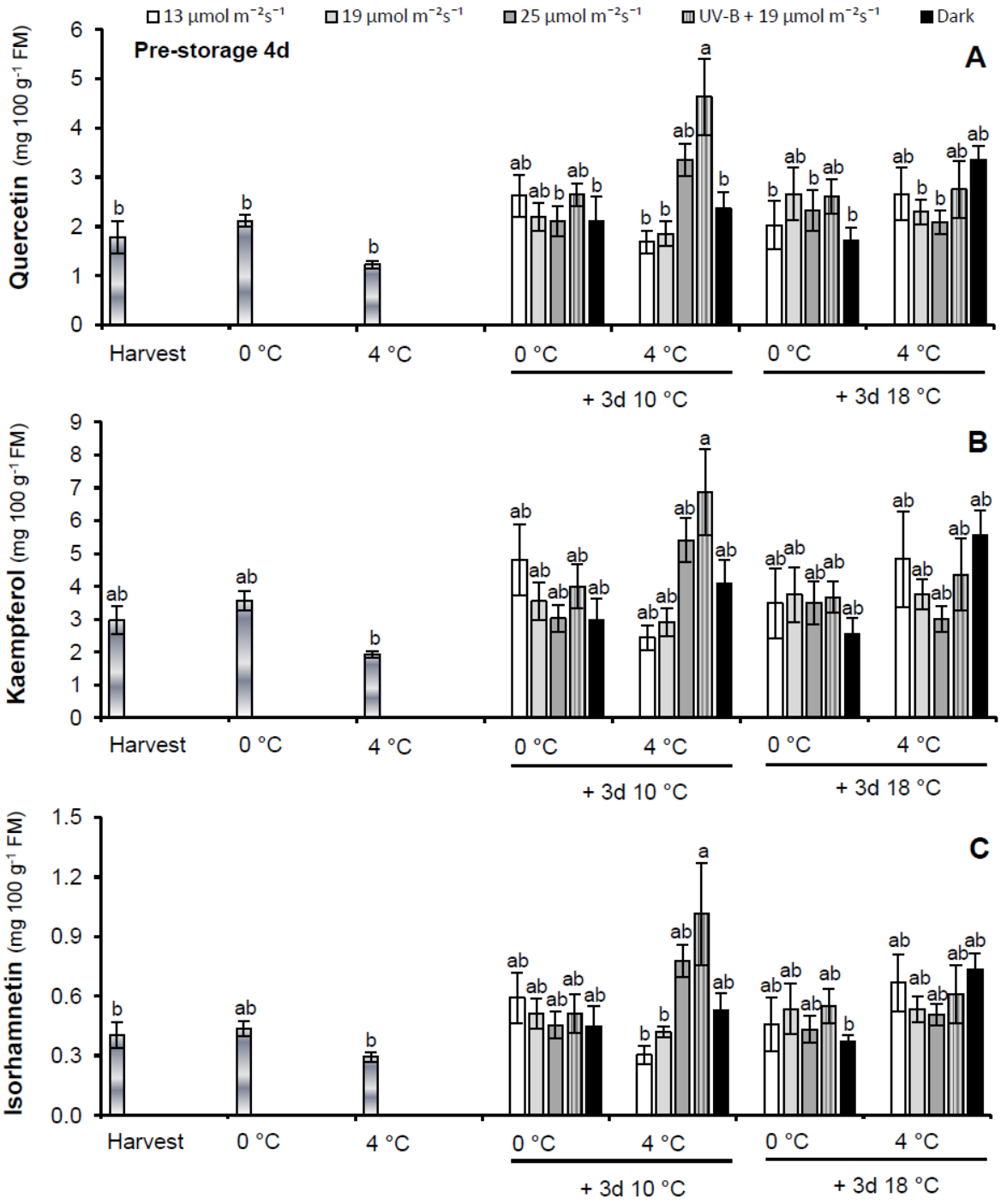

13 µmol m⁻²s⁻¹
19 µmol m⁻²s⁻¹
25 µmol m⁻²s⁻¹
UV-B + 19 µmol m⁻²s⁻¹
Dark
Pre-storage 4d
A
Quercetin (mg 100 g⁻¹ FM)
B
Kaempferol (mg 100 g⁻¹ FM)
C
Isorhamnetin (mg 100 g⁻¹ FM)
Harvest
0 °C
4 °C
0 °C
4 °C
0 °C
4 °C
+ 3d 10 °C
+ 3d 18 °C

Figure 2

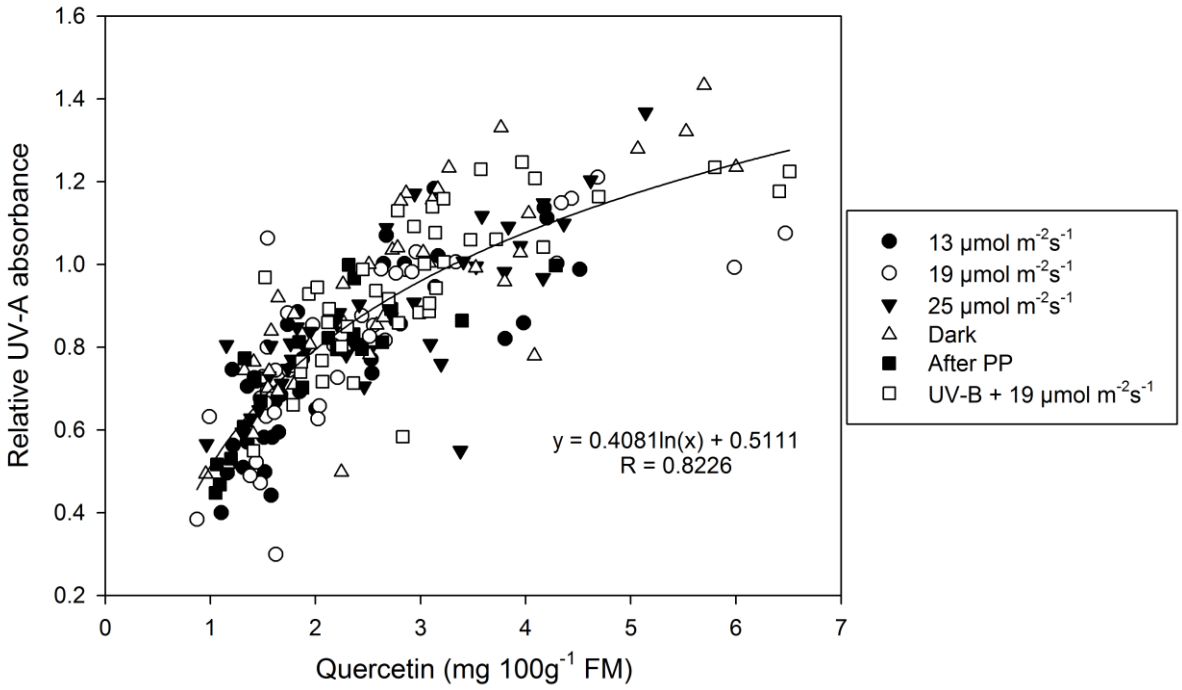

Relative UV-A absorbance
Quercetin (mg 100g-1 FM)
y = 0.4081ln(x) + 0.5111
R = 0.8226
13 µmol m-2s-1
19 µmol m-2s-1
25 µmol m-2s-1
Dark
After PP
UV-B + 19 µmol m-2s-1

Figure 3

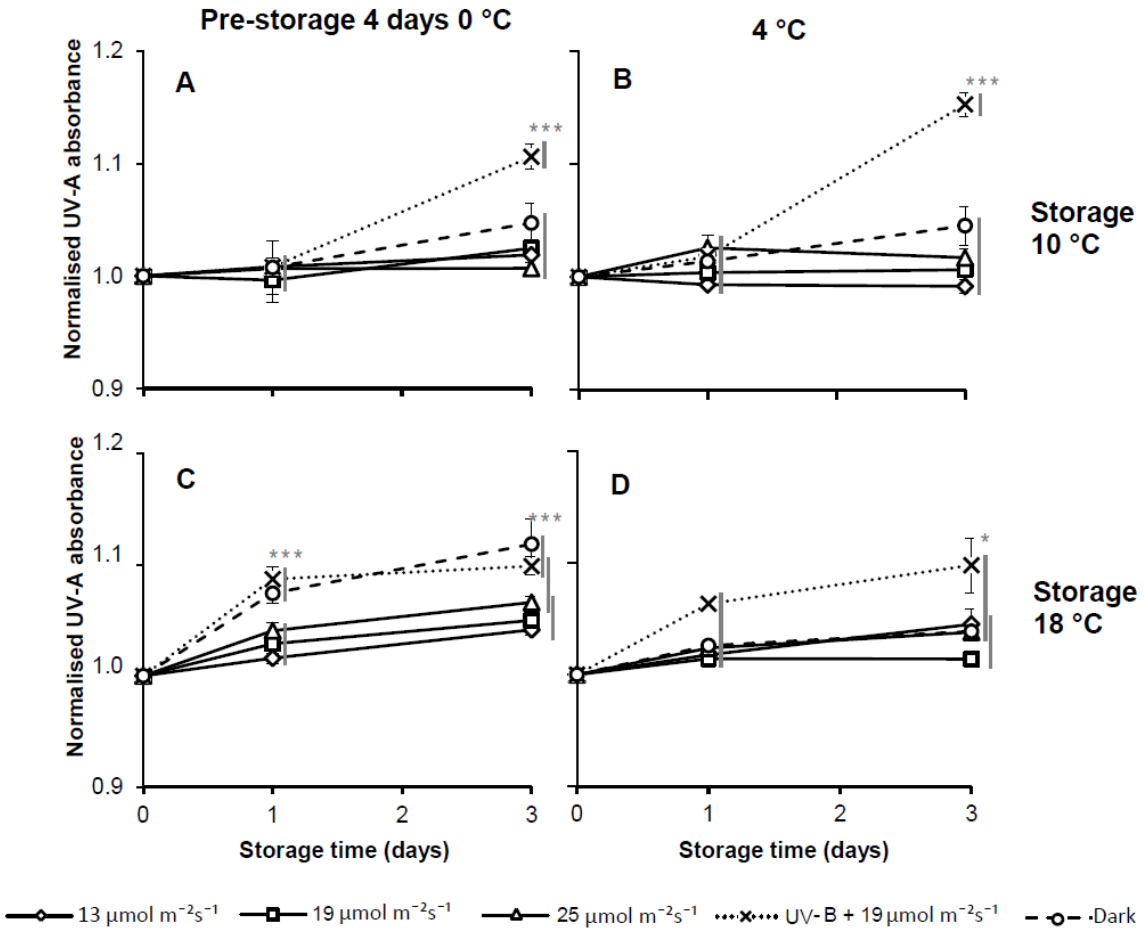
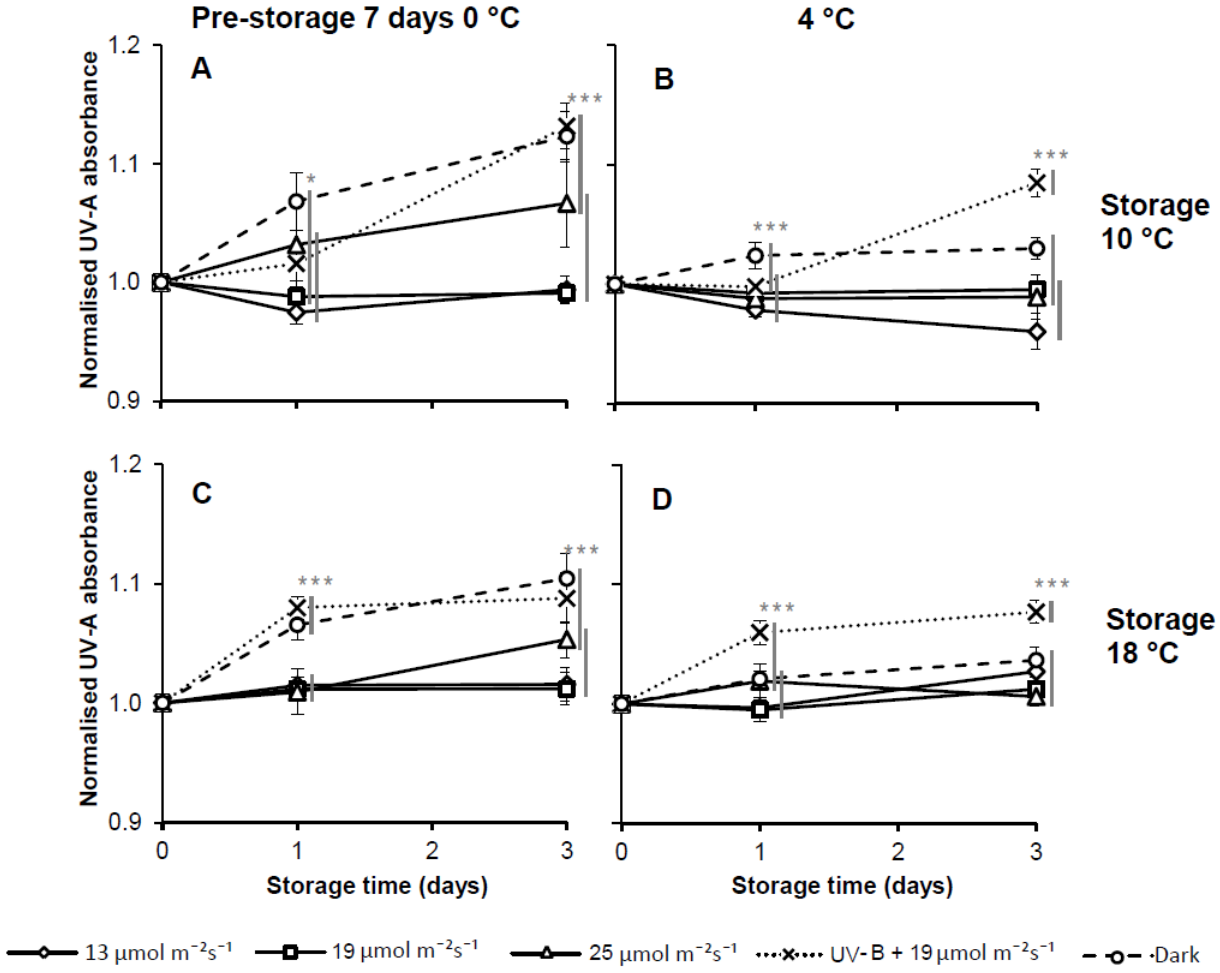

Pre-storage 4 days 0 °C
4 °C
A
B
C
D
Normalised UV-A absorbance
1.2
1.1
1.0
0.9
***
*
Storage 10 °C
Storage 18 °C
0
1
2
3
Storage time (days)
13 µmol m⁻²s⁻¹
19 µmol m⁻²s⁻¹
25 µmol m⁻²s⁻¹
UV-B + 19 µmol m⁻²s⁻¹
Dark

Figure 4


Pre-storage 7 days 0 °C
4 °C
A
B
C
D
Normalised UV-A absorbance
1.2
1.1
1.0
0.9
0
1
2
3
Storage time (days)
Storage 10 °C
Storage 18 °C
*
***
13 µmol m⁻²s⁻¹
19 µmol m⁻²s⁻¹
25 µmol m⁻²s⁻¹
UV-B + 19 µmol m⁻²s⁻¹
Dark

Paper III

Glucosinolates in broccoli (*Brassica oleracea* L. var. *italica*) as affected by postharvest temperature and radiation treatments

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Abstract

The purpose of the present study was to examine how glucosinolate (GLS) levels in broccoli flower buds are affected by storage time, temperature and radiation treatments in an experiment with multi-level design simulating mapped commercial storage conditions from harvest until consumer purchase. The GLS content in broccoli flower buds was examined during pre-storage and storage of broccoli heads, representing refrigerated transport with wholesale distribution and retail, respectively. The GLS analysis was conducted also for floret stalks of chosen broccoli. Broccoli heads were pre-stored for four or seven days at 0 or 4 °C in the dark and then stored for three days at 10 or 18 °C. During storage, the broccoli heads were exposed for 12 h per day to three different levels of visible light (13, 19 or 25 μmol m^{-2} s^{-1}) or a combination of visible light (19 μmol m^{-2} s^{-1}) and UV-B irradiation (20 kJ m^{-2} d^{-1}), or they were stored in the dark. The GLS contents were not changed in broccoli flower buds during pre-storage at low temperatures (0 °C or 4 °C) for four or seven days. However, general linear model analysis revealed that pre-storage period significantly influenced total GLS, total indolic and total aliphatic GLS, and almost all individual GLS levels, except for 4-methoxyglucobrassicin (4-Me-GB) in broccoli flower buds after storage at higher temperatures (10 °C or 18 °C). Higher levels of these compounds were found in broccoli pre-stored for seven days than in those pre-stored for four days. Storage temperature affected GLS levels in broccoli flower buds significantly. 4-Hydroxyglucobrassicin (4-OH-GB) content was increased after storage at 18 °C, while the levels of all other individual GLS, total GLS and total aliphatic and indolic GLS were higher in flower buds of broccoli after storage at 10 °C than at 18 °C. The effect of storage temperature on aliphatic GLS in broccoli flower buds depended on pre-storage temperature and it was negligible for broccoli heads pre-stored at 4 °C but significant for those pre-stored at 0 °C. Radiation treatments applied during storage did not influence total and individual GLS levels in broccoli flower buds. However, the accumulation of aliphatic GLS in broccoli pre-

stored for seven days at 0 °C and stored for three days at 10 °C under visible light of 25 μmol $m^{-2}s^{-1}$ appeared both in flower buds and floret stalks. Significant increases in indolic 4-OH-GB and 4-Me-GB contents were observed only in flower buds or floret stalks of some broccoli heads after storage at 18 °C upon exposure to visible light of 25 μmol $m^{-2}s^{-1}$.

Keywords: Glucosinolates, postharvest handling, storage period, light treatment, UV-B irradiation

1. Introduction

Broccoli (*Brassica oleracea* L. var. *italica*) is a well-known brassicaceous crop, which is cultivated worldwide with increasing production rate (FAOSTAT, 2013). Epidemiological studies gave convincing evidence that consumption of *Brassica* vegetables, including broccoli, is associated with reduced risk of several types of cancer and cardiovascular disease mortality (Zhang et al., 2011; Bosetti et al., 2012; Liu et al., 2013; Liu and Lv, 2013). These health-promoting properties, anti-carcinogenic in particular, have been mostly attributed to high contents of glucosinolates (GLS) present in *Brassica* vegetables (Latté et al., 2011; Dinkova-Kostova and Kostov, 2012; Fahey et al., 2012). Apart from GLS broccoli contains other health related secondary metabolites such as polyphenols and carotenoids, and is also a good source of vitamins, minerals and dietary fibre (Moreno et al., 2006; Podsędek, 2007; Björkman et al., 2011). Broccoli inflorescences are harvested immature that leads to rapid senescence during storage with chlorophyll degradation within sepals as a visual sign (Page et al., 2001). This vegetable is therefore difficult to handle postharvest due to a short maximal storage length, which is 2-3 days at 20 °C and 3-4 weeks at 0 °C (Dixon and Dickson, 2007).

Glucosinolates are sulphur- and nitrogen-containing plant secondary metabolites reported almost exclusively for the plants belonging to the Capparales/Brassicales order, which includes the Brassicaceae family (Halkier and Gershenzon, 2006; Clarke, 2010). They are recognized

as (Z)-*N*-hydroximinosulfate esters with sulphur-linked β-D-glucopyranose and a variable side chain (R-group) (Halkier and Gershenzon, 2006). The 200 known GLS are divided into three main groups, aliphatic, aromatic and indolic GLS according to their amino acid precursor and they can be further classified by the type of modification of the R group including hydroxylation, methylation, desaturation, glycosylation and acylation (Halkier and Gershenzon, 2006; Clarke, 2010). In plant cells GLS are localized in vacuoles and by tissue damage they can be mixed with myrosinase, a β-thioglucosidase present in myrosin cells that leads to GLS breakdown (Grubb and Abel, 2006). There are several end-products of GLS hydrolysis, including the most common isothiocyanates, thiocyanates, nitriles, epithionitriles and oxazolidines, the final composition of which is determined by various chemical conditions (Bones and Rossiter, 2006). The glucosinolate-myrosinase system plays roles in plant's defence, as some GLS breakdown products have been found to be toxic or deterrent towards herbivores and pathogens, but also mediates interactions between plant and adapted herbivores (Halkier and Gershenzon, 2006; Bednarek et al., 2009; Björkman et al., 2011). Isothiocyanates, with the extensively studied sulforaphane, derived from glucoraphanin (GRA), have been recognized as agents affecting carcinogenesis by blocking tumour initiation and promotion, and by suppressing progression, angiogenesis and metastasis (Juge et al., 2007; Traka and Mithen, 2009; Dinkova-Kostova and Kostov, 2012). GLS have also been found to contribute to taste and flavour of *Brassica* vegetables (Schonhof et al., 2004).

GLS levels in *Brassica* crops at harvest are determined mostly by genetic factors, but also by environmental conditions during growing season and further by postharvest factors including storage conditions and processing (Jones et al., 2006; Verkerk et al., 2009). Storage temperature was shown to have substantial impact on GLS content in broccoli with stable levels of total GLS or GRA during storage at 4 °C, but a decline observed during storage at 20 °C (Rodrigues and Rosa, 1999; Rangkadilok et al., 2002b). However, other authors reported either

no change in total GLS or GRA levels (Song and Thornalley, 2007; Winkler et al., 2007) or reduction in total GLS (Vallejo et al., 2003) in broccoli during storage at both low and higher temperature in the range of 1 - 22 °C. Moreover, a 42% increase in total GLS was reported for broccoli florets after storage at 10 °C (Hansen et al., 1995). Therefore, the effect of storage temperature on GLS content in broccoli is not yet fully understood. Although the biosynthesis of GLS has been found to be controlled by visible light in *Arabidopsis* (Huseby et al., 2013) and pre-harvest UV-B irradiation to induce accumulation of some GLS in *Brassica* plants (Reifenrath and Müller, 2007; Mewis et al., 2012), the evidence on the effect of postharvest light and UV-B irradiation on GLS content is scarce. To our knowledge one study has been conducted on the effect of fluorescent light on total GLS content in broccoli florets during storage at 25 °C (Jin et al., 2015) and one that evaluated how postharvest UV-B irradiation affected glucotropaeolin content in nasturtium (Schreiner et al., 2009).

The aim of the present study was to evaluate the effect of storage time, temperature and radiation treatments on GLS levels in broccoli flower buds in an experiment with multi-level design simulating mapped commercial storage conditions from harvest until consumer purchase. Two lengths of pre-storage period at two temperatures were applied to simulate transport with wholesale distribution, followed by storage at two temperatures and with four different light treatments, including three levels of visible light and darkness as a control, to simulate retail. A combination of visible light with UV-B irradiation was applied, as a fifth treatment during storage, to test whether it has a potential to alter the GLS contents in broccoli flower buds. Additionally, the broccoli heads that showed significant increase in GLS levels in flower buds after storage were chosen for the GLS analysis in floret stalks.

2. Materials and methods

2.1. Chemicals

Glucotropaeolin of HPLC grade was purchased from AppliChem GmbH (Darmstadt, Germany). Type H-1 sulfatase from *Helix pomatia*, (-)-sinigrin hydrate and sodium hydroxide of analytical grade were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Sephadex DEAE ™ A-25 was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Acetonitrile and methanol of HPLC grade and glacial acetic acid of analytical grade were purchased from Merck KGAa (Darmstadt, Germany). Milli-Q quality water (Millipore, Bedford, MA, USA) was used for chemical analysis.

2.2. Plant material and storage experiment

Broccoli (*Brassica oleracea* L. var. *italica*, cv. Marathon) inflorescences were grown and the storage experiment was conducted as previously described by Rybarczyk-Plonska et al. (2014).

In brief, a total of 360 broccoli heads were pre-cooled in a storage room at 0 °C immediately after harvest. Eight broccoli heads comprised the control sample at harvest and were prepared for chemical analysis. The remaining broccoli heads were wrapped with 0.01 mm polyvinylchloride food film and put into polypropylene plug trays. For each postharvest treatment there were eight broccoli heads representing biological replicates. Half the number of the broccoli heads were pre-stored in the dark at 0 °C and the other half at 4 °C for the same periods. Samples were collected and prepared for chemical analysis after 4 and 7 days of pre-storage, and after storage for three more days. During the 3-day storage period, two different temperatures, 10 °C or 18 °C, and five different radiation treatments were applied. Broccoli heads were weighed before and after the experiment.

The radiation treatments were applied 12 h per day as follows: visible light of 0, 13, 19 and 25 μmol m^{-2} s^{-1} and a combination of 19 μmol m^{-2} s^{-1} visible light and UV-B irradiation in the range of 19 - 22 kJ m^{-2} d^{-1} (0.22 - 0.25 W m^{-2}). The irradiation levels were obtained by regulating the distance between the lamps and broccoli heads. Additionally, 0.18 mm cellulose diacetate

foil (Rachow, Hamburg, Germany) was placed above broccoli heads treated with both visible light and UV-B radiation to exclude possible UV-C stray light.

The temperature and visible light levels used during the 3-day storage were chosen according to the results of a survey conducted in seven Norwegian retail stores. The surface temperature of broccoli heads during display, measured with a Raynger® MX4^{TM} non-contact infrared thermometer (Raytek® GmbH, Berlin, Germany), was found to be in the range from 10 °C to 18 °C. The visible light level varied between 1000 lux and 2000 lux in the different stores, as determined just above the area of broccoli display using a Lu-Ex 02 digital luxmeter (Ecom Rolf Nied GmbH, Assamstadt, Germany). The level of UV-B irradiation chosen in the study was according to Hagen et al. (2007), who found the treatment effective in increasing the content of flavonols in the peel of shade-grown apples.

2.3. Sample preparation

Florets from a 10 cm diameter circular top area of individual broccoli heads were cut off from the main stem and immediately frozen in liquid nitrogen. Flower buds were scraped off the stalks and separated from the small branches using a sieve with 4 mm diameter holes under freezing conditions. The weight of flower buds per floret was 24.8 ± 3.7 g (mean ± SD, n = 214). The mean bud mass fraction of the florets was 38.7% (range 25.9 – 44.2%). The buds and stalks were placed in separate 50 mL polypropylene tubes (VWR International, Arlington Heights, IL, US) and stored at -80 °C until analyses. Five out of eight replicates (n = 5) for each treatment and for selected treatments were randomly chosen for glucosinolate analysis in flower buds and in stalks, respectively.

2.4 Extraction and desulfation of glucosinolates

The extraction procedure, which is described briefly below, was according to Steindal et al. (2013) with some modifications. Approximately 10 g of frozen broccoli flower buds or stalks,

was freeze dried using GAMMA 1-16 LSC laboratory freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until constant weight of the material was obtained. Dry matter was determined after freeze drying and the material was ground to a fine powder in a mortar with a pestle. In order to perform glucosinolate extraction, 200 mg of freeze dried flower buds or stalks was placed into a 15 mL polypropylene tube (VWR International, Arlington Heights, IL, US) and thermostated at 73 °C for 3 min. Then 3 mL of hot (73 °C) 70% aqueous methanol was added and the sample was hand mixed at 73 °C for 3 min. After extraction 0.1 mL of glucotropaeolin (1 mg / mL of methanol) was added as internal standard and the samples were centrifuged at 4400 rpm for 15 min at 4 °C (Heraeus® Multifuge® 4KR, Thermo Electron Corp., Waltham, MA, US). The pellets were re-extracted under the same conditions as above and the supernatants obtained from two-step extraction procedure were combined. Such prepared extracts were stored at -80 °C until desulfation procedure. The procedure was according to International Standard Method: ISO 9167-1:1992(E) (ISO, 1992) with some modifications as described below. In brief, 30 mg of glass wool was well packed in a 1 mL HSW SOFT-JECT® syringe (Henke-Sass Wolf GmbH, Tuttlingen, Germany) and rinsed with 0.5 mL of water. Then 0.5 mL of DEAE™ Sephadex A-25 suspension in 0.02M sodium acetate buffer (pH 5.0) was added and the column was rinsed twice with 1 mL of water. One mL of extract was transferred to such prepared column and allowed to drain before 1 mL of water was added. The pH of the column was adjusted to 5.0 with addition of 1 mL of sodium acetate buffer and left to drain completely. In order to desulfate glucosinolates, 75 µL ethanolic solution of purified sulfatase was added onto a column and left at room temperature overnight. The desulfated GLS were eluted with 1.25 mL of water, the eluate was filtered through 0.45 µm Millex-HA filters (Millipore, Molsheim, France), and high performance liquid chromatography (HPLC) analysis was performed shortly after.

2.5 Quantitative HPLC analysis of glucosinolates

The HPLC analysis of desulfated GLS was conducted using an Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany), equipped with a quaternary pump, an in-line degasser, a thermo-stated autosampler (4 °C), a column heater and a photodiode array detector. The desulfated GLS were separated on a Spherisorb ODS2 column (250 mm × 4.6 mm i.d., 5 μm particle size) equipped with a Spherisorb ODS2 guard cartridge (10 mm × 4.6 mm i.d., 5 μm particle size), both obtained from Waters Corporation (Milford, MA, USA), and operated at 30 °C. Eluent A consisted of ultra-pure water, whereas eluent B consisted of 20% v/v acetonitrile in water. Flow rate was set to 1.5 mL min^{-1}, the injection volume was 30 μL, run time was 39 min and detection was at 229 nm. The gradient elution profile used was as follows: 1% B for 1 min, 1-99% B in 20 min, 99% B for 3 min followed by 99-1% B in 5 min and finally 10 min of 1% B as column equilibration step. The desulfated GLS were quantified on a basis of relative response factors towards internal standard (ISO, 1992) and results were expressed as μmol per 1 g dry matter of broccoli flower buds or stalks. Identification of desulfated GLS was based on liquid chromatography – mass spectrometry (LC-MS) analysis.

2.6 LC-MS analysis of glucosinolates

The identification of desulfated GLS was carried out using an Agilent 1100 Series HPLC system as described above, with an electrospray ionisation (ESI) interface and MSD XCT ion trap mass spectrometer (Agilent Technologies, Waldbronn, Germany). The HPLC conditions used were as described above except for the flow and the run time, which were changed to 1 mL min^{-1} and 45 min, respectively. The gradient elution profile was as follows: 1% B for 1 min, 1-99% B in 30 min, 99% B for 3 min followed by 99-1% B in 5 min and finally 6 min of 1% B as column equilibration step. After UV-vis detection the effluent was introduced directly, without splitting, to the ESI interface where ionization in positive mode was performed. The nebulizer pressure was 50 psi, dry gas flow 12 L/min, dry temperature 350 °C, and capillary voltage 3.5 kV. Ultra scan mode was used (50-2200 m/z and 26,000 m/z per sec),

and ions with *m/z* 40 to 600 were measured in the ion trap detector. Fragmentation (MS^2) was performed in automatic mode of the two most abundant ions in MS^1 using MS/MS fragmentation amplitude voltage 1.0 V and helium as the collision gas.

2.5. Statistical analyses

The experimental design chosen for the study was four factorial fixed effect model. Factors used were as follows: pre-storage period (two levels), pre-storage temperature (two levels), storage temperature (two levels) and light treatment (five levels). One way analysis of variance (ANOVA), general linear model (GLM) and Tukey's multiple comparison test with significance level $\alpha = 0.05$ were performed using Minitab® 16 Statistical Software (Minitab Inc., State College, PA, USA). Standard error of the mean (SEM) was calculated for each mean value by dividing obtained standard deviation by square root of the number of biological replicates analysed.

3. Results and discussion

3.1. Identification of desulfated glucosinolates using LC-MS

The desulfated GLS were characterized according to their chromatographic behaviour and mass spectra obtained under electron spray ionisation (ESI) conditions at positive ion scan mode. The identity of all compounds was confirmed by their quasi-molecular ions $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$, and by the presence of a 162 Da neutral fragment loss proving glucosyl structure. For all GLS $[M+Na]^+$ quasi-molecular ion was found, which was according to Sun et al. (2011), together with two other ions: $[M+Na]^+$ and $[M+K]^+$ (Table 1). The loss of a neutral fragment of 162 Da of the $[M+Na]^+$ ions was observed for all compounds in MS^2 and in MS^1 except for desulfoglucoerucin (Table 1).

The chromatographic profile of GLS extracted from flower buds of freshly harvested broccoli is shown in Figure 1. Eight glucosinolates were identified in broccoli flower buds, four

aliphatic: glucoiberin (GIB), glucoraphanin (GRA), glucoalyssin (GAL) and glucoerucin (GER), and four indolics: 4-hydroxyglucobrassicin (4-OH-GB), glucobrassicin (GB), 4-metoxyglucobrassicin (4-Me-GB) and neoglucobrassicin (NGB). GAL and GER were however present in broccoli buds as traces. Except for GAL and GER, these GLS have been previously identified in inflorescences of the same broccoli cultivar (Aires et al., 2011). However, low levels of progoitrin, gluconapin, glucobrassicanapin, gluconasturtiin and glucobarbarin have been detected by others (Hansen et al., 1995; Rosa and Rodrigues, 2001; Vallejo et al., 2003). The discrepancies in GLS composition in freshly harvested broccoli of the same cultivar observed in different studies can be explained by differences in growing conditions. Climatic and agronomic conditions during plant's growth have been reported to influence both GLS content and profile in *Brassica* vegetables including broccoli (Schreiner, 2005; Aires et al., 2011).

3.2. Glucosinolate profile in flower buds and floret stalks of broccoli at harvest

Total GLS content in broccoli flower buds at harvest was 21.14 ± 3.48 μmol g^{-1} dry matter. Aliphatic GRA and indolic GB and NGB were predominant and accounted for 35%, 38% and 18% of the total GLS content, respectively (Table 2). GIB, 4-OH-GB and 4-Me-GB were found in broccoli flower buds in amounts less than 1 μmol g^{-1} dry matter. Similar GLS profile and total GLS level have been previously reported for inflorescences of the same broccoli cultivar (Rosa and Rodrigues, 2001; Vallejo et al., 2003). Stalks of broccoli florets, which contained 13.59 ± 0.42 μmol g^{-1} dry matter of total GLS at harvest, had a different GLS profile from buds (Table 2). This was in accordance with previous studies showing variation in GLS profile among different organs of *Brassica* plants (Brown et al., 2003; Velasco et al., 2007; Sotelo et al., 2014). GRA and GB were predominant in stalks of broccoli florets in the present study, representing 56% and 27% of the total GLS content, respectively. NGB, 4-OH-GB, 4-Me-GB and GIB were found at low levels (< 1 μmol g^{-1} dry matter). There was no significant difference

in total GLS content in stalks and buds, although total GLS content in stalks was 36% lower than in buds. This was probably due to a large biological variation in contents of individual GLS observed in broccoli flower buds from individual heads. However, the levels of total indolic GLS, GB and NGB were significantly higher in broccoli flower buds than in stalks. The content of aliphatic GLS was at the same level in both stalks and buds of broccoli florets.

3.3. Effects of storage time, temperature and radiation treatments on glucosinolate contents in broccoli flower buds

Pre-storage time and temperature. During pre-storage at 0 °C or 4 °C for four or seven days there were no significant changes in the contents of total GLS, total aliphatic, total indolic, or individual GLS in broccoli flower buds (Tables 3, 4 and 5). This is in accordance with several other studies, where total GLS level did not change in broccoli after refrigerated storage for five days at 4 °C (Rodrigues and Rosa, 1999), for seven days at 4-8 °C (Song and Thornalley, 2007), or for 21 days at 1-2 °C (Fernández-León et al., 2013). GRA was found stable in packaged broccoli inflorescences during storage for seven days at 4 °C (Rangkadilok et al., 2002b) and for 28 days at 1 °C or 4 °C (Winkler et al., 2007). However, Fernández-León et al. (2013) reported increased aliphatic GLS and decreased indolic GLS content, while Rodrigues and Rosa (1999) found stable indolic GLS level, but a 31% reduction in GRA level. Vallejo et al. (2003) found a 71% decrease in total GLS content in packaged broccoli inflorescences after pre-storage for seven days at 1 °C. Those findings are in contrast with our results, and may be explained by differences in pre-harvest and harvest conditions, storage times, or lack of packaging in several studies. The use of packaging has been previously demonstrated to preserve GRA in broccoli during storage (Rangkadilok et al., 2002b).

Storage temperature and irradiation. After storage at both temperatures (10 °C and 18 °C) there were no significant differences in contents of total GLS, individual GLS and total aliphatic and total indolic GLS for broccoli heads pre-stored for four days, as compared with

the contents at harvest (Tables 3, 4 and 5). This is in accordance with Winkler et al. (2007), who found stable levels of GRA in broccoli after storage for three days at 8 °C, 15 °C or 20 °C with cold (1 °C or 4 °C) pre-storage. Different results were however reported by (Vallejo et al., 2003), who found that the total GLS level was reduced by 80% in broccoli pre-stored for seven days at 1 °C and then stored for three days at 15 °C. In the present study, broccoli heads pre-stored for seven days at 0 °C and stored for three days at 10 °C under visible light of 13 μmol $m^{-2}s^{-1}$ and 25 μmol $m^{-2}s^{-1}$ had significantly higher contents of GIB, GRA and total aliphatic GLS than at harvest (Table 4). The respective increases of these contents in flower buds were of 130%, 99% and 102%. GRA content per plant has been reported to increase during broccoli development from seedling to vegetative stage with the highest value observed for green head (180 days after sowing), and then rapidly decline when flowering initiated (Rangkadilok et al., 2002a). Moreover, a postharvest increase in GRA in broccoli has been previously recognized as an indicator of vegetative stage (Schouten et al., 2009). Therefore the increases in individual and total aliphatic GLS levels in the present study suggest that these broccoli heads were still vegetative.

A significant increase in 4-OH-GB, as compared with harvest, was found in broccoli pre-stored for seven days at 0 °C and exposed to visible light of 25 μmol $m^{-2}s^{-1}$ during storage for three days at 18 °C (Table 5). Also, 4-Me-GB increased significantly, but after pre-storage for seven days at 4 °C and exposure to visible light of 13 μmol $m^{-2}s^{-1}$ during storage for three days at 10 °C (Table 5). Postharvest increases in indolic GLS were observed for 4-OH-GB in broccoli stored under controlled atmosphere at three different temperatures (Schouten et al., 2009) and for both 4-OH-GB and 4-Me-GB in chopped broccoli florets after 48 h of storage at room temperature (Verkerk et al., 2001). These increases, including those observed in the present study, were probably stress-related, as suggested previously by Verkerk et al. (2001).

General linear model (GLM) analysis revealed that pre-storage period (PP) and storage temperature (ST) significantly influenced contents of total GLS, total indolic and total aliphatic GLS, and all individual GLS, except for 4-Me-GB, in broccoli flower buds after storage (Table 6). Broccoli heads that were pre-stored for seven days had significantly higher levels of the above mentioned compounds than those pre-stored for four days. Moreover, total GLS, total aliphatic and total indolic GLS, and all individual GLS, except for 4-OH-GB, were higher in flower buds of broccoli after storage at 10 °C than at 18 °C. The opposite was found for 4-OH-GB, contents of which were significantly increased after storage at 18 °C compared to 10 °C. This in turn suggests that storage at 18 °C was a stress factor for broccoli heads. Although pre-storage temperature (PT) did not influence GLS levels in broccoli flower buds during storage significantly, the interaction PT × ST was found to be significant for total and individual aliphatic GLS (Table 6). This indicates that the effect of storage temperature on aliphatic GLS in broccoli flower buds depends on pre-storage temperature. The effect was negligible for broccoli pre-stored at 4 °C but significant for those pre-stored at 0 °C with higher levels of total and individual aliphatic GLS levels found after storage at 10 °C than at 18 °C. Consequently, the content of total aliphatic GLS in flower buds of broccoli pre-stored at 0 °C was 35% higher after storage at 10 °C than at 18 °C, and 23% higher after storage at 10 °C than after storage at both temperatures with pre-storage at 4 °C. Thus, the combination of pre-storage at 0 °C and storage at 10 °C was the most favourable in keeping broccoli in the vegetative stage and delaying senescence.

None of the radiation treatments (R), applied in the experiment during storage, influenced GLS levels in broccoli flower buds significantly (Table 6). Similarly UV-B radiation of an ecologically realistic dose (5.5 kJ m^{-2}) during plant growth was found to lack effect on GRA and GB contents and on the expression of the key gene involved in indolic GLS synthesis in *Arabidopsis thaliana* (Demkura and Ballaré, 2012). Also in broccoli plants, pre-harvest UV

radiation has been shown to have no influence on GLS levels (Kuhlmann and Müller, 2009). Moreover, fluorescent light had no effect on total GLS content in broccoli florets during storage for two days at 25 °C (Jin et al., 2015). However, in contrast to our results, increased levels of glucotropaeolin were observed in nasturtium organs upon postharvest exposure to UV-B radiation at 0.075 and 0.15 Wh m^{-2} corresponding to 0.0018 and 0.0036 kJ m^{-2} d^{-1}, respectively (Schreiner et al., 2009), and increased levels of both aliphatic and indolic GLS in broccoli sprouts after pre-harvest UV-B radiation of low to moderate ecologically relevant doses at 0.3 – 1 kJ m^{-2} d^{-1} (Mewis et al., 2012). In the study by Mewis et al. (2012) the GLS increases depended on the UV-B dose, time after the low dosage treatment and on the length of exposure to moderate dose of UV-B. Therefore these diverging results could be explained by differences between UV-B levels applied and plant material used in our and those studies. Also, natural UV radiation during plant growth has been previously demonstrated to influence GLS content in species-specific and tissue-age dependent manners (Reifenrath and Müller, 2007). Although there was no effect of radiation treatments in the present study, a three-factor interaction between pre-storage temperature, storage temperature and radiation treatments (PT × ST × R) was found to be significant for total and individual aliphatic GLS and for the indolic 4-OH-GB and GB.

3.4. Effects of chosen postharvest treatments on glucosinolate contents in broccoli flower buds and floret stalks

Due to significant increases in total and individual aliphatic GLS and in 4-OH-GB contents in flower buds after storage, broccoli pre-stored for seven days at 0 °C and stored for three days at 10 °C and 18 °C under visible light of 25 μmol $m^{-2}s^{-1}$, were chosen for GLS analysis in floret stalks. This was done in order to determine if the increase observed in flower buds appeared also in floret stalks. In addition, GLS in floret stalks of broccoli pre-stored for four days at 0 °C and stored for three days at identical conditions were analysed. Total and individual

aliphatic GLS contents were found to be significantly higher than at harvest in both flower buds and floret stalks of broccoli pre-stored for seven days at 0 °C and stored for three days at 10 °C under visible light of 25 μmol $m^{-2}s^{-1}$ (Table 7). This suggests that aliphatic GLS in broccoli are synthesised both in flower buds and floret stalks, which is according to (Li et al., 2011), who localized the enzymes involved in side chain elongation of aliphatic GLS both in flower buds and flower stalks of *Arabidopsis thaliana*. However in the present study, the increase in 4-OH-GB content in flower buds of broccoli pre-stored for seven days at 0 °C after storage at 18 °C upon visible light of 25 μmol $m^{-2}s^{-1}$ exposure as compared to harvest, was not observed in floret stalks (Table 7). Moreover, broccoli heads pre-stored for four days at 0 °C and stored under identical conditions contained significantly higher levels of 4-Me-GB in floret stalks than at harvest. However, this increase did not appear in flower buds of the same broccoli heads (Table 7). This in turn could indicate that indolic GLS in broccoli during storage accumulate either in flower buds or in floret stalks and confirm the conclusion by Sarsby et al. (2012) that accumulation of indolic and aliphatic GLS is not dependent on each other.

4. Conclusions

The present study has demonstrated that although GLS levels were not changed in broccoli flower buds during pre-storage at low temperatures (0 °C and 4 °C), pre-storage period affected the contents of almost all GLS during storage at higher temperatures (10 °C and 18 °C) significantly. Moreover, pre-storage temperature was found to influence the effect of storage temperature on aliphatic GLS levels in broccoli flower buds. Therefore pre-storage treatment should be an important aspect for both postharvest research and industry. Increased levels of 4-OH-GB after storage at 18 °C, and increased levels of all other GLS after storage at 10 °C, with significant interaction between pre-storage and storage temperature (PT × ST) found for aliphatic GLS, suggest that the combination of pre-storage at 0 °C and storage at 10 °C should

preferably be applied to promote high GLS levels and to keep broccoli inflorescences longer in the vegetative stage and delay senescence.

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Figure captions

Fig. 1. HPLC-DAD chromatogram of desulfated glucosinolates profile of broccoli flower buds detected at 229 nm. Peak numbers correspond to Table 1. (IS) internal standard, glucotropaeolin.

Tables

Table 1
Desulfated glucosinolates identified in broccoli flower buds using LC-MS.

Peak No[a]	Compounds identified			Rt (min)	MW of desulfo-GLS	MS^1, *m/z* [ID]	MS^2 $[M+Na]^+$, *m/z* [ID]
	Trivial name	Abbreviation	Chemical name of R group				
Aliphatic glucosinolates							
1	Glucoiberin	GIB	3-Methylsulfinylpropyl	7.59	343	164.9, 181.9 $[M\text{-}G^b+H]^+$, 344.1 $[M+H]^+$, 366.1 $[M+Na]^+$, 382.0 $[M+K]^+$, 540.0, 579.7	153.8, 169.8, 185.7, 203.8 $[M\text{-}G+Na]^+$, 218.8, 301.9
2	Glucoraphanin	GRA	4-Methylsulfinylbutyl	9.95	357	195.8 $[M\text{-}G+H]^+$, 358.1 $[M+H]^+$, 380.0 $[M+Na]^+$, 395.9 $[M+K]^+$	138.8, 167.8, 183.8, 199.8, 217.9 $[M\text{-}G+Na]^+$, 316.0
3	Glucoalyssin	GAL	5-Methylsulfinylpentyl	13.07	371	62.6, 89.2, 98.2, 116.2, 129.0, 167.9, 183.9, 215.8, 371.8 $[M+H]^+$, 394.1 $[M+Na]^+$, 410.0 $[M+K]^+$, 510.3	181.9, 197.9, 213.9, 231.8 $[M\text{-}G+Na]^+$, 330.0
5	Glucoerucin	GER	4-Methylthiobutyl	21.35	341	58.6, 110.0, 342.1 $[M+H]^+$, 364.0 $[M+Na]^+$, 380.0 $[M+K]^+$, 395.9	167.8, 184.9, 201.9 $[M\text{-}G+Na]^+$, 218.8
Indolic glucosinolates							
4	4-Hydroxyglucobrassicin	4-OH-GB	4-Hydroxyindol-3-ylmethyl	16.91	384	222.9 $[M\text{-}G+H]^+$, 385.0 $[M+H]^+$, 407.0 $[M+Na]^+$, 423.0 $[M+K]^+$, 437.0, 452.9, 554.1, 579.1	145.9, 167.9, 184.9, 210.8, 218.8, 244.8 $[M\text{-}G+Na]^+$, 330.1
6	Glucobrassicin	GB	Indol-3-ylmethyl	24.47	368	206.9 $[M\text{-}G+H]^+$, 369.0 $[M+H]^+$, 391.1 $[M+Na]^+$, 406.9 $[M+K]^+$, 565.1	129.9, 151.8, 184.9, 218.8, 228.9 $[M\text{-}G+Na]^+$
7	4-Methoxyglucobrassicin	4-Me-GB	4-Methoxyinol-3-ylmethyl	39.01	398	236.9 $[M\text{-}G+H]^+$, 399.0 $[M+H]^+$, 421.0 $[M+Na]^+$, 437.0 $[M+K]^+$, 459.2	131.9, 159.9, 181.8, 202.8, 218.8, 242.9, 258.9 $[M\text{-}G+Na]^+$, 300.6
8	Neoglucobrassicin	NGB	1-Methoxyindol-3-ylmethyl	35.51	398	236.9 $[M\text{-}G+H]^+$, 399.0 $[M+H]^+$, 421.1 $[M+Na]^+$, 437.0 $[M+K]^+$	184.9, 202.9, 216.8, 259.0 $[M\text{-}G+Na]^+$, 274.0, 390.0

[a] Peak numbers correspond to peaks in Figure 1.
[b] Glucosyl

Table 2
Glucosinolate (GLS) contents (μmol g^{-1} dry matter) in flower buds and floret stalks of broccoli heads at harvest [a].

Compound	Part of broccoli floret		*P* [b]
	Buds	Stalks	
Glucoiberin	0.71 ± 0.10	0.84 ± 0.07	NS
Glucoraphanin	7.16 ± 0.92	7.67 ± 0.39	NS
Total aliphatic GLS	7.87 ± 1.02	8.50 ± 0.45	NS
4-Hydroxyglucobrassicin	0.20 ± 0.04	0.38 ± 0.06	*
Glucobrassicin	8.18 ± 1.50	3.61 ± 0.30	*
4-Metoxyglucobrassicin	0.82 ± 0.11	0.75 ± 0.04	NS
Neoglucobrassicin	4.07 ± 1.22	0.34 ± 0.03	*
Total indolic GLS	13.27 ± 2.80	5.08 ± 0.36	*
Total GLS	21.14 ± 3.48	13.59 ± 0.42	NS

[a] Data presented as mean values ± standard error of the mean (n=5).
[b] NS Non-significant, * significant at $0.01 < P < 0.05$.

Table 3
Total glucosinolate contents (µmol g^{-1} dry matter) in flower buds of broccoli heads pre-stored for four or seven days in the dark at 0 °C and 4 °C and then stored for three days with different radiation treatments at 10 °C or 18 °C [a].

PP[b] (days)	PT (°C)	ST (°C)	Harvest	Pre-storage	R with light (13-25 µmol m^{-2} s^{-1}) and UV-B (20 kJ m^{-2} d^{-1})				
					13	19	25	19 + UV-B	Dark
4	0	10	21.14 a[c]	24.64 a	21.89 a	22.55 a	23.57 a	21.14 a	22.18 a
		18			13.86 a	21.56 a	16.53 a	21.83 a	11.90 a
	4	10		24.36 a	20.00 a	23.68 a	18.61 a	19.60 a	28.11 a
		18			19.34 a	21.01 a	20.47 a	13.40 a	17.54 a
7	0	10	21.14 a	26.38 a	28.42 a	29.30 a	26.79 a	25.14 a	31.00 a
		18			23.83 a	23.91 a	25.55 a	21.93 a	21.13 a
	4	10		21.37 a	29.93 a	26.66 a	27.09 a	26.70 a	24.17 a
		18			22.55 a	22.35 a	24.36 a	23.94 a	19.87 a

[a] Data presented as mean values (n = 5) of calculated sums of all individual glucosinolate contents.
[b] Abbreviations: PP, pre-storage period; PT, pre-storage temperature; ST, storage temperature; R, radiation treatments.
[c] Values for each PP followed by different letters are significantly different at $\alpha = 0.05$.

Table 4
Aliphatic glucosinolate (GLS) contents (µmol g^{-1} dry matter) in flower buds of broccoli heads pre-stored for four or seven days in the dark at 0 °C and 4 °C and then stored for three days with different radiation treatments (R) at 10 °C or 18 °C [a].

PP[b] (days)	PT (°C)	ST (°C)	Harvest	Pre-storage	R with light (13-25 µmol m^{-2} s^{-1}) and UV-B (20 kJ m^{-2} d^{-1})				
					13	19	25	19 + UV-B	Dark
			Glucoiberin						
4	0	10	0.71 ab[c]	1.04 ab	0.98 ab	1.36 a	1.06 ab	1.00 ab	0.76 ab
		18			0.52 b	0.91 ab	0.64 ab	0.96 ab	0.52 b
	4	10		0.94 ab	0.70 ab	1.02 ab	1.20 ab	0.97 ab	1.08 ab
		18			0.89 ab	0.94 ab	0.86 ab	0.69 ab	0.86 ab
7	0	10	0.71 b	1.25 ab	1.58 a	1.48 ab	1.70 a	1.44 ab	1.45 ab
		18			0.91 ab	1.03 ab	1.51 ab	1.01 ab	1.18 ab
	4	10		1.10 ab	0.98 ab	0.92 ab	1.26 ab	1.17 ab	1.32 ab
		18			1.21 ab	1.32 ab	1.10 ab	1.14 ab	0.92 ab
			Glucoraphanin						
4	0	10	7.16 ab	9.70 ab	9.40 ab	12.69 a	10.20 ab	10.00 ab	8.11 ab
		18			5.28 b	8.63 ab	6.73 ab	9.21 ab	5.54 b
	4	10		8.54 ab	6.77 ab	9.77 ab	10.00 ab	7.97 ab	10.24 ab
		18			8.54 ab	8.70 ab	8.63 ab	6.53 ab	8.77 ab
7	0	10	7.16 b	11.00 ab	14.47 a	12.12 ab	14.05 a	13.01 ab	12.54 ab
		18			8.43 ab	9.79 ab	13.03 ab	9.74 ab	10.34 ab
	4	10		9.98 ab	8.92 ab	8.22 ab	11.80 ab	10.54 ab	11.58 ab
		18			11.74 ab	12.01 ab	10.21 ab	10.51 ab	8.41 ab
			Total aliphatic GLS[d]						
4	0	10	7.87 ab	10.74 ab	10.38 ab	14.06 a	11.26 ab	11.00 ab	8.87 ab
		18			5.80 b	9.54 ab	7.36 ab	10.16 ab	6.06 b
	4	10		9.47 ab	7.47 ab	10.79 ab	11.20 ab	8.94 ab	11.31 ab
		18			9.43 ab	9.63 ab	9.49 ab	7.22 ab	9.63 ab
7	0	10	7.87 b	12.25 ab	16.05 a	13.60 ab	15.75 a	14.45 ab	13.98 ab
		18			9.34 ab	10.82 ab	14.54 ab	10.75 ab	11.52 ab
	4	10		11.08 ab	9.90 ab	9.14 ab	13.05 ab	11.72 ab	12.90 ab
		18			12.96 ab	13.33 ab	11.31 ab	11.65 ab	9.33 ab

[a] Data presented as mean values (n = 5).
[b] Abbreviations: PP, pre-storage period; PT, pre-storage temperature; ST, storage temperature; R, radiation treatments.
[c] Values for each compound and for each PP followed by different letters are significantly different at $\alpha = 0.05$.
[d] The sum of individual aliphatic glucosinolates.

Table 5
Indolic glucosinolate (GLS) contents (µmol g^{-1} dry matter) in flower buds of broccoli heads pre-stored for four or seven days in the dark at 0 °C and 4 °C and then stored for three days with different radiation treatments at 10 °C or 18 °C [a].

PP[b] (days)	PT (°C)	ST (°C)	Harvest	Pre-storage	R with light (13-25 µmol m^{-2} s^{-1}) and UV-B (20 kJ m^{-2} d^{-1})				
					13	19	25	19 + UV-B	Dark
			4-Hydroxyglucobrassicin						
4	0	10	0.20 abc[c]	0.18 abc	0.14 bc	0.16 abc	0.12 c	0.12 c	0.11 c
		18			0.40 abc	0.20 abc	0.43 abc	0.23 abc	0.19 abc
	4	10		0.17 abc	0.14 bc	0.15 bc	0.12 c	0.11 c	0.15 bc
		18			0.21 abc	0.52 ab	0.24 abc	0.56 a	0.34 abc
7	0	10	0.20 b	0.35 b	0.32 b	0.33 b	0.28 b	0.24 b	0.28 b
		18			0.59 ab	0.35 b	1.03 a	0.27 b	0.36 b
	4	10		0.28 b	0.25 b	0.26 b	0.32 b	0.30 b	0.26 b
		18			0.57 b	0.40 b	0.51 b	0.37 b	0.59 b
			Glucobrassicin						
4	0	10	8.18 a	9.02 a	6.91 a	5.70 a	7.70 a	6.73 a	8.11 a
		18			5.14 a	8.10 a	5.43 a	7.68 a	4.02 a
	4	10		9.98 a	8.14 a	8.24 a	5.06 a	6.63 a	9.91 a
		18			6.53 a	6.31 a	7.16 a	3.70 a	4.87 a
7	0	10	8.18 ab	9.32 ab	7.88 ab	9.77 ab	6.64 ab	7.17 ab	9.89 ab
		18			8.32 ab	8.60 ab	6.33 b	7.19 ab	6.31 b
	4	10		6.74 ab	12.16 ab	10.61 ab	8.69 ab	9.52 ab	6.84 ab
		18			6.13 b	5.52 b	8.46 ab	7.36 ab	6.19 b
			4-Methoxyglucobrassicin						
4	0	10	0.82 a	1.10 a	1.07 a	0.94 a	1.22 a	0.94 a	1.17 a
		18			0.97 a	1.37 a	1.12 a	1.38 a	0.76 a
	4	10		1.26 a	1.31 a	1.11 a	0.89 a	1.02 a	1.49 a
		18			1.16 a	1.26 a	1.18 a	0.79 a	1.09 a
7	0	10	0.82 b	1.22 ab	1.20 ab	1.38 ab	0.92 b	0.92 ab	1.22 ab
		18			1.40 ab	1.13 ab	1.18 ab	1.02 ab	1.05 ab
	4	10		1.06 ab	1.76 a	1.41 ab	1.23 ab	1.41 ab	1.11 ab
		18			1.13 ab	1.06 ab	1.36 ab	1.16 ab	1.04 ab
			Neoglucobrassicin						
4	0	10	4.07 ab	3.59 ab	3.39 ab	1.69 ab	3.26 ab	2.34 ab	3.93 ab
		18			1.56 ab	2.35 ab	2.19 ab	2.38 ab	0.87 b
	4	10		3.48 ab	2.93 ab	3.39 ab	1.34 ab	2.90 ab	5.25 a
		18			2.00 ab	3.29 ab	2.41 ab	1.14 ab	1.61 ab

7	0	10	4.07 a	3.25 a	2.96 a	4.21 a	3.19 a	2.36 a	5.63 a
		18			4.18 a	3.01 a	2.48 a	2.69 a	1.90 a
	4	10		2.21 a	5.86 a	5.24 a	3.79 a	3.76 a	3.07 a
		18			1.76 a	2.04 a	2.71 a	3.39 a	2.72 a
			Total indolic GLS[d]						
4	0	10	13.27 a	13.90 a	11.51 a	8.49 a	12.31 a	10.14 a	13.31 a
		18			8.07 a	12.02 a	9.17 a	11.67 a	5.84 a
	4	10		14.89 a	12.53 a	12.88 a	7.41 a	10.66 a	16.80 a
		18			9.90 a	11.38 a	10.98 a	6.19 a	7.91 a
7	0	10	13.27 ab	14.13 ab	12.37 ab	15.70 ab	11.04 ab	10.69 ab	17.02 ab
		18			14.49 ab	13.08 ab	11.01 ab	11.18 ab	9.61 b
	4	10		10.29 ab	20.03 a	17.52 ab	14.04 ab	14.99 ab	11.28 ab
		18			9.59 b	9.02 b	13.04 ab	12.29 ab	10.55 ab

[a] Data presented as mean values (n = 5).
[b] Abbreviations: PP, pre-storage period; PT, pre-storage temperature; ST, storage temperature; R, radiation treatments.
[c] Values for each compound and for each PP followed by different letters are significantly different at $\alpha = 0.05$.
[d] The sum of individual indolic glucosinolates.

Table 6

Influence of pre-storage period and temperature, storage temperature, and radiation treatments on glucosinolate contents in broccoli flower buds after pre-storage in the dark and then stored for three days with radiation treatments (GLM analysis).

Source of variation	GIB [a]	GRA	Total aliphatic GLS	4-OH-GB	GB	4-Me-GB	NGB	Total indolic GLS	Total GLS
	F value and significance level [b]								
Pre-storage period (PP)	44.08 ***	37.25 ***	38.24 ***	32.29 ***	12.31 ***	2.43 NS	10.49 ***	13.65 ***	35.8 ***
Pre-storage temperature (PT)	2.10 NS	2.72 NS	2.67 NS	0.14 NS	0.32 NS	1.83 NS	0.61 NS	0.59 NS	0.07 NS
Storage temperature (ST)	18.07 ***	15.05 ***	15.47 ***	53.42 ***	17.68 ***	0.90 NS	21.2 ***	16.18 ***	25.7 ***
Radiation treatments (R)	1.97 NS	1.58 NS	1.63 NS	1.84 NS	0.96 NS	1.09 NS	0.81 NS	0.86 NS	0.74 NS
PP × PT [c]	5.87 *	2.82 NS	3.13 NS	1.25 NS	0.09 NS	0.58 NS	0.01 NS	0.02 NS	0.64 NS
PP × R	1.36 NS	1.57 NS	1.54 NS	2.83 *	0.35 NS	0.52 NS	0.26 NS	0.35 NS	0.66 NS
PT × ST	8.38 **	11.90 ***	11.57 ***	0.28 NS	3.27 NS	2.57 NS	0.97 NS	2.44 NS	0.33 NS
PT × R	0.56 NS	0.86 NS	0.81 NS	3.83 **	0.69 NS	0.30 NS	0.33 NS	0.12 NS	0.32 NS
ST × R	0.29 NS	0.35 NS	0.35 NS	1.56 NS	1.89 NS	1.41 NS	2.61 *	2.38 NS	1.76 NS
PT × ST × R	2.74 *	2.84 *	2.84 *	3.98 **	2.60 *	1.14 NS	1.83 NS	2.21 NS	0.37 NS
PP × PT × ST × R	0.56 NS	0.75 NS	0.72 NS	1.16 NS	1.54 NS	0.82 NS	2.74 *	2.02 NS	1.21 NS
	Content (µmol g^{-1} dry matter) [d]								
Pre-storage period									
4 days	0.90 b	8.58 b	9.48 b	0.23 b	6.60 b	1.11 a	2.51 b	10.46 b	19.94 b
7 days	1.23 a	11.07 a	12.30 a	0.39 a	7.98 a	1.20 a	3.35 a	12.93 a	25.23 a
Pre-storage temperature									
0 °C	1.10 a	10.17 a	11.27 a	0.31 a	7.18 a	1.12 a	2.83 a	11.44 a	22.70 a
4 °C	1.03 a	9.49 a	10.52 a	0.32 a	7.40 a	1.20 a	3.03 a	11.95 a	22.47 a
Storage temperature									
10 °C	1.17 a	10.62 a	11.79 a	0.21 b	8.12 a	1.19 a	3.53 a	13.04 a	24.83 a
18 °C	0.96 b	9.03 b	9.99 b	0.42 a	6.47 b	1.13 a	2.34 b	10.35 b	20.34 b
Radiation treatments									
13 µmol m^{-2} s^{-1}	0.98 a	9.19 a	10.17 a	0.33 a	7.65 a	1.25 a	3.08 a	12.31 a	22.48 a
19 µmol m^{-2} s^{-1}	1.12 a	10.24 a	11.36 a	0.30 a	7.86 a	1.21 a	3.15 a	12.51 a	23.88 a
25 µmol m^{-2} s^{-1}	1.17 a	10.58 a	11.75 a	0.38 a	6.94 a	1.14 a	2.67 a	11.12 a	22.87 a
UV-B + 19 µmol m^{-2} s^{-1}	1.05 a	9.69 a	10.74 a	0.28 a	7.00 a	1.08 a	2.62 a	10.97 a	21.71 a
Dark	1.01 a	9.44 a	10.45 a	0.29 a	7.02 a	1.12 a	3.12 a	11.54 a	21.99 a

[a] Abbreviations: GIB, glucoiberin; GRA, glucoraphanin; GLS, glucosinolates; 4-OH-GB, 4-hydroxyglucobrassicin; GB, glucobrassicin; 4-Me-GB, 4-methoxyglucobrassicin; NGB, neoglucobrassicin.

[b] NS, *, **, *** Non-significant or significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

[c] Only interactions with at least one significant value are shown.

[d] Mean values (n = 40 for radiation treatments, n = 100 for pre-storage period, pre-storage temperature and storage temperature). Values that do not share a common letter are significantly different at $\alpha = 0.05$.

Table 7

Glucosinolate contents (μmol g^{-1} dry matter) in floret stalks or flower buds of broccoli heads pre-stored for four or seven days in the dark at 0 °C and then stored for three days with visible light of 25 μmol m^{-2} s^{-1} treatment at 10 °C or 18 °C [a].

Harvest or PP (d), PT (°C), ST (°C)[b]	GIB[c]	GRA	Total aliphatic GLS	4-OH-GB	GB	4-Me-GB	NGB	Total indolic GLS	Total GLS
Stalks									
Harvest	0.84 b[d]	7.67 b	8.50 b	0.38 a	3.61	0.75 b	0.34	5.08	13.59
PP 4	1.00 ab	9.03 ab	10.03 ab	0.35 a	3.52	0.83 ab	0.46	5.17	15.20
PP 4, ST 10	1.04 ab	9.07 ab	10.11 ab	0.27 a	2.97	0.84 ab	0.37	4.44	14.56
PP 4, ST 18	0.96 ab	8.58 ab	9.54 ab	0.21 a	4.21	1.06 a	0.85	6.33	15.87
PP 7	0.88 b	7.32 b	8.20 b	0.35 a	2.76	0.86 ab	0.23	4.20	12.39
PP 7, ST 10	1.38 a	11.11 a	12.50 a	0.25 a	2.77	0.85 ab	0.45	4.32	16.82
PP 7, ST 18	1.16 ab	9.67 ab	10.83 ab	0.23 a	2.53	0.89 ab	0.36	4.01	14.84
P [e]	**	*	*	*	**NS**	*	**NS**	**NS**	**NS**
Buds									
Harvest	0.71 b	7.16 b	7.87 b	0.20 b	8.18	0.82	4.07	13.27	21.14
PP 4	1.04 ab	9.70 ab	10.74 ab	0.18 b	9.02	1.10	3.59	13.90	24.64
PP 4, ST 10	1.06 ab	10.20 ab	11.26 ab	0.12 b	7.70	1.22	3.26	12.31	23.57
PP 4, ST 18	0.64 b	6.73 b	7.36 b	0.43 b	5.43	1.12	2.19	9.17	16.53
PP 7	1.25 ab	11.00 ab	12.25 ab	0.35 b	9.32	1.22	3.25	14.13	26.38
PP 7, ST 10	1.70 a	14.05 a	15.75 a	0.28 b	6.64	0.92	3.19	11.04	26.79
PP 7, ST 18	1.51 a	13.03 a	14.54 a	1.03 a	6.33	1.18	2.48	11.01	25.55
P	***	***	***	***	**NS**	**NS**	**NS**	**NS**	**NS**

[a] Data presented as mean values (n = 5).

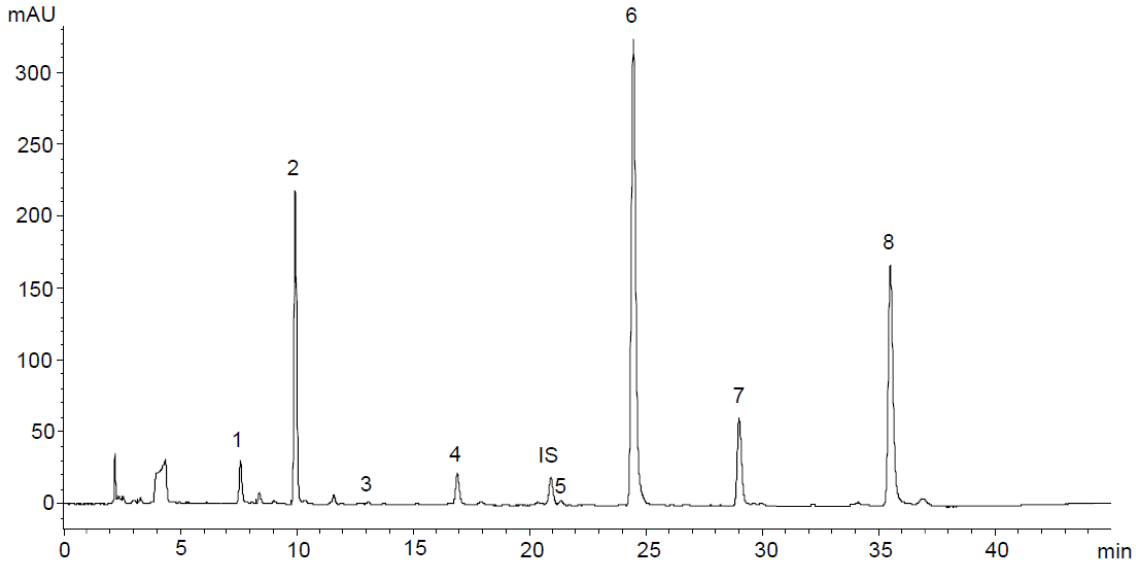
[b] Abbreviations: PP, pre-storage period; PT, pre-storage temperature; ST, storage temperature.

[c] Abbreviations: GIB, glucoiberin; GRA, glucoraphanin; GLS, glucosinolates; 4-OH-GB, 4-hydroxyglucobrassicin; GB, glucobrassicin; 4-Me-GB, 4-methoxyglucobrassicin; NGB, neoglucobrassicin.

[d] Values for each compound and for each part of broccoli head followed by different letters are significantly different at $\alpha = 0.05$.

[e] NS, *, **, *** Non-significant or significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Figure 1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